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Presence of Lipids in Urinary Stones: Results of Preliminary Studies

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Summary: The presence of lipids in urinary stones was determined by histochemical and biochemical methods. When crystals of calcium oxalate, made by mixing calcium chloride and potassium oxalate solutions and sections of human calcium oxalate urinary stones, were exposed to osmium vapors, there was no staining of the pure crystals whereas the stone sections were stained. De-paraffinized sections of demineralized calcium oxalate stones showed positive sudanophilia on staining with Sudan black B. Both these experiments indicate the presence of lipids in calcium oxalate stones. Lipids were extracted from uric acid, struvite, and calcium oxalate stones using standard techniques. Phospholipids were separated by one-dimensional thin layer chromatography. All the stones studied contained lipids. In calcium oxalate stones they accounted for 10.15% of the matrix. Calcium oxalate and struvite stones contained more phospholipids than uric acid stones. Cardiolipin, sphingomyelin, phosphatidyl choline, phosphatidyl inositol, phosphatidyl ethanolamine, phosphatidyl serine, and phosphatidyl glycerol were identified in lipid extracts. Demineralization by ethylenediaminetetraacetate (EDTA) treatment increased lipid output from calcium oxalate stones by 15.5%.

Key words: Lipids — Phospholipids — Urinary stone — Cellular membrane — Crystal nucleation — Stone matrix.

The presence of lipids has been demonstrated histochemically [1] and biochemically [2–6] at physiological as well as pathological calcification sites. *In vitro,* acidic phospholipids and lipid extracts from various calcified tissues have been shown to initiate calcium phosphate formation from metastable solutions [7–11]. Boyce [12] demonstrated osmiophilic material in stone matrices but interpreted the material as glycoproteins, and Kim and Johnson [13] suggested that the positive staining of urinary stones by osmium was caused by lipids. We are interested in crystal-matrix interaction in urinary stones and have initiated a study of the role of lipids in urolithiasis. The purpose of this study was to histochemically demonstrate and localize the lipids in urinary stones, and to biochemically extract and identify them.

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Materials and Methods

Scanning Electron Microscopic (SEM) Histochemistry

Calcium oxalate urinary stones were sectioned using diamond wafering saw, and the sections were exposed to osmium vapors. Some of the sections were embedded in 1% agar, demineralized by treatment with EDTA, stained with osmium tetroxide, and processed for scanning electron microscopy [14]. In addition, calcium oxalate monohydrate was precipitated from a supersaturated calcium oxalate solution, dried, and exposed to osmium vapors. All these samples were then analyzed by energy dispersive X-ray microanalysis.

Light Microscopic Histochemistry

Calcium oxalate stone sections were embedded in agar and demineralized by treatment with EDTA and glutaraldehyde/paraformaldehyde mixture [14]. Fixed and demineralized samples were impregnated with paraffin and sectioned. These sections were de-paraffinized, hydrated to 70% alcohol, and stained in a saturated solution of Sudan black B in 70% ethanol for 3 hours at 50°C. Some of the de-paraffinized sections were treated with chloroform-methanol mixture to extract the lipids and then

stained with Sudan black B. The sections were examined with or without counter staining with either saffranin or nuclear fast red. Other controls included examining sections with only counterstain or Sudan black B.

Extraction and Identification of Lipids

The stones used in this study were of known crystalline composition and were obtained from Louis C. Herring Lab, Orlando, Florida. All calcium oxalate and struvite stones contained some apatite; from traces to 24% in oxalate stones and 5–35% in struvite stones. Lipids were extracted from these stones by methods based on Folch et al. [15] Wutheir [2], and Boskey et al. [6] and are outlined below. Phospholipid contents of various lipid extracts were determined by the method of Rouser et al. [16]. The phospholipids were separated by one-dimensional thin layer chromatography using Fisher brand Redi/plate silica gel G250 μm plates (Fisher, Orlando, FL.) or HPTLC-GHL silica gel 250 μm plates (Analtech, Neward, DL). Extracted lipids were dissolved in 100% chloroform and spotted onto plates at a concentration of 100 μg of phospholipids. Chromatography chambers were allowed to equilibrate for 30 minutes prior to run, and plates were activated at 160°C for 30 minutes prior to spotting. Various phospholipids were visualized with iodine vapor and identified by comparison with chromatograms of known standards spotted at a concentration of 20–30 μg and run in parallel with the unknowns [17]. Lipid standards were obtained from Sigma Chemicals, St. Louis, MO and Avanti Polar Lipids, Birmingham, AL.

