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Experimental Induction of Crystalluria in Rats Using Mini-Osmotic Pumps

Saeed R. Khan, Birdwell Finlayson and Raymond L. Hackett

Department of Pathology and Department of Surgery/Urology, College of Medicine, University of Florida, Gainesville, Florida, USA

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Summary. Crystalluria was induced in male Sprague-Dawley rats by implanting potassium-oxalate-containing mini-osmotic pumps. Urine of all experimental animals had abundant crystals of calcium oxalate, hydroxypatite, struvite, and calcium phosphate. These crystals were similar in morphology to the crystals found in human crystalluria. Histological examination of kidneys and tissue samples from other organs revealed no abnormality. One group of animals was injected with sodium oxalate in addition to implantation of potassium-oxalate-containing mini-osmotic pumps. Bladder urine from these animals contained calcium oxalate microstones and their kidneys had deposits of calcium oxalate crystals.

Key words: Calcium oxalate, Crystalluria, Urinary crystal morphology, Mini-osmotic pumps.

Introduction

Crystalluria is usually present in the urine of stone-formers, and is indicative of previous urinary supersaturation with stone-forming salts. Although stone forming patients cannot be identified on the basis of crystalluria alone, both qualitative [3, 11, 15–17] and quantitative [21, 22] differences exist in the crystalluria of stone-formers and normal controls. Moreover, effective stone therapy has been shown to decrease crystalluria as well as to stop stone growth. Intuitively, the formation of crystals is essential for stone formation, and in our view, any precipitate occurring in the urinary system is urolithiasis [7]. Accordingly, crystalluria is urolithiasis. We view urinary stone disease as urolithiasis with subsequent retention of precipitates resulting in macropathology. With this in mind, we explored the development of a relatively non-toxic animal model for calcium oxalate (CaOx) crystalluria.

Calcium oxalate crystal formation in the urinary tract can be induced in various ways including manipulation of the diet, administration of a toxic lithogen by injection, or by its addition to the drinking water. Because of variations in the amount of water and food consumed, and in the rate at which some lithogens are metabolized, feeding and drinking of a lithogen can give inconsistent results. Methods utilizing single injections of lithogen have the serious defect that they produce sudden, acute, overloading surges causing high supersaturation of urinary salts and only transient crystalluria. In an attempt to meet some of these objections, we implanted mini-osmotic pumps (Alza Corporation, California) loaded with a saturated solution of potassium oxalate (KOx). These pumps, when implanted in the animal's body, deliver their contents at a specific rate for a given period of time.

Materials and Methods

Mini-Osmotic Pumps. To study and induce crystalluria, mini-osmotic pumps, model #2002, were used; these have a mean fill volume of 225 μl and an average pumping rate of 0.5 μl/h. To test their efficiency, mini pumps were loaded with ^{14}C labelled KOx and subcutaneously implanted in the interscapular region of male Sprague-Dawley rats. The amount of ^{14}C was measured in 24 h urine collections over a period of 17 days. The scintillation counts were highest on the first day after implantation, stayed elevated for 12 to 14 days, and declined rapidly thereafter (unpublished results).

Animals and Surgical Techniques. The experimental animals were male Sprague-Dawley rats weighing 150–200 g which had been acclimatized for at least a week. They were housed in animal quarters, fed rat chow, and allowed to drink water ad libitum.

Mini-osmotic pumps, loaded with a solution of 1.2 M KOx, were implanted intraperitoneally. The implantations were done under ether anesthesia with sterile techniques. Skin clips were used to close the incisions. Our calculations indicated that one pump would not deliver sufficient oxalate fast enough; therefore, four pumps were implanted simultaneously. In order to maintain crystalluria for several weeks, the discharged pumps were replaced with fully-loaded new pumps every 10 to 12 days.

At the end of each experiment all animals were autopsied. Urine was aspirated from their bladders for scanning electron microscopy (SEM), pH measurement, and bacterial culture. Tissue samples taken

Table 1. Experimental protocol

Groups	No of Animals	Experiment Day														
		0	4	7	12	14	19	21	23	25	27	29	32	36	40	43
#1 Pumps with saline	3	4 Pumps implanted		1st Urine collection	1st Pump replacement		2nd Urine collection			2nd Pump replacement			3rd Urine collection		Autopsy	
#2 Pumps with saturated KOx followed soon after by NaOx injection	3	Na-oxalate injected				1st Pump replacement		2nd Urine collection			2nd Pump replacement			3rd Urine collection		Autopsy
#3 Pumps with saturated KOx	3								2nd Urine collection			2nd Pump replacement				

from kidneys, heart, lung, liver, spleen, ureters, urinary bladder, and peritoneal soft tissues were fixed in alcoholic formalin and processed for light microscopy (LM). The pumps were recovered and examined by SEM for crystal deposition and identification.

