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Membranes and Their Constituents as Promoters of Calcium Oxalate Crystal Formation in Human Urine

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Abstract. We have proposed that membranes of cellular degradation products are a suitable substrate for the nucleation of calcium oxalate (CaOx) crystals in human urine. Human urine is generally metastable with respect to CaOx. To demonstrate that cellular membranes present in the urine promote nucleation of CaOx we removed these substrates by filtration or centrifugation and induced crystallization by adding sodium oxalate, before and after filtration or centrifugation. In a separate experiment, membrane vesicles isolated from rat renal tubular brush border were added into the filtered or centrifuged urine before crystal induction. Crystals were counted using a particle counter. Urine, the pellet, and retentate were analyzed for the presence of membranes, lipids, and proteins. Lipids were further separated into different classes, identified, and quantified. Both filtration and centrifugation removed lipids, proteins, and membrane vesicles, causing a reduction in lipid and protein contents of the urine. More crystals formed in whole than in filtered or centrifuged urine. The number of crystals significantly increased when filtered urine was supplemented with various urinary components such as the retentate and phospholipids, which are removed during filtration. We also determined the urinary metastable limit with respect to CaOx. Filtration and centrifugation were associated with increased metastable limit which was reduced by the addition of membrane vesicles. These results support our hypothesis that urine normally contains promoters of CaOx crystal formation and that membranes and their constituents are the most likely substrate for crystal nucleation in the urine.

Key words: Calcium oxalate — Nephrolithiasis — Cell membrane — Matrix Vesicles — Phospholipids.

Human urine is generally metastable with respect to calcium oxalate (CaOx) [1, 2], the most common constituent of urinary stones. As a result, crystal nucleation, the first step towards stone formation, requires a substrate. We have proposed that in the human urine, membranes of cellular degradation products act as nucleators of CaOx crystals [3]. Membranes and their lipids have also been implicated in the nucleation of calcium phosphate (CaP) crystals in biological fluids [4–15]. *In vitro* studies have shown that acidic phospholipids, lipid extracts from various calcified tissues, membranes of so-called matrix vesicles, and liposomes all can initiate the formation of CaP crystals in metastable solutions. Lipids can also induce CaP crystallization *in vivo.* We have demonstrated that membranes of renal tubular origin and lipids of CaOx stones promote the precipitation of CaOx crystals *in vitro* in metastable solutions [16–18]. In addition we have shown that hyperoxaluric rats develop CaOx crystalluria and nephrolithiasis only in the presence of sloughed cellular membranes [19].

The studies described here were carried out to determine the role of membranes and their constituents in the formation of CaOx crystals in the urine. Urine samples were filtered and/or centrifuged and analyzed for lipids and membranes. Metastable limit with respect to CaOx was determined for various samples with or without the addition of membrane vesicles. Nucleation of CaOx was induced by addition of sodium oxalate. Results indicate that human urine contains promoters of CaOx crystal nucleation and, that cellular membranes are the most likely nucleators. Removal of these nucleators from the urine through filtration and centrifugation changes the CaOx crystallization activity in the urine, and reintroduction of membranous substances into the processed urine boosts their crystallization potential.

Materials and Methods

Urine Collection for Crystallization Studies

Twenty-four-hour urine samples were collected from normal healthy human males. During collection, the specimens were maintained at room temperature (approximately 24°C). One milliliter of 20% sodium azide, an antibacterial agent, was placed in the collection bottles prior to collection. The pH and total urinary volume were recorded. The total urinary protein was determined using dipsticks and/or a Bio-Rad (Bio-Rad Laboratories, Hercules, CA) protein assay kit. Urine was examined microscopically. Absence of proteinuria, overt crystalluria, and blood cells in the urine were considered to indicate the absence of kidney diseases.

Following a generally accepted protocol of urine processing [2, 20–22] before performing crystallization studies, aliquots were filtered through 5 μm or 0.22 μm filters and/or centrifuged at 10,000 *g* at 20°C for 20 minutes using the J-14 rotor of the J2-21 centrifuge. Both the pellets and the retentate on the filters were processed for and examined by transmission electron microscopy (TEM) as described in our earlier publications [18]. Calcium and oxalate contents of sample urine were determined both before and after various manipulations to verify that filtration and centrifugation did not alter such urinary constituents.