Twelve calcium oxalate, nine struvite, and seven uric acid urinary stones were ground with mortar and pestle after washing with deionized water and were then dried under vacuum to a constant weight. The ground stones were sonicated, 1 min/g of stone, and extracted with chloroform/methanol/water (2:1:1), 30 ml/g of stone. Phase separation was allowed overnight at 22°C. The organic fraction was dried under N_2; complete dryness was accomplished by lyophilization. Dried lipids were dissolved in chloroform and their phospholipids separated by one-dimensional thin layer chromatography using a chloroform/methanol/water (65:25:1) mobile phase.

In the literature on calcified tissues, it has been demonstrated that some phospholipids are closely bound to the calcium and can be extracted only after demineralization [1–4]. Our next step was to determine whether or not this was true for urinary stones as well. In a separate experiment, four calcium oxalate urinary stones were first treated with 0.83% ammonium chloride for 15 minutes and then rinsed with glass-distilled deionized water to wash off any surface contaminants. The stones were dried, ground, sonicated, and extracted overnight with chloroform/methanol/water (2:1:1) at 4°C. The solid phase following extraction was separated. Aqueous layers were re-extracted three times and organic layers were washed three times in one-third volume Tris buffer, pH 7.4. Various organic layers from individual stones were combined and final aqueous layers were discarded. The organic material was dried under N_2 and stored in fresh chloroform at −20°C. The solid residue obtained following initial lipid extraction was demineralized in 0.5 M EDTA, pH 7.4 for 16 hours at 4°C. EDTA insoluble material was dialyzed against glass-distilled deionized water for 24 hours at 4°C to wash off EDTA and then extracted with chloroform/methanol/Tris, and further processed as in earlier extractions. Various phospholipids from individual lipid extracts were separated by one-dimensional thin layer chromatography using chloroform/methanol/glacial acetic acid/water (25:15:4:2) as the mobile phase.

The total lipid content of calcium oxalate stones and phospholipid/total lipid/ stone matrix ratios were determined as follows: Calcium oxalate stones were washed as above, dried, ground, sonicated, and extracted with chloroform/methanol/Tris buffer, pH 7.4 (2:1:1). Solid phase was dialyzed against 0.5 M EDTA, pH 7.4 at 4°C until calcium was negligible in the dialysate. EDTA-insoluble matrix material was dialyzed against glass-distilled deionized water at 4°C. Various organic and aqueous phases were processed and their phospholipids separated and identified on HPTLC silica plates in a solvent system containing chloroform/methanol/glacial acetic acid/water (75:45:3:1). Phospholipid contents was determined, as in previous extractions [16]. To determine the total lipid content of the stone samples, various lipid fractions were dried under vacuum and weighed. The EDTA insoluble residues were also dried and weighed to determine the matrix contents of the stones.

Results

SEM Histochemistry

Calcium oxalate stone sections turned brown on exposure to osmium vapors whereas the calcium oxalate crystals precipitated *in vitro* remained white. Energy-dispersive X-ray microanalysis of the exposed crystals showed no peaks for osmium whereas stones showed osmium peaks, indicating that stone samples reacted with the osmium whereas the *in vitro* precipitated crystals did not. Demineralized stones treated with osmium also showed peaks for osmium indicating that it is the stone matrix that is osmiophilic.

LM Histochemistry

Sections of decalcified stones showed distinct sudanophilia. Intense sudanophilia was seen in the central areas of the stones between ghosts of spherulitic calcium oxalate monohydrate crystals (Fig. 1). The material between dipyramidal calcium oxalate dihydrate crystals was also intensely sudanophilic (Fig. 2). Sudan black B also stained the concentric laminations and material adhering to stone surfaces (Fig. 3). Prior extraction with chloroform/methanol mixture resulted in total absence of sudanophilia.

