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ETIOLOGY OF CALCIUM OXALATE NEPHROLITHIASIS IN RATS. II. THE ROLE OF THE PAPILLA IN STONE FORMATION

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Abstract

In kidneys of healthy rats submitted to a crystal-inducing diet (CID) with ethylene glycol (EG) and NH_4Cl, the fate of retained crystals in the papillar region is studied during a recovery period of one, five or ten days, as model system for human nephrolithiasis. Scanning electron microscopy (SEM) shows, at papillary tips bulging into the calycine space, crystal masses covered either by the epithelium or a thin fibrous veil, or by unidentified mobile cuboidal cells. After CID plus one or five days recovery, small sub-epithelial swellings are seen of large sub-epithelial crystals at or around the papillary tip. After CID plus ten days, massive sub-surface crystal-containing micrometer-sized stones are seen in which the presence of calcium is confirmed by X-ray microanalysis. The papillary tip of rats after a re-challenge with an oxalate load from 0.1 vol% EG for twelve or forty-two days shows minor lesions. But a re-challenge with 0.3 vol% EG for thirty-seven days induces large sub-epithelial papillary millimeter-sized stones. The Von Kossa section staining converts the crystals into a black precipitate, but large peri-tubular or peri-vascular calcium deposits are absent. A new hypothesis about the etiology of an inductive calcium oxalate monohydrate nephrolithiasis is formulated which differs from the one proposed by Randall based on his deductive human kidney studies.

Key Words: Nephrolithiasis, calcium oxalate monohydrate, rat model system, transmission electron microscopy, scanning electron microscopy.

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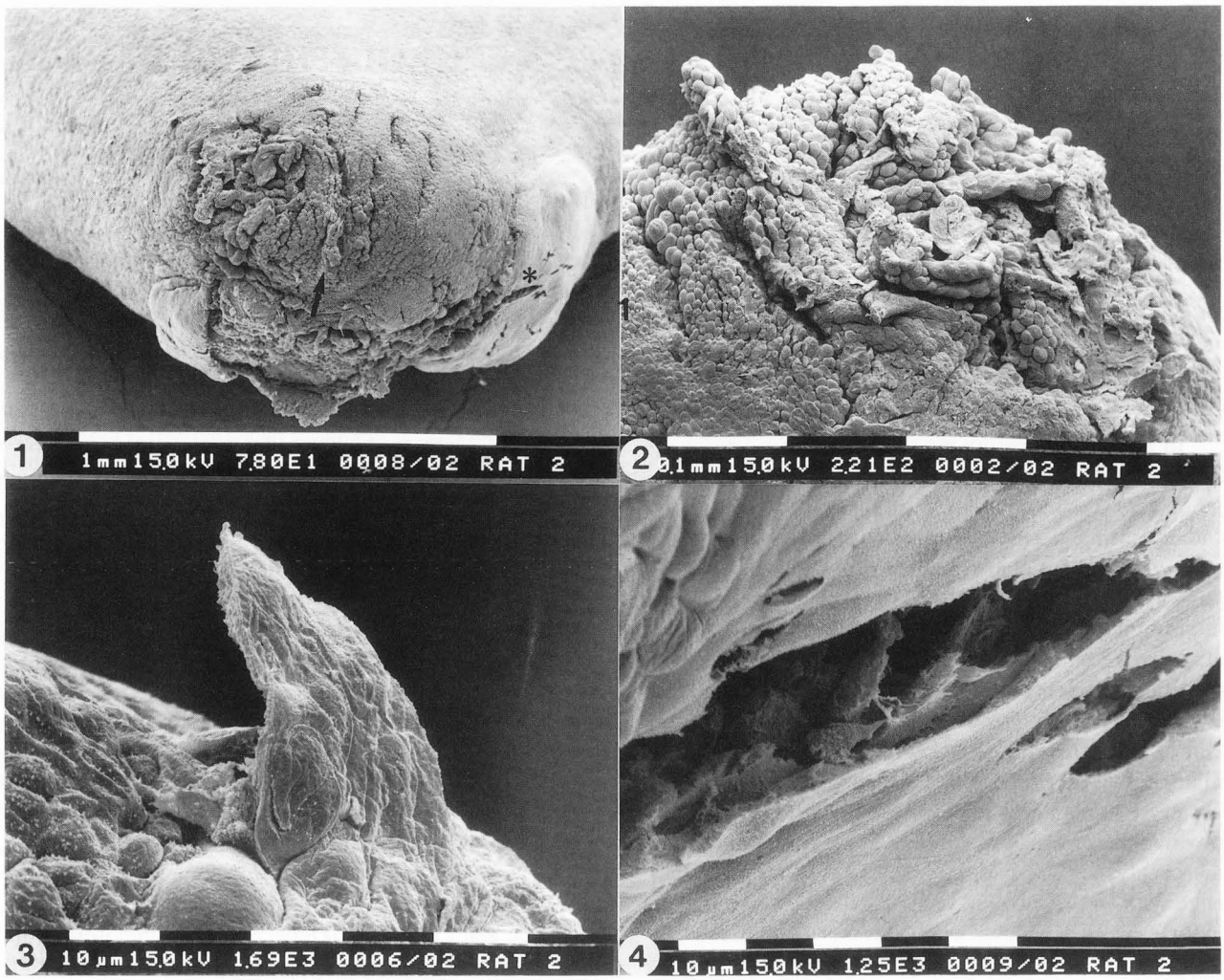
Introduction