Crystal Induction and Particle Counting

Crystallization of CaOx was induced by the addition of 30 μl of 0.1 M sodium oxalate to 2 ml of native or filtered urine. The mixture was incubated for 30 minutes in a shaking water bath after

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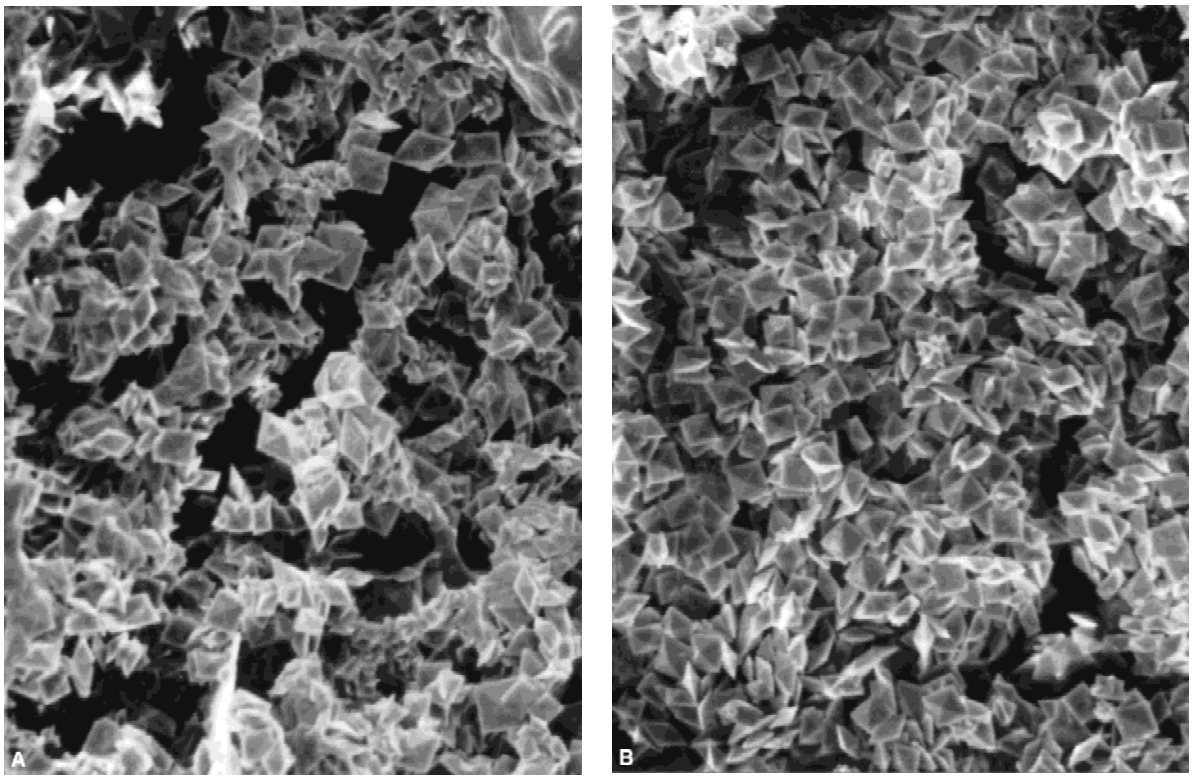

Fig. 1. Crystals produced in whole urine **(A)** or in the urine filtered through a 0.22 μm filter **(B).** ×400.

which crystals were counted using an Elzone 80 XY Model 112 PC particle counter (Particle Data Inc., Elmhurst, IL). The mixture was filtered through a 0.22 μm filter and the crystals were identified by x-ray diffraction, SEM, and energy dispersive X-ray microanalysis [16–18]. The effect of centrifugation on number of crystals formed was similarly investigated.

Determination of Metastable Limit

Metastable limit of whole, filtered, and filtered and centrifuged urine with respect to CaOx was determined using a 96-well microplate [22]. The urine was centrifuged at 10,000 *g* and/or filtered through a 0.22 μm filter. Sodium oxalate (10 μl) at concentrations ranging from 0.1 to 1.5 mM was added to 200 μl of urine. After 20 minutes of incubation at room temperature, microplates were examined using a phase contrast light microscope equipped with polarizing optics. The minimum amount of oxalate required to produce microscopically detectable CaOx crystals was judged as the metastable limit of the sample.