Lipid Extraction and Identification

All three types of stones, calcium oxalate, struvite, and uric acid contained lipids. The lipid extracts separated into a number of individual phospholipids

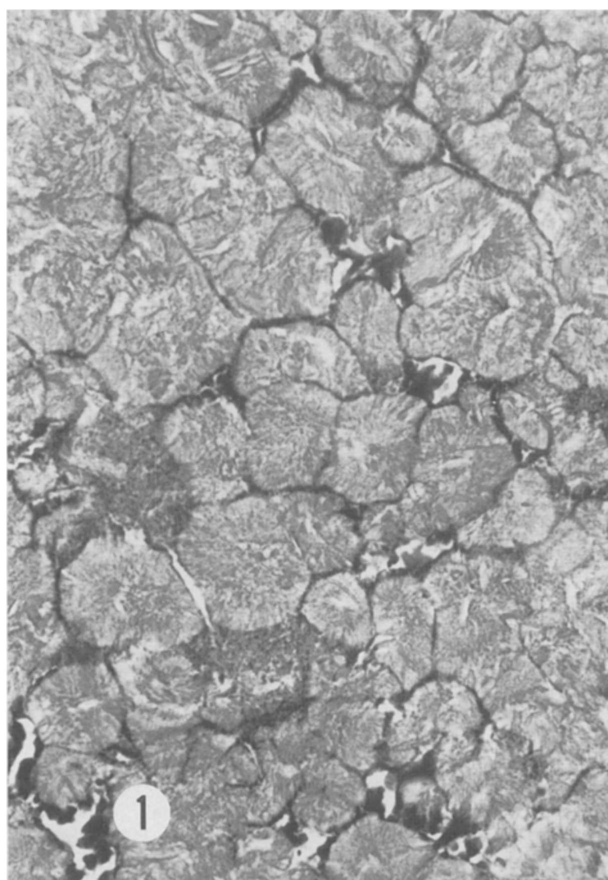

Fig. 1. Sudan black B-stained section of the center of a demineralized calcium oxalate stone. Intercrystalline material present between spherulitic calcium oxalate monohydrate crystal ghosts is intensely sudanophilic. ×130.

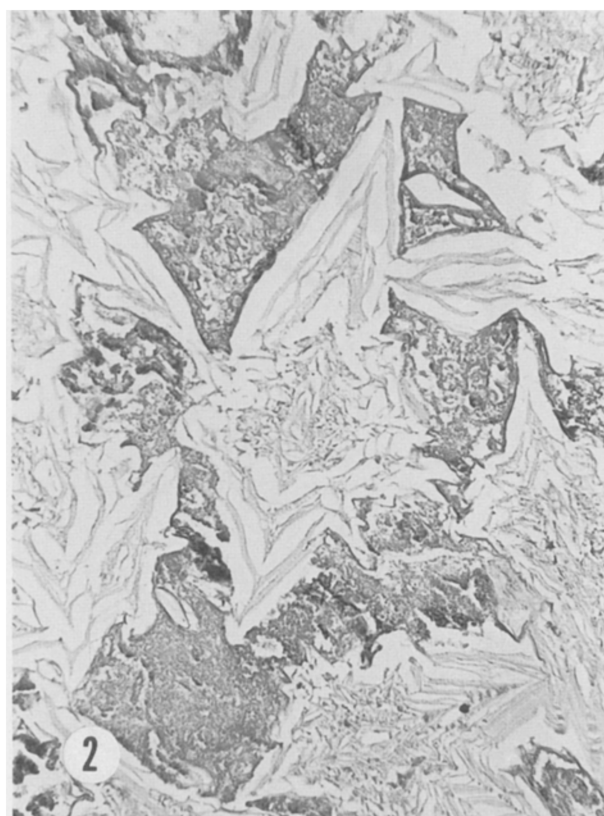

Fig. 2. Sudan black B-stained section of a demineralized calcium oxalate dihydrate stone. Note the dipyramidal ghosts of the crystals and sudanophilia of intercrystalline material. ×52.

(Table 1). All the stones had phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), sphingomyelin (SPM), and cardiolipin (CL). Phosphatidyl serine (PS), and phosphatidyl glycerol (PG) were present in the extracts from some CaOx, struvite, and UA stones but were absent in extracts from others. Free fatty acids were present at the solvent front. In every chromatogram there were one or two spots that remained unidentified. Calcium oxalate and struvite stones, on the average, had more phospholipids than uric acid stones.

In the experiment where lipids were extracted from urinary CaOx stones both before and after demineralization, 15.5% (±6.2) additional phospholipids were obtained after demineralization (Table 2). All the stones had PE, PC, and SPM which were present in extracts before as well as after demineralization. Cardiolipin was also present in all the stones but apparently all of it was released during extraction prior to demineralization. Phosphatidyl inositol (PI) and PS, on the other hand, were mostly released after demineralization. In addition, some of the stones had PG, lyso-PC, lyso-PE, lyso-PI, and lyso-PS.