Control rats were implanted with pumps containing either 0.9% saline or 1.2 M potassium chloride solution. For every group of experimental rats, a group of control rats with the same average weight was purchased simultaneously from the same source, and housed under identical conditions.

Urine Collection and Analysis. Urine collections were done in metabolic cages. Freshly-voided three hour urine samples were used for counting crystals and to study crystal morphology. Urine was filtered through 0.2 μm nucleopore or millipore filters, and these were coated with gold-palladium or left uncoated, and examined by SEM equipped with an energy dispersive x-ray microanalyser (EDX). The EDX results were confirmed by x-ray diffraction when enough crystals were present. For LM, crystals were studied using both polarizing and bright field optics.

Experimental Design. The main object of our experiments was to induce crystalluria in rats without crystal deposition in the kidneys or other organs and to study the nature and morphology of the various crystals that formed. We also wanted to study the effects of an oxalate surge upon the size and structure of crystalline deposits in the urine. Such an oxalate surge was obtained by intraperitoneally injecting 0.22 M solution of sodium oxalate (NaOx) in 0.9% saline at the rate of 7 mg/100 g rat body weight. The temporal sequence of the experimental protocol is detailed in Table 1.

Results

The urine of all experimental animals had abundant crystals of calcium oxalate (CaOx), hydroxyapatite (HAP), or struvite type mixed with amorphous calcium phosphate (CaP) and an amorphous viscid material which was mostly CaP (Table 2). The urine had a number of casts, some of which appeared cylindrical. No bacteria were found by culture in the aspirated bladder urine. The pH of collected urine averaged 7.5.

Calcium oxalate crystals were present as monohydrates and dihydrates. Calcium oxalate monohydrate crystals were generally in the form of biconcave ovals ranging in size between 3–13 μm, with 6–8 μm size being more common. Such crystals appeared dumbbell shaped (Fig. 1) in side view and as rosettes (Fig. 2) when viewed enface. Interpenetrant biconcave oval twins were also present. The oval crystals were composed of smaller crystallites. Calcium oxalate dihydrate crystals were generally dipyramidal

Table 2. Urinary crystal morphology

Group #	1st Urine Collection	2nd & 3rd Urine Collection	Bladder Urine
1	Few calcium phosphate (CaP) and struvite crystals	Similar to first collection	Similar to first collection
2	Considerable amorphous material covering the crystals: small CaOx mono- as well as dihydrate crystals along with their aggregates; some larger rosettes of monoclinic CaOx monohydrate crystals; amorphous and spherulitic CaP and large struvite crystals also common	Crystals well dispersed with less amorphous substance; CaOx dihydrate crystals with bipyramidal shape most common; CaOx monohydrate as small oval biconcave discs, few rosettes of monoclinic crystals also present; few small interpenetrant twins of CaOx dihydrate along with CaP and struvite also present	Large deposits of CaOx monohydrate and dihydrate crystals; spherulitic as well as amorphous CaP also present
3	Well dispersed crystals; CaOx mono- and dihydrate crystals generally small; monohydrate as biconcave ovals and dihydrates as bipyramids; both also present as interpenetrant twins; aggregates of small crystals of CaOx also present; some CaOx crystals appear attached to urinary casts; CaP present as spherulitic units or amorphous forms; struvites also common	Similar to first collection	Similar to first collection but with lesser CaP and struvite crystals

in shape with some being dodecahedral in habit. Most of these crystals showed 011 faces only and had smooth surfaces. Rarely the crystals also showed 100 faces. These crystals ranged in size between 2–25 μm, most being 8–12 μm. Single as well as multiple interpenetrating twins (Fig. 6) of dipyramidal CaOx dihydrate crystals were also common. Some of these twins were up to 50 μm in their largest dimensions. Both types of CaOx crystals were seen attached to urinary casts (Fig. 3). Aggregates of CaOx monohydrate (Fig. 4) and/or dihydrate crystals were also seen in which individual crystals appeared to be attached to each other by some organic substance (Fig. 4).