It is our hypothesis that filtration and centrifugation remove membranous substances from the urine and cause a reduction in its crystallization activity. Thus, addition of membranous material into the filtered and/or centrifuged urine should increase the crystallization activity to its original state. To check this hypothesis we isolated brush border membrane from the proximal tubules of the rat kidney cortex [18] and tested the urinary metastable limit in the presence of added membranes. The brush border membrane vesicles were added to each well as 10 μl of a 1.01 mg protein/ml of the vesicle suspension.

Isolation, Identification, and Quantification of Lipids

To demonstrate the loss of lipids by filtration and centrifugation, we isolated lipids from the urine. The methods are described in detail in earlier publications [23, 24]. In brief, 24-hour urine specimens were collected from normal subjects and stored at 4°C; sodium azide was added as an antibiotic. Urine was filtered through 5 μm or 0.22 μm filters or centrifuged at 10,000 *g* at 20°C for 20 minutes using the J-14 rotor of the J2-21 centrifuge. To isolate lipids, 400 ml was taken from the native or processed urine specimen and 1.2 liter of 2:1 chloroform:methanol was added. The mixture was shaken and placed on an end-over-end mixer for 24 hours at 4°C. The samples were then centrifuged at 7000 r.p.m. to achieve phase separation. The top portion was removed and combined as the aqueous layer; the middle layer was recovered as the interface; and the lower phase was collected and pooled as the organic layer. After evaporation to a smaller volume, the organic sample was Folch-washed twice, pooling the respective phases with the previous ones. The organic phase was then lyophilized, weighed and reconstituted with 2:1 chloroform:methanol to a final volume of 1 ml with 0.1% butyl hydroxytoluene (BHT) as a preservative, blanketed with dry nitrogen, and stored in the dark at −70°C until further analysis.

The organic extract was separated into various lipid classes with Bio Sil A silicic acid. The sample was applied to a 1.5 × 15 cm column equilibrated with chloroform. Neutral lipids were eluted with 230 ml of chloroform, glycolipids with 900 ml of acetone, and phospholipids with 230 ml of methanol. Each lipid class was concentrated with a rotary evaporator, dried under nitrogen gas, lyophilized, weighed, and re-suspended in a known volume of chloroform:methanol (2:1) with 0.1% BHT and stored at −70°C.

Phospholipids were further quantitated with ammonium ferrothiocyanate and Victorian Blue R (VBR) methods [24]. Neutral lipids were analyzed for total and free cholesterol, cholesterol esters, and triglycerides. The glycolipids were analyzed for glucose. The lipid classes were analyzed further for identification of individual lipids by one-dimensional, thin-layer chromatography (TLC). Neutral lipid standards were purchased from Nu Chek Prep (Elysian, MN); glycolipid standards from Sigma Chemical Ltd. (St. Louis, MO.); and phospholipid standards from Avanti Polar