The quantities and proportions of matrix, total lipids, and phospholipid contents of CaOx stones are given in Table 3. On the average, 4.34% of the stone was EDTA-insoluble organic matrix, total lipids were 0.457% of the stone and 10.25% of the stone matrix; phospholipids were 0.0375% of the stone and 0.84% of the stone matrix. Although phospholipid and total lipid amounts of the stone samples showed great variations, phospholipid to total lipid ratio at mean value of 8.6% appeared very consistent.

Discussion

This study confirms reports of the earlier workers [12, 13] about the presence of osmiophilic material in urinary stone matrix, and for the first time demonstrates that osmiophilia could be caused by the presence of lipids in the urinary stones. Although sudanophilic material has been demonstrated at

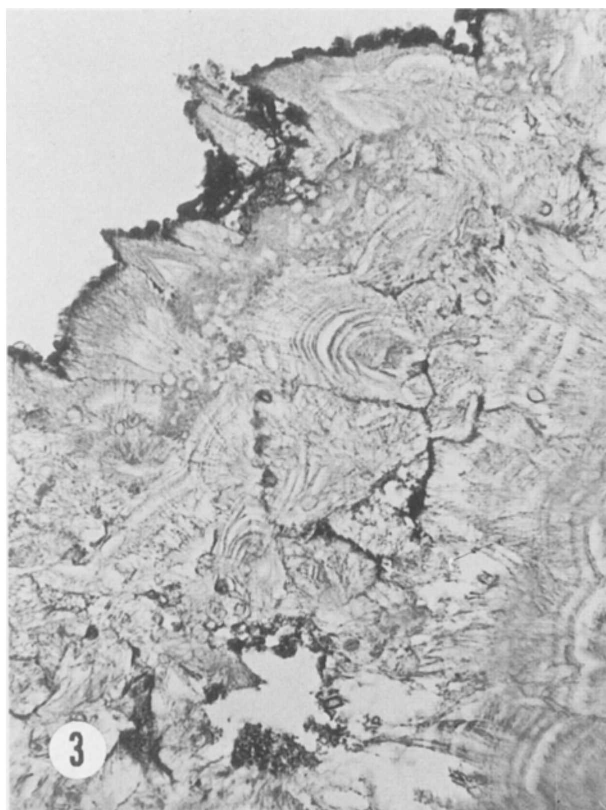

Fig. 3. Sudan black B-stained section of the periphery of a demineralized calcium oxalate stone. Note the intense sudanophilia of the surface-adhering material and some inner laminations. ×130.

various calcification sites [1] its presence in stone matrix is demonstrated here for the first time. Loss of sudanophilia following chloroform/methanol treatment and biochemical identification of various phospholipids in the stone matrix indicates that sudanophilia was caused by the presence of lipids.

Earlier studies have shown that demineralization was necessary for total lipid extraction from mineralized tissues and that some of the phospholipids including PE and PS would only be released after demineralization [1, 2, 18]. Later it was shown that even though demineralization helped in total phospholipid extraction, both PE and PS could be extracted without demineralization if sonication was applied during the extraction [6]. Our results confirm that both demineralization and sonication help the phospholipid extraction. There was on the average an increase of 15.5% in the extraction of phospholipids after demineralization and PE and PS were present in both pre- as well as postdemineralization extracts. In a study of mineralized tissues, Shapiro [18] found that all the cardiolipin of mature bovine bone was present in the predemineralized lipid extract. In this study too, it appears that cardiolipin is the only phospholipid to be totally extractable without demineralization.

Table 1. Phospholipids of urinary stones

Type of stone	Phospholipid content (mg/g of stone)	Type of phospholipids
Calcium oxalate (N = 12)	1.28 ± 1.2	PE, PG, PS, SPM, CL, PC
Struvite (N = 9)	1.305 ± 0.76	PE, PG, PS, SPM, CL, PC
Uric acid (N = 7)	0.124 ± 0.057	PE, PS, SPM, CL, PC

A study of the results of our first experiment (Table 1) shows a very high standard deviation for phospholipid contents, especially in calcium oxalate stones. This deviation is probably due to incomplete removal of surface contaminants because of insufficient washing. Subsequent improvement of our washing procedure gave us more consistent results.