The search for human kidney-stone etiology, which has occupied many investigators for years, is spinning around Randall's deductive observations and definitions, summarized in 1940 [18] about an initiating or pre-calculus lesion and nephrocalcinosis. His description of such initiating lesions in the outer surface of the human papilla is confirmed later by others [1, 17, 19, 20, 21] and is since named "Randall's plaques". Rosenow [19] has described the presence of papillar peri-tubular or peri-vascular calcium deposits, some located in the sub-epithelial region. Vermooten [22] has emphasized the localization of calcium in fibrillar material. Laminated electron dense calcium (phosphate?) containing spherules have been identified ultrastructurally in the interstitium, in thickened tubular basement membranes, and between collagen fibres by Haggitt and Pitcock [13], whereas, Öhman and Larsson [16] have claimed white ultra-violet (UV) fluorescing fibrous material, named lithofibrin, to form the funiculus of the stone to the Randall's plaque on the basis of a single calcium peak from the stalk's periphery detected by scanning electron microscopy (SEM) and X-ray microanalysis. Other authors, investigating removed stones by SEM, demonstrated, in some, the presence of the Randall's plaque material attached to stone material of a different composition [5 and the literature cited therein, 6, 12, 14]. The complex chemical nature of stones is demonstrated among others by Blijenberg *et al.* [2]. Recently, new aspects have been disclosed about their mechanism of formation [12].

Randall's rather clinical definition [18] of idiopathic calculi, "for which no apparent causal factor in the kidney, pelvis or ureter can be recognized", excludes hyper-excretion of chemical compounds involved (e.g., calcium, oxalate, urate and phosphates) as an etiological factor. Also, alkalinization of the urine by bacterial infections was excluded as an etiological source for this type of stone formation. This aspect might be criticized from the point of view that the described peri-tubular or peri-vascular calcium deposits lead to plaques when located at the sub-epithelial border and can be considered



Figures 1 to 4. Scanning electron micrographs of rat papillary tip after CID plus one day recovery. **Figure 1.** Sub-epithelial material below the tip is seen as flat elevations with large cracks (*). Bar = 1 mm. **Figure 2.** Papillary tip area, with open ends of the ducts of Bellini, no crystals present. Bar = 0.1 mm. **Figure 3.** Sub-surface material (?) completely covered by epithelial cells, standing up from the papillary surface. Bar = 10 μm. **Figure 4.** Inside a crack (Figure 1*), no sub-epithelial crystals are seen. Bar = 10 μm.