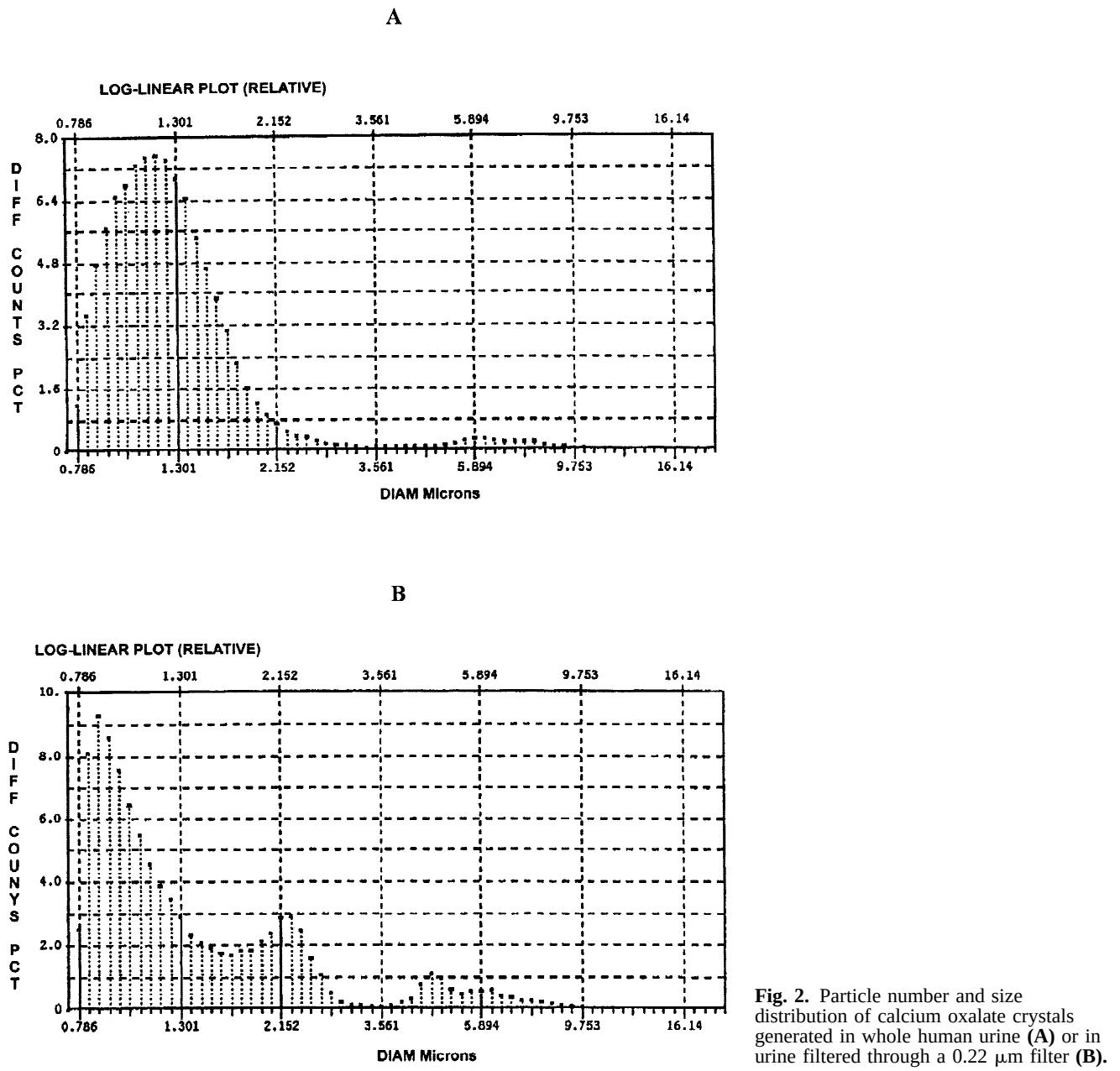

Fig. 2. Particle number and size distribution of calcium oxalate crystals generated in whole human urine **(A)** or in urine filtered through a 0.22 μm filter **(B).**

Lipids (Birmingham, AL). Individual lipid spots were visualized by exposure to iodine vapor for 30 minutes and identified by comparison to known standards. Individual phosphlipids were quantified by scraping the spots from the iodine-stained plates and analyzing them for phosphate using Bartlett's method.

Statistical analyses of the data were performed using a computer-based program (MS-Excel). Statistical comparisons were made by Student's one-tailed paired *t*-test. A P value of <0.05 was considered significant.

Reconstitution Studies

Lipids and proteins were isolated from the urine using the procedure described above. The aqueous and interface were combined to represent proteins fraction and lipids were obtained from the organic layer. Total lipids were further separated into various classes. Crystallization was induced in the whole urine and urine filtered through a 0.22 μm filter before and after the addition of retentate, total lipids, neutral lipids, or phospholipids. Various fractions were added in the amounts needed to equal that in the whole urine. The number of crystals formed was counted as in the other experiments.

Results

Crystallization in the Urine

Filtration and centrifugation did not appear to significantly alter the calcium and oxalate contents of the urine. Whole urine ($n = 6$) contained 0.738 ± 0.384 mMol/liter of calcium and 0.087 ± 0.004 mMol/liter of oxalate. Urine filtered