Lipids have been isolated from matrices of all types of mineralized tissues and have been suggested to play an important role in the mineralization process. Of various matrices, lipids make up 7–14% of bone, 2–6% of dentin, and 12–22% of newly mineralized enamel [19]. Thus the percentage of lipid in calcium oxalate stone matrix at 10.25% is comparable to that of the other mineralized systems. Still better comparison can be made with lipids in dental calculi which may be closer to renal calculi in their etiology than any other system. Total lipids are 9.6% of the matrix of submandibular salivary gland calculi [20] and 10.2% of the matrix of supragingival calculi [21]. Phospholipids are 10.2% of the total lipids in supragingival calculi and 3% of submandibular salivary gland calculi. In calcium oxalate urinary stones as determined in this study, the phospholipids account for approximately 8.6% of the total lipid.

Since most of the lipids encountered in urinary stones are those that are present in cellular membranes [22, 23], and cellular degradation products have been observed in human calcium oxalate urinary stones by transmission electron microscopy [24], presence of lipids may represent passive incorporation of sloughed epithelial cells in growing stones. Conversely, membranes and membrane-associated lipids may play an active role in urolithiasis by being involved in the heterogeneous nucleation of crystals as they do in other biomineralization systems [5–11, 19–21, 25–28]. According

Table 2. Phospholipids from CaOx stones before and after demineralization (weight in mg/g of stone)

	Pre-EDTA		Additional post-EDTA		
Stone no.	Weight	Types	Weight	Types	% increase
1	0.788	SPM, PC, CL	0.062	SPM, PC, PE	7.3
2	0.567	SPM, PC, CL, PE, PG	0.106	PE, PS	15.8
3	0.458	SPM, PC, CL, PE, lyso-PE, PS, lyso-PC	0.09	SPM, PC, PI, PS, PE, lyso-PI	16.4
4	0.351	SPM, PC, CL, PE, PS, PG, lyso-PI	0.102	SPM, PC, PI, PS, lyso-PE	22.5
Mean ± S.D.	0.417 ± 0.187		0.09 ± 0.02		15.5 ± 6.2

Table 3. Total lipids (TL), phospholipids (PL), and matrix of CaOx stones

Sample no.	Matrix[a]	TL[a]	PL[a]	PL:TL	PL:Matrix	TL:Matrix
0520	47.3	7.26	0.517	7.13	1.09	15.33
0527[b]	45.4	3.81	0.376	9.87	0.80	8.38
0605	37.5	2.64	0.232	8.79	0.62	7.04
Mean ± S.D.	43.4 ± 5.2	4.57 ± 2.40	0.375 ± 0.142	8.60 ± 1.38	0.84 ± 0.24	10.25 ± 4.45

[a] mg/g of stone
[b] Fragments from five stones

to current concepts, initial calcium phosphate deposition in both physiological and pathological calcification occurs on cellular membranes. One of the suggested reasons for this is the presence of proteo- and phospholipids in the cell membranes and their affinity for calcium. How does the affinity between membrane lipids and calcium result in the initiation of calcification? It is hypothesized that initially a calcium-phospholipid-phosphate complex is formed. Such complexes have been isolated from various normal and pathological calcification sites and both extracted and synthetic calcium-phospholipid-phosphate complexes induce *in vitro* apatite nucleation from metastable calcium phosphate solutions. In addition, it has been shown that all pathological and normal calcified tissues studied so far contain a calcifiable proteolipid. It has recently been shown that in *Bacterionema matruchotii*, acidic phospholipids involved in the formation of calcium-phospholipid-phosphate complexes are intimately associated with proteolipids [29]. These and other observations clearly implicate the membrane-associated proteolipids and phospholipids in nucleation of calcium phosphate crystals in various biomineralizations systems. Nucleation of calcium phosphate crystals in urine may similarly be catalyzed by cellular membranes and their associated lipids. What about the other types of crystals such as calcium oxalate, uric acid, and struvite which are common constituents of a number of urinary stones? There are a number of possibilities: (1) Similar to calcium phosphate, crystallization of all other urinary crystals is also aided by the cellular membranes and membranous lipids; (2) lipids and membranes play a direct role in the formation of only some of the urinary crystals; (3) calcium phosphate is formed first which then induces the nucleation of other crystals [30, 31]. All these possibilities are being currently investigated. Results of these studies should provide us with the insight into the role of membranes and lipids in the formation of urinary crystals.

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