The typical habit of CaP was spherulitic (Fig. 5), similar to the known habit of HAP [22]. Most of the amorphous coating on the filter paper was also identified by EDX as CaP.

A large number of crystals in the urine had pentahedral (Fig. 10) or rhombohedral habits and appeared similar in morphology to struvite crystals [5]. These crystals were approximately 100–175 μm in their largest dimensions with some of them up to 250 μm. EDX analysis of a number of these crystals demonstrated the presence of potassium together with magnesium and phosphorus. X-ray diffraction identified them as magnesium ammonium phosphate hexahydrate (MAPH) and/or magnesium potassium phosphate hexahydrate (MKPH). Long exposure to the electron beam resulted in cracking of their surfaces as described by Hesse et al. [11].

The crystals from three hour urine collections, and from urine aspirated from the bladder of the animals at the time of autopsy, were similar in nature and appearance (Table 2) except from the group of animals that received an oxalate surge in the form of intraperitoneal injection of NaOx. The first urine collected from NaOx-injected animals had more amorphous substance coating the urinary crystals and the filter paper, rosettes of plate-like CaOx monohydrate crystallites, and aggregates of small CaOx crystals in addition to other crystal types. Bladder urine from this group had large deposits of CaOx crystals (Figs. 7, 8) up to 175 μm in the largest dimensions. Most of the deposits had both CaOx monohydrate and dihydrate crystals showing intergrowth and twinning, as well as aggregation. Calcium oxalate monohydrate crystals in such deposits appeared as ellipsoidal plates with smooth broad faces. Their narrow faces appeared etched and showed 295A thick subunits (Fig. 9). The surfaces of CaOx dihydrate crystals were also rough (Fig. 7), which could either be the result of etching or represent a growth phenomenon.

Histological examination of the kidneys and other organs from experimental animals revealed no abnormality except for the animals that received NaOx injections. In these animals, scattered CaOx crystals were present in some tubules, mainly the loops of Henle; the renal papilla often appeared jagged showing apical or lateral deposition of CaOx crystals.