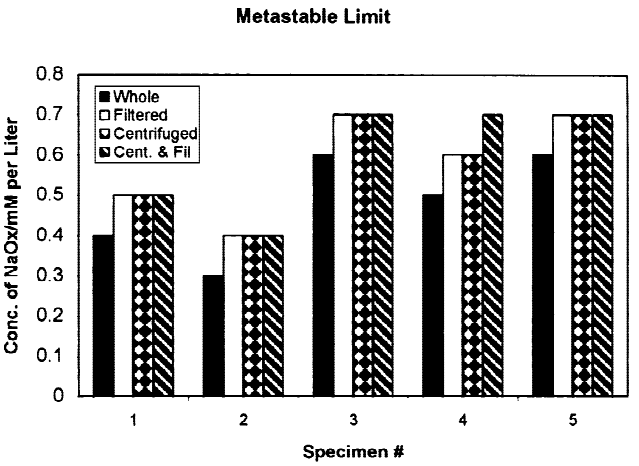

Fig. 3. Metastable limit of whole urine, urine filtered through a 0.22 μm filter or centrifuged at 10,000 *g*, or both centrifuged and filtered. The limit was determined by adding sodium oxalate at increasing concentrations and microscopic examination of the samples for precipitation of calcium oxalate crystals after a 20-minute incubation period. Both filtration and centrifugation increased the metastable limit.

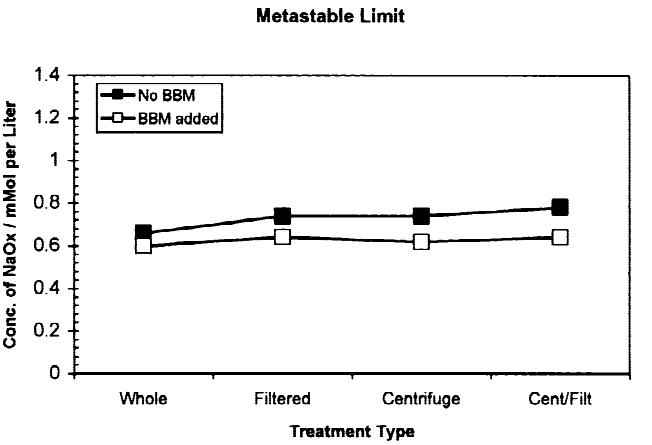

Fig. 4. Metastable limit of the similar samples as in Figure 4, after addition of brush border membrane vesicles. There was a definite reduction in the amount of oxalate needed for discernable precipitation of calcium oxalate.

through a 0.22 μm filter (n + 3) contained 0.771 ± 0.031 mMol/liter of calcium and 0.098 ± 0.01 mMol/liter of oxalate. Centrifuged urine (n = 3) contained 0.836 ± 0.036 mMol/liter of calcium and 0.084 ± 0.016 mMol/liter of oxalate.

Filtration of urine through a 5 μm filter was associated with a reduction in the number of CaOx crystals formed, from 109,062 ± 10,665 to 60,968 ± 9,180 (n = 5, $P < 0.0002$). Filtration through 0.22 μm filter further reduced the number of crystals formed to 10, 902 ± 3842 (n = 5, $P < 0.0002$). Crystals were identified as CaOx dihydrate (Fig. 1). Individual crystals had the typical bipyramidal habit and many of them appeared twinned. Some CaOx monohydrate crystals were also seen and appeared to be nucleating on the surfaces of CaOx dihydrate crystals. Filtration did not appear to cause a change in mean crystal size, but crystals formed in whole urine showed unimodal particle size distribution whereas those formed in filtered urine demonstrated a multimodal distribution (Fig. 2). Centrifugation was similarly associated with a decrease in the number of crystals formed—from 108,504 to 12,546.

Metastable Limit of the Urine

Processing of the urine in which urine was filtered and/or centrifuged resulted in a significant increase ($P < 0.05$) in the metastable limit of the processed urine compared with the whole urine (Fig. 3). More oxalate was needed to precipitate CaOx in processed urine than in the native whole urine. No significant differences were noticed between various treatments examined. Addition of membranous vesicles to the urinary specimens resulted in significant lowering ($P < 0.05$) of the metastable limit (Fig. 4).

Reconstitution Studies

Table 1 shows results of the studies in which crystallization was induced in whole or filtered urine. Crystals were generated in the filtered urine before and after the addition of urinary components removed during filtration. Filtration resulted in a significant reduction in the number of crystals formed, and a highly significant (<0.001) increase occurred in the number of crystals when components were added back into the filtered urine. Addition of every component—total lipids, phospholipids, proteins, and retentate—was associated with increased crystallization, not only compared with the filtered urine, but also with the original whole urine. The highest and most significant increase was, however, observed when phospholipids were added. Addition of the retentate or the proteins had similar outcomes.