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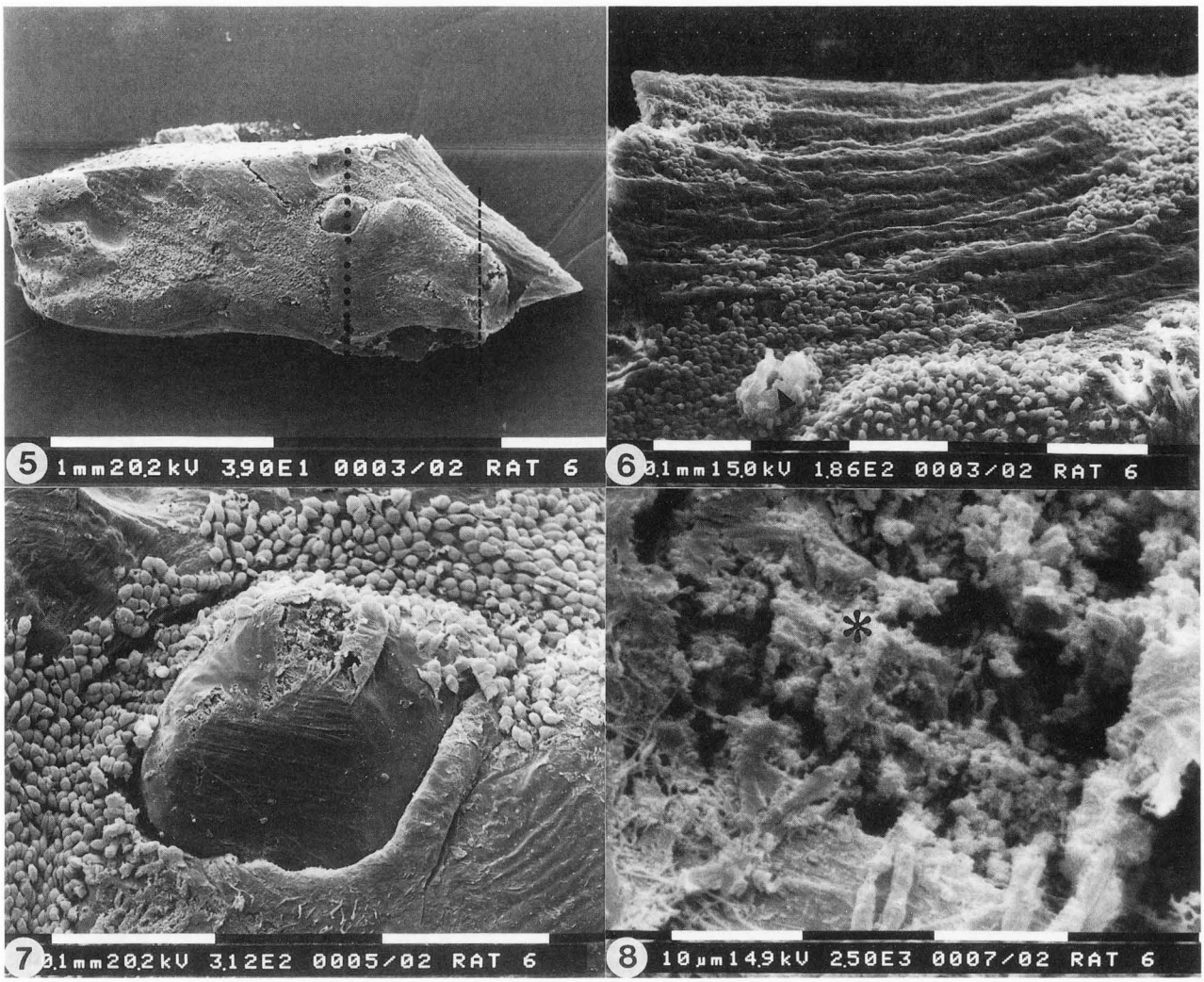
signs from abnormal excretion in the tissue. However, in calcium oxalate stone formers, both normo- and hyper- calci- and oxaluria are present. So the question might be raised whether etiologically different pathways of stone formation are present.

Previously, we have described an inductive rat-model system for calcium oxalate monohydrate (COM) nephrolithiasis [3, 7, 9, 10]. We have observed tubular epithelial cells to overgrow crystals retained by the apex of the lining cells. Following new basement membrane formation, this process leads to crystal exotubulosis [2, 7, 9, 10]. As a result, numerous crystals are observed by light (LM) and transmission electron microscopy (TEM) in the interstitium along the whole nephron including the papillar region. Moreover, we have observed crystals: (1) free in the calycine space, (2) at the papillary surface surrounded by cuboidal cells, and (3) in sub-epithelial spaces in the interstitium [10].

In the present study, kidneys from animals similarly treated as before [9] with and without post-fixation with osmium tetroxide with ferrocyanide [8] are investigated by SEM and X-ray microanalysis in combination with LM observations from the same specimen following reembedding in epon.

The aim of this investigation is to establish: (**1**) a possible relation between the interstitial crystals observed before [9, 10] and the presence of previously described Randall's plaque material [18]; (**2**) possible



Figures 5 to 8. Scanning electron micrographs of rat papillary tip after CID plus five days recovery. **Figure 5.** A large elevation is present above the papillary tip. Dotted lines represent the position of Figures 19 and 20 (shown later). Bar = 1 mm. **Figure 6.** Original papillary surface structure showing sub-surface collecting ducts. In the left-hand corner a surface perforation is seen (▲). The area is covered by mobile cells. Bar = 0.1 mm. **Figure 7.** Detail of the dome-shaped area where the original covering is broken. The area is covered by mobile cells with pseudopods. Bar = 0.1 mm. **Figure 8.** No crystals are seen in the interior of that dome, note the fibres in and at the surface. (*) X-ray microanalysis site shown in Figure 9. Bar = 10 mm.