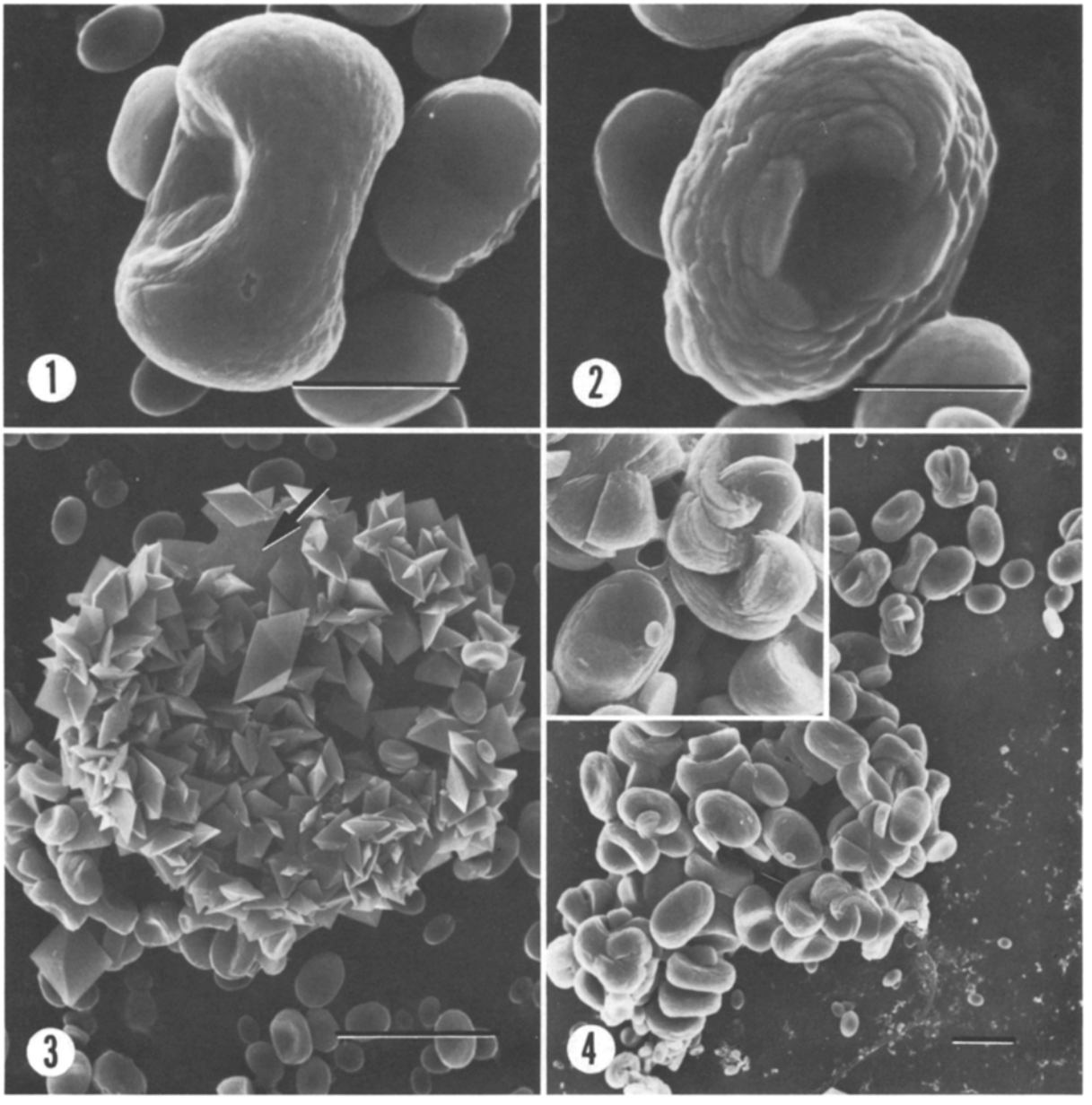

Fig. 1. Side view of a CaOx monohydrate crystal from first urine collection. KOx only. Bar = 5 μm

Fig. 2. Crystal similar to the shown in Fig. 1, viewed enface. Bar = 5 μm

Fig. 3. Calcium oxalate crystals attached to a urinary cast (*arrow*). First urine collection. KOx only. Bar = 10 μm

Fig. 4. An aggregate of CaOx monohydrate crystals. Individual crystals are attached to each other by an organic substance *(inset)*. First urine collection. KOx only. Bar = 10 μm

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Fig. 5. Calcium phosphate. Bladder urine. KOx followed by NaOx injection. Bar = 5 μm ▶