Table 1. Number of particles produced by addition of 0.1 M sodium oxalate to whole or filtered urine[a] (n = 3)

Urine	No. of crystals
Whole urine	141,600 ± 14,212
Filtered urine	19,113 ± 2,293
Filtered urine + retentate	210,803 ± 6,008
Filtered urine + proteins	208,727 ± 8,800
Filtered urine + neutral lipids	231,123 ± 1,680
Filtered urine + lipids	336,600 ± 10,842
Filtered urine + phospholipids	415,517 ± 14,966

$P < 0.001$

[a] Whole urine vs filtered urine with or without additives

Change in Protein and Lipid Contents of Urinary Specimens

Filtration as well as centrifugation altered both the protein and lipid contents of the urine (Table 2). Reduction in the amounts of both protein and lipids was higher and in some cases, significant after filtration through a 0.22 μm filter than after centrifugation at 10,000 *g*. Both processes resulted in a reduction in the amounts of various types of urinary lipids including neutral lipids, glycolipids, and phospholipids. Filtration through a 0.22 μm filter almost always resulted in more significant reduction than centrifugation. Amount of total cholesterol, triglycerides, and glycolipids, as determined by glucose contents, was signifi-

Table 2. Effect of filtration and centrifugation on urinary lipids and proteins

Protein and lipids Mg/liter	Whole urine	Centrifuged urine	Filtered urine
Protein	21.65 ± 10.94	19.42 ± 10.43	17.98 ± 9.47
Lipids	296.6 ± 50.80	249.8 ± 81.43	203.2 ± 47.62
Cholesterol	1.209 ± 0.527	0.993 ± 0.442	0.863 ± 0.296[c]
Triglycerides	2.034 ± 1.171	1.242 ± 0.334	1.059 ± 0.546
Glycolipids	1.982 ± 0.538	1.188 ± 0.334	1.087 ± 0.290
Phospholipids (AMF)*	1.061 ± 0.680	0.775 ± 0.358[a]	0.651 ± 0.330[b]
Phospholipids (VBR)†	0.333 ± 0.202	0.242 ± 0.140[a]	0.173 ± 0.089

Both lipids and proteins were significantly reduced ($P < 0.05$) on centrifugation or filtration except [a], between whole and centrifuged; [b], whole and filtered; and [c], centrifuged and filtered

* Ammonium ferrothiocyanate, †Victorian Blue R assay

cantly reduced after centrifugation. Filtration resulted in reduction of total cholesterol, triglycerides, glycolipids, and phospholipids. We also examined individual phospholipids. Sphingomyelin (SM), phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidic acid (PA), and phosphatidyl glycerol (PG) were detected in almost all the urine samples. Some samples also contained phosphatidyl serine (PS), lyso-PG, lyso-PE, and lyso-PC. Filtration and centrifugation both resulted in significant reduction in the amounts of almost all the phospholipids listed above (Table 3).

Both lipids and proteins were present in the pellets as well as the retentate on the filters (Tables 4, 5). All classes of lipids found in the urine were identified in the corresponding pellets and retentates. Lesser amounts of lipids and proteins were found on the filters than in the pellets apparently because retentate was removed from the filters by washing with aqueous solution, which resulted in incomplete recovery. Ultrastructural analysis of the pellets and filters showed an abundance of membrane-bound vesicles (Fig. 5).

Discussion

It is currently acknowledged that crystallization in the urine is controlled by two equally important but opposing forces—urinary supersaturation with respect to the stone salts and presence of inhibitors of crystallization [1, 2, 25, 26]. Most urinary macromolecular inhibitors of CaOx crystal formation are either glycoproteins or glycosaminoglycans [25, 26]. Human urine is metastable with respect to CaOx, i.e., nucleation can occur in the presence of favorable substrates, but there is no consensus as to the nature of nucleation substrate [3]. Since most pathological as well as physiological calcification processes have been suggested to be aided by membranes and their lipids [4–15], we have proposed that they may also assist crystallization in the urinary system [3].