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mechanisms which allow such sub-epithelial crystals to grow into large crystalline masses or stones; and (3) whether bare crystals/mini-stones are present on the papillary surface to give a clue to the etiology of primary papillary calculus formation.

Materials and Methods

Male Wistar rats (weighing 250 g) from the Erasmus Animal Science Centre were acclimatized and kept on a normal rat chow. At the start of the experiment, their drinking water was supplemented with 0.8 vol.% ethylene glycol (EG) plus 1 wt.% NH_4Cl for nine days. Control rats were sacrificed at the end of that induction period to confirm the presence of the intra-tubular crystals as described before [3, 7]. After this crystal induction period, all rats were allowed a crystalluria/recovery phase (called "post-induction recovery phase" in this paper) on fresh drinking water for one, five and ten days respectively; this regime allows renal cells to recover from the oxalate and ammonium chloride challenge.

After a three day post-induction recovery phase, rats were given an additional oxalate challenge by re-supplementing the drinking water with 0.1 vol% EG

without NH_4Cl for twelve or forty-two days respectively. Another rat was given an increased oxalate re-challenge with solely 0.3 vol% EG for thirty-seven days. In all cases, three days of normal drinking water was included in the regime at the end of that challenge.

All rat kidneys were fixed by perfusion with glutaraldehyde in cacodylate/HCl buffer as described before [3]. All removed kidneys were examined by X-ray analysis *in toto* for possible crystal/stone locations.

For SEM, papillae were excised macroscopically. Some remained only aldehyde-fixed; while others were postfixed with osmium tetroxide plus ferrocyanide [8], dehydrated in ethanol series up to 100% and critical-point dried (Balzers CPD 030K, Liechtenstein), carbon-, and later gold-coated for observation in a Philips SE 5103 SEM operating at accelerating voltages of 15 or 20 kV (Philips, Einhoven, The Netherlands). The SEM is equipped with a Tracor-Northern X-ray microanalyzer. After SEM analysis, the dry specimens were wetted in epoxy propane and embedded in epon and reinvestigated by LM observations. Toluidine Blue-stained LM-sections were examined with a Zeiss Axiophote light microscope (Zeiss, Oberkochen, Germany) and routinely recorded in an analogue way on film [3]. The results are related to previously reported ultrastructural TEM observations [9, 10].

Results

Scanning electron microscopy (SEM)

A rat papilla after one day post-induction recovery is shown in Figures 1 to 4. In Figure 1, the tip area is shown with some open ends of the ducts of Bellini; other ducts are damaged by local swellings (↓). At some distance from the very tip, dome-shaped elevations (*) are seen. In Figure 2, at the tip, no crystals are seen attached to or in the lumen of the Bellini ducts. Irregularly shaped cells are dominating this area forming small irregularly-shaped protrusions. In Figure 3, about 20 μm from the tip, sub-epithelial material is seen standing up from the cell-covered papillary surface. In Figure 4, the large surface elevation marked in Figure 1 (*), is shown to be covered by epithelial cells, but at places the surface shows small cracks. Inside cracks, sub-epithelial cells but no crystals are seen.

Figures 5 to 8 show a rat papilla after crystal-inducing diet (CID) plus five days post-induction recovery. In Figure 5, a large elevation is present about 30 μm above the patent-looking papillary tip. The broken and dotted line indicate the places where Figures 19 and 20 (shown later) are acquired. In Figure 6, details of the original surface of the papilla are shown, possibly representing sub-surface collecting ducts. The area is covered by a population of apparently mobile single cells.

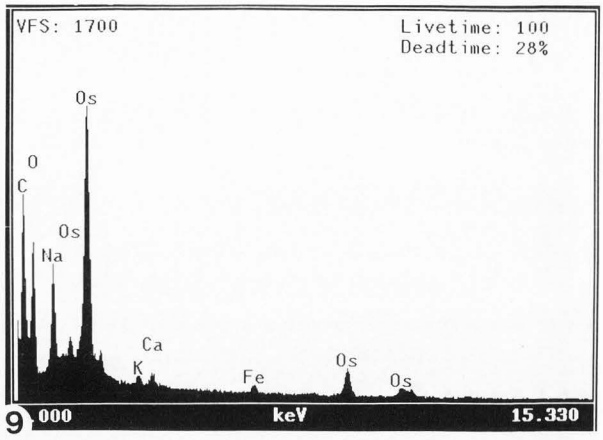
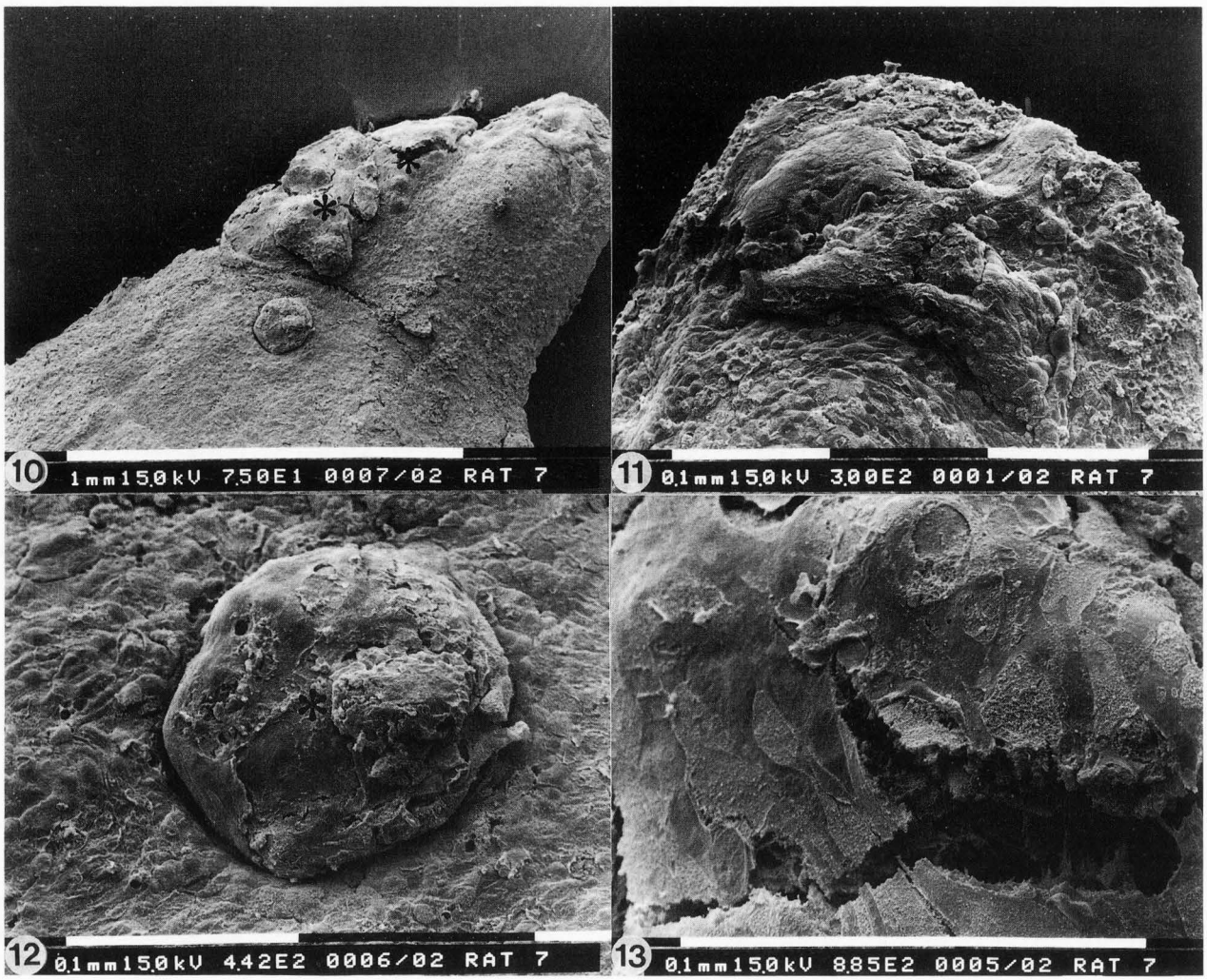


Figure 9. X-ray microanalysis of the marked area (*) in Figure 8 shows very little calcium.

In the left-hand corner, a small, cell-covered surface perforation is seen, which appears to be empty (▲), as confirmed by later TEM examination after re-embedding the specimen. In Figure 7, a dome-shaped area is seen, apparently breaking through the original surface, but still covered by a thin veil. The area is crowded by a cell population with clear pseudopodia. At the top, where the original covering is broken, Figure 8 shows the material underneath. No crystals are recognized in the interior. Fibres are seen in and at the surface. The energy dispersive X-ray (EDX) spectrum (Fig. 9) acquired at this site shows some calcium, the presence of some phosphorus and sulphur (unmarked), and some sodium, plus osmium and iron from the post-fixation procedure.

The ten days post-induction recovery phase papillar changes are shown in Figures 10 to 13. In Figure 10, large elevations are present between 20-70 μm above the tip, all covered by epithelial cells, but with cracks and holes (*). In Figure 11, elevations are seen in the tip area, covered by flattened and irregularly shaped cuboidal cells, masking the exits of the Bellini ducts. Figure 12 shows a rounded elevation in a dimple in the original papillary surface still completely covered by a veil with small holes. Figure 13 shows one of the large cracks in the elevation marked in Figure 10 (*). Also, in this case, crystalline material is not seen through the holes in the cover. Figure 14 shows an EDX-spectrum of the area in Figure 12 (*) with much calcium and some phosphorus and sulphur. Figures 15 and 16 show the papillary tip of a rat after a three day post-induction recovery, re-challenged with an additional oxalate load from 0.1 vol% EG for twelve (Fig. 15) and forty-two (Fig. 16) days. Only in the latter case, some minute sub-epithelial changes are present at the tip around the ducts of Bellini.



Figures 10 to 13. Scanning electron micrographs of rat papillary tip after CID plus ten days recovery. **Figure 10.** Large elevations are present above the tip, all covered by epithelial cells (*). Bar = 1 mm. **Figure 11.** Detail of the tip with numerous sub-epithelial elevations. Bar = 0.1 mm. **Figure 12.** Detail of the dome-shaped lesion, showing holes and cracks in the cover. (*) site of X-ray microanalysis shown in Figure 14. Bar = 0.1 mm. **Figure 13.** No crystals are seen through the holes in the epithelial cover. Bar = 0.1 mm.

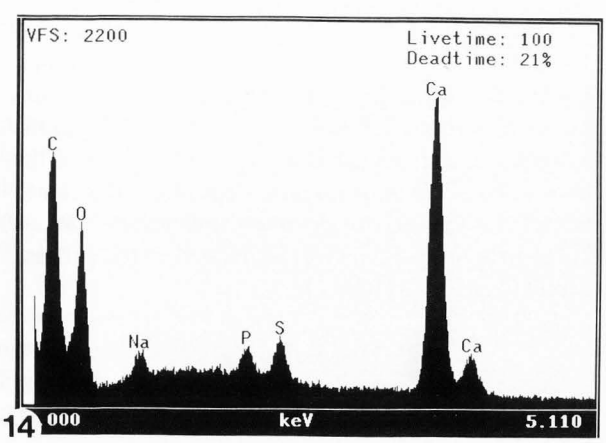
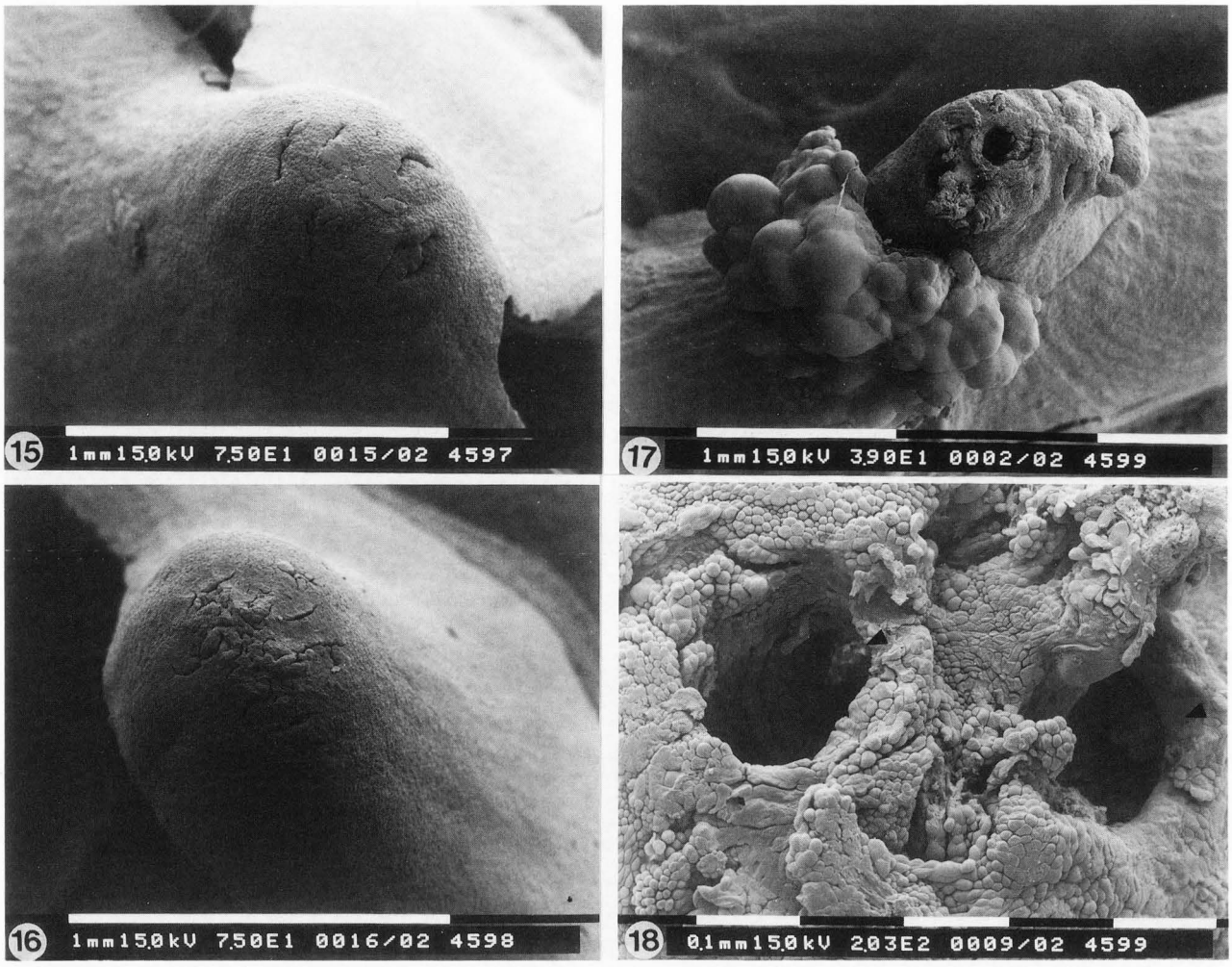


Figure 14 (at left). X-ray microanalysis of the marked area (*) in Figure 12 with much calcium.

Figures 17 and 18 show the changes after a re-challenge with 0.3 vol% EG for thirty-seven days. Figure 17 shows the large sub-epithelial cauliflower-like mm-sized protrusions at the cranial and caudal sites of the tip area. Figure 18 shows the interior of the enlarged ducts of Bellini at the very tip of the papilla surrounded by protrusions of irregularly shaped cuboidal cells. Inside the holes crystal agglomerates might be present (▲).

SEM observations from the surfaces of the papillary beds from which the papillae are removed did not show any free crystals or lesions (not shown).



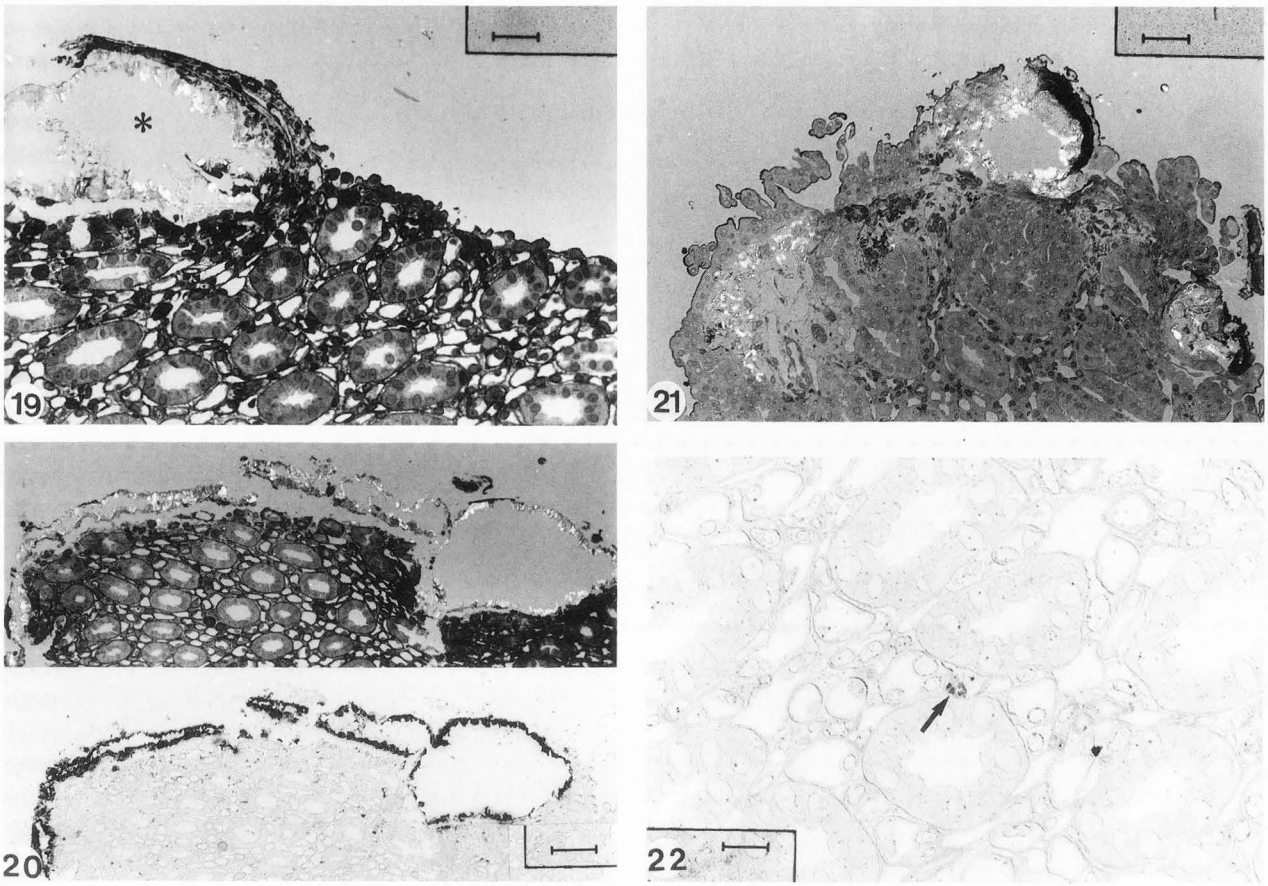
Figures 15 to 18. Scanning electron micrographs of a rat's papilla which after a three day post-induction recovery phase is re-challenged with an additional oxalate load. **Figure 15.** Shows the absence of serious papillary damage after twelve days 0.1 vol% EG. Bar = 1 mm. **Figure 16.** Shows the rather minute papillary changes at the very tip around the ducts of Bellini after forty-two days 0.1 vol% EG. Bar = 1 mm. **Figure 17.** Shows the mm-sized stone formed at some distance from the papillary tip from a re-challenge with 0.3 vol% EG for thirty-seven days. Bar = 1 mm. **Figure 18.** Shows the interior of the enlarged ducts of Bellini in which crystal agglomerates might be present (▲) and the cuboidal cells covering the tip. Bar = 0.1 mm.