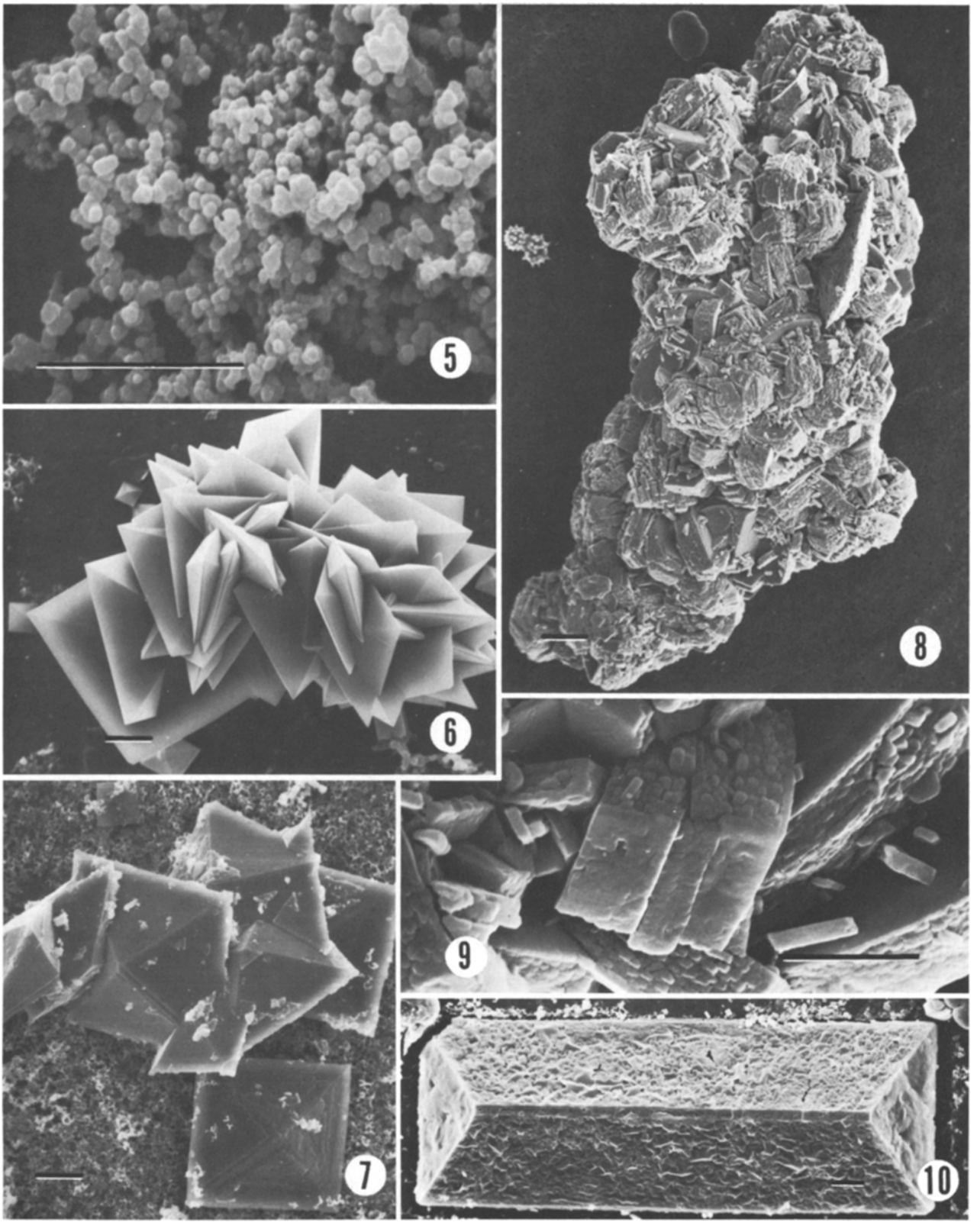
Fig. 6. Multiple interpenetrating twin of dipyramidal CaOx dihydrate. First urine collection. KOx only. Bar = 5 μm

Fig. 7. Calcium oxalate dihydrate crystals from bladder urine. KOx followed by NaOx injection. Bar = 10 μm

Fig. 8. Calcium oxalate crystalline deposit from bladder urine. KOx followed by NaOx injection. Bar = 10 μm

Fig. 9. Enlargement of an area in Fig. 8. Individual crystals show subunits. Bar = 5 μm

Fig. 10. A struvite crystal from second urine collection. KOx only. Bar = 10 μm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Calcium oxalate crystal deposition and calcification were present in the peritoneum adjacent to the pumps. Examination of mini-osmotic pumps extracted from the experimental animals at the time of autopsy revealed deposition of CaOx monohydrate crystals around the heads of the pumps.

The urine of control animals contained HAP, amorphous CaP, and struvite-type crystals. An amorphous viscid material was also present on the crystals and the filter. Occasionally a few small CaOx dihydrate crystals between 2–5 μm in size were also present. The urine was free of bacteria. Kidneys and other organs of control animals appeared normal on histological examination. Some calcification was evident in the peritoneum and at the heads of mini-osmotic pumps.