Initial CaP deposition in a number of calcific diseases occurs on cellular membranes which are present at the calcification sites either as limiting membranes of the so-called matrix vesicles [5, 7, 9] or constituent of cellular degradation products [8]. Calcification of bioprosthetic devices such as heart valves fabricated from porcine aortic valves or bovine pericardium, is also membrane mediated [27]. Initial calcification starts with nucleation by membrane fragments of the cells from cusp or pericardium. Dental plaque and calculus formation is yet another example of calcification initiated by cell membrane [28]. Membranes of microorganisms present in the dental plaque nucleate CaP and start calculus formation. One of the main reasons for cellular membranes acting as nucleators of CaP is suggested to be the presence of lipids, particularly acidic phospholipids. Lipids have been identified in matrices of all types of calcified tissues and at most pathological calcification sites [4, 6]. They have also been isolated from all such tissues. *In vitro*, the membranes, acidic phospholipids, lipid extracts from various calcified tissues, and liposomes [15] all have been shown to initiate CaP crystallization in a metastable solution.

Our ultrastructural and biochemical studies have demonstrated the presence of lipids and membranes in the matrices of CaOx kidney stones [23, 29]. Membranous profiles were seen tightly bound to the ghosts of CaOx crystals. Matrices of CaOx crystals induced *in vitro* in human urine also showed the presence of membranes and lipids. In addition, an analysis of human urine showed that significantly more phospholipids were present in the urine of CaOx stone formers than in the normal individuals [24]. Phospholipids are suggested to be the principal lipids involved in calcification. Membrane vesicles isolated from the rat renal tubular brush border promoted the formation of CaOx crystals in a buffered metastable solution [17, 18]. We have recently demonstrated the growth of CaOx monohydrate crystals at Langmuir monolayers of various phospholipids [30].

Our animal model studies have also shown an involvement of cell membranes in crystallization. Crystals of CaOx experimentally induced in rat kidneys were always seen in association with cellular degradation products [31]. Some of the crystals appeared to nucleate on epithelial cell surfaces while others appeared to be aggregating with the assistance of cellular membrane fragments. In a series of experiments, hyperoxaluria and shedding of the membranes from the renal tubular epithelial cells were induced in male rats. Crystals of CaOx formed only when both membrane shedding and hyperoxaluria were simultaneously produced [19]. Urinary CaOx crystals were seen connected to the shed membranes [3, 19]. Hyperoxaluria alone did not result in crystallization.

Results presented here demonstrate that both filtration and centrifugation change the crystallization activity of the urine. Filtration even through a 5 μm filter significantly reduced the number of crystals formed. The number of crystals was drastically reduced after filtration through a 0.22 μm filter. There was also a change in the urine's metastable

Table 3. Effect of filtration and centrifugation on urinary phosphoipids

Phospholipids Mg/liter	Whole urine	Centrifuged urine	Filtered urine
Sphingomyelin	0.625 ± 0.422	0.431 ± 0.274	0.273 ± 0.208
Phosphatidylcholine	0.511 ± 0.338	0.382 ± 0.205	0.189 ± 0.154
Phosphatidylethanolamine	0.630 ± 0.282	0.424 ± 0.201	0.247 ± 0.172
Phosphatadic acid	0.239 ± 0.094	0.135 ± 0.078[a]	0.113 ± 0.085
Phosphatidylglycerol	0.377 ± 0.276	0.243 ± 0.160	0.137 ± 0.090

Almost all major phospholipids were significantly reduced ($P < 0.05$) on filtration or centrifugation except [a], between whole and centrifuged

Table 4. Total protein and lipids in the pellet and on the filter

Protein and lipids Mg/liter	Pellet	Filter
Protein	17.98 ± 9.47	12.48 ± 7.02
Lipids	101.4 ± 32.95	120.0 ± 34.88
Cholesterol	0.653 ± 0.213	0.624 ± 0.217
Triglycerides	0.729 ± 0.428	0.537 ± 0.516
Glycolipids	0.852 ± 0.209	0.747 ± 0.238
Phospholipids (AMF)*	0.356 ± 0.286	0.227 ± 0.229
Phospholipids (VBR)†	0.106 ± 0.043	0.077 ± 0.045

* Ammonium ferrothiocyanate, †Victorian Blue R assay

Table 5. Different types of phospholipids identified in the pellets and on filters