Light microscopy

Figures 19 to 22 show images from the osmium tetroxide plus ferrocyanide postfixed cross-sectioned papillary tip after a five days recovery phase at the places marked in Figure 5. Figure 19 shows crystalline masses to be present in the sub-epithelial space. Figure 20 compares the Von Kossa stain of this area with the Toluidine Blue staining, demonstrating that a reaction product is present at the places where previously the bi-refringent material was present. Figure 21, from an area at the apex of the cross-sectioned papilla of Figure 11, after ten days recovery, shows the presence of sub-epithelial crystal masses about 15 μm from the tip. Figure 22 shows the virtual absence of calcium deposits in the peri-tubular or peri-vascular spaces in the centre of the papilla. The absence of calcium deposits in the centre of the tubular lumen is in the mean time shown. When deposits are present, they can be related to the process of exotubulosis of crystals (↓).

Discussion

The SEM observations show the presence of large



Figures 19 and 20. Light micrograph of cross-sectioned rat papillary tip after CID plus five days recovery, at places marked in Figure 5 by a broken or dotted line. **Figure 19**. large sub-epithelial crystal masses (*), marked in Figure 5, by a broken line. Epon, Toluidine Blue, Crossed Nicol prisms. Bar = 50 μm. **Figure 20 (top)**. Large sub-epithelial crystal masses, marked in Figure 5 by a dotted line. Epon, Toluidine Blue, Crossed Nicol prisms. **Figure 20 (bottom)**. Calcium deposits in this area. Von Kossa, Crossed Nicol prisms. Bar = 50 μm.

Figures 21 and 22. Light micrographs showing details of Figure 11, from the cross-sectioned rat papillary tip after CID plus ten days recovery. **Figure 21**. Crystalline masses around the tip of the papilla. Note the irregular layer of cuboidal cells. Epon, Toluidine Blue, Crossed Nicol prisms. Bar = 50 μm. **Figure 22**. Absence of peri-tubular or peri-vascular calcium deposits in the centre of the papilla. Arrow points to a small interstitial deposit after exotubulosis. Von Kossa. Bar = 25 μm.

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epithelial-cell covered elevations and domes between about 30-100 μm from the tip. In combination with the observations on the sectioned material from the same epon-embedded specimens shown here and previously [9], the following can be summarized:

(1) After CID plus one day recovery, sub-epithelial elevations are seen. The papillary tip shows some swellings and cuboidal cells over the original papillary cover (