Discussion

Urinary stone disease is multifactorial, but regardless of the underlying reasons, it is the result of a supersaturation of urine with certain urinary salts and subsequent nucleation of their crystals [7]. Once crystals nucleate, further mass accretion is by crystal growth or aggregation or both. As long as these accreting units stay small and are excreted, the result is crystalluria, but when they are retained and become large enough to obstruct urinary passages, the result is urinary stone disease. Although crystallization of urinary salts is not diagnostic for stone disease, it is essential for the induction of urolithiasis. Crystalluria is as frequent in stone-formers as in non-stone-formers. In a study of 122 hospital patients of whom 94% had no evidence of past stone disease, Elliot and Robinowitz [6] found crystalluria in about one of three voidings. In an earlier study involving male hypercalciuric stone-formers and normal controls, Dyer and Nordin [4] could not demonstrate a clear difference in crystalluria between these two groups. However in recent years, a number of studies have documented differences between crystalluria of urinary stone disease and normal subjects. Robertson et al. [16, 17] demonstrated that urine of recurrent CaOx stone-formers has larger crystals and crystal aggregates than the urine of normal subjects. Robertson and Nordin [15] showed that controls or normal subjects pass only single uniformly small CaOx crystals, while patients with recurrent CaOx stone disease also pass some larger crystals of 20–50 μm diameter as well. These crystals were often fused into polycrystalline aggregates up to 200–300 μm diameter. Such large crystals and crystal aggregates were never observed in freshly voided urine of normal subjects. In a study of normal subjects and stone-formers with idiopathic hypercalciuria, Hallson and Rose [11] reported crystalluria as more common in stone-formers than in normal subjects with almost complete absence of CaOx crystal aggregation in the latter group. Werness et al. [21, 22] studied 4,835 voidings from 162 patients with primary hyperparathyroidism (with and without stones), primary hyperoxaluria, and idiopathic calcium urolithiasis, as well as normal subjects. Although all groups had crystalluria, patients with stone disease had a greater number of crystals in their urine than normal subjects. The authors also showed that the therapy which effectively controls stone disease also decreases crystalluria. Crystal poisons which inhibit CaOx crystal growth and aggregation in vitro [18], also increase the inhibitory activity of urine and reduce the average size of CaOx crystals [19]. It is clear then that although crystalluria can occur in the absence of stone disease, both qualitative and quantitative differences exist between the crystalluria of patients with and without stone disease.

The results of our experiments described above clearly demonstrate that implantation of mini-osmotic pumps filled with solution of KOx induces a sustained crystalluria in rats without pathological changes in the kidneys and other tissues. Deposits of CaOx crystals in renal tissue were seen only in rats which were injected intraperitoneally with NaOx in addition to implantation of mini-osmotic pumps. This probably resulted from the acute overloading surge of oxalate with increased supersaturation of CaOx in renal tubular fluids [13]. The crystals induced in rat urines were generally similar in nature and habit to the crystals found in human crystalluria [1, 2, 5, 6, 21, 22], except for the presence of struvite crystals which are uncommon in sterile human urine. The large number of struvite crystals in these experiments is probably due to the high urinary pH [15]. The reasons for higher urinary pH are unclear. Although urinary cultures were negative, we are not convinced of the total absence of urease-splitting microbes from the collected urine. The presence of both magnesium ammonium phosphate hexahydrate and magnesium potassium phosphate hexahydrate crystals and the inability to differentiate between the two morphologically, or by x-ray diffraction, adds to the uncertainty.

The presence of large CaOx crystals and microstones in bladder urine of animals that received a NaOx injection in addition to KOx in mini-osmotic pumps can be attributed to an increase in CaOx supersaturation within a very short time since the more saturated a solution, the faster the rate of crystal growth and the larger the crystal size [15]. It is likely that a rapid change in urinary supersaturation of CaOx resulted in an imbalance between precipitability and inhibitory activity [15] causing rapid crystal growth. This is similar to Vermeulen's "triggering period" [20] of stone formation. Robertson and Peacock [14] have shown that the addition of oxalate to the diet of human stone-formers resulted in an increase in aggregation and size of CaOx crystals, and that lower inhibitory activity in stone-formers' urine was responsible for this occurrence.

This model was developed with the aim of producing a relatively non-toxic, experimental, sustained crystalluria. We have shown that by implanting KOx-containing mini-osmotic pumps, a persistent crystalluria can be induced for up to 6 weeks. It is assumed that oxalate appears in the urinary system at a uniform rate maintaining increased levels of urinary oxalate, although this must be confirmed

by determining urinary oxalate levels. Because a correlation has been shown to exist between crystalluria and urinary supersaturation in humans [17, 22], we are in the process of determining supersaturations of various urinary salts and studying their correlation with crystal numbers. In addition, this technique can be used as a test system for various stone therapies, study of inhibitors and crystal poisons, as well as for testing various hypotheses concerning stone disease. For example, we intend to apply the method in studying the role of urothelial injury on the development of stone disease [9, 10] in the presence of sustained crystalluria. Additionally we plan to utilize this method in studying the process of stone growth on foreign bodies implanted in urinary bladders. In our opinion this model provides a number of advantages over other rat models of urolithiasis and offers a novel means to experimentally study urinary stone formation.

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Prof. Dr. R. L. Hackett
Department of Pathology
University of Florida
Gainesville, FL 32610
USA