Phospholipids Mg/liter	Pellets	Filters
Sphingomyelin	0.219 ± 0.146	0.125 ± 0.152
Phosphatidylcholine	0.093 ± 0.058	0.055 ± 0.67
Phosphatidylethanolamine	0.159 ± 0.132	0.067 ± 0.055
Phosphatadic Acid	0.108 ± 0.087	0.020 ± 0.018
Phosphatidylglycerol	0.089 ± 0.058	0.063 ± 0.045

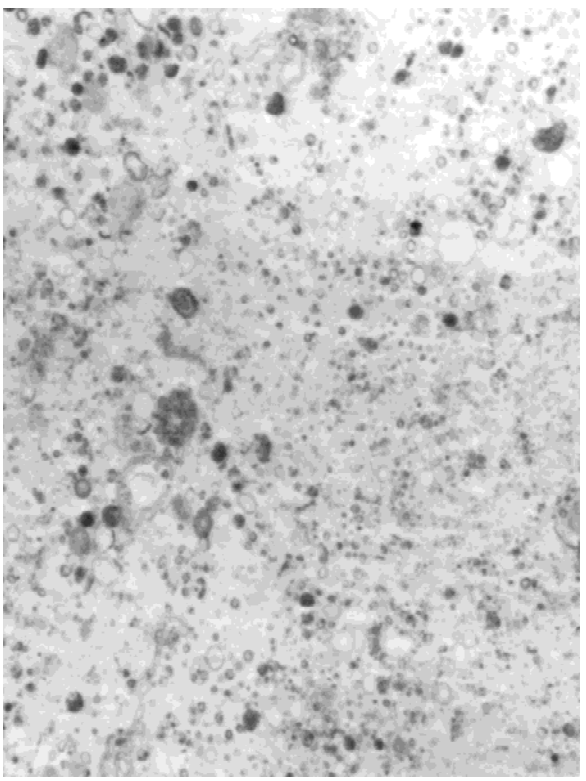

Fig. 5. Transmission electron micrograph of the retentate on a 0.22 μm filter showing an abundance of membrane-bound vesicles. ×6300.

limit with respect to CaOx. Both filtration and centrifugation resulted in an increase in the metastable limit, i.e., more oxalate was required to produce microscopically detectable CaOx crystals within a specified time. These results indicate that urine contains promoters of crystallization, especially nucleation substrates, which are removed through processing such as filtration and centrifugation.

Transmission electron microscopic analysis of the filter and the pellet showed that processing resulted in the removal of membranous vesicles and fibrillar material. Biochemical analysis showed that processing caused a reduction in both the urinary proteins and lipids and that both the pellets and filters contained lipids and proteins. All classes of lipids were similarly affected indicating that most lipids were probably removed as constituent of the membranes. Thus, substances eliminated during processing included proteins, perhaps some glycosaminoglycans, and membranous vesicles. Crystallization in the urine is modulated by a variety of macromolecules [25, 26]. Many of the normally occurring, well characterized urinary macromolecules such as osteopontin [32, 33], bikunin [34, 35], nephrocalcin [36], prothrombin fragment-1 [37], calgranulin [38, 39], Tamm-Horsfall protein [40] and various glycosaminoglycans [41] are all currently considered as inhibitors of CaOx crystallization. Membranes and their constituents are perhaps the only urinary component with the potential to promote CaOx crystal nucleation. Removal of membranes through processing is most probably the cause of a decline in nucleation activity as illustrated by the significant reduction in crystal numbers. Our reconstitution studies in which reintroduction of various urinary components to the filtered urine caused a highly significant increase in the number of crystals formed also support the notion of membrane-associated nucleation of calcium oxalate crystals. The highest and most significant increase was noticed when lipids and particularly phospholipids were added to the urine. The importance of cellular membranes in CaOx crystallization is further emphasized by the results showing a reversal in the crystallization activity of the processed urine on addition of the membranous vesicles.

It is generally acknowledged that human urine contains inhibitors of CaOx crystallization. Promotion of crystallization is, however, not a generally accepted phenomenon. Results of studies described here suggest that promoters of crystallization, particularly of crystal nucleation, are also present in the normal human urine. Lack of evidence for crystallization promotion is perhaps a result of the commonly practiced procedures of filtration and centrifugation which, as we showed here, can remove the substances that may be involved in crystal nucleation. The results also indicate that cellular membranes and their constituents are the likely promoters of crystal nucleation in the urine.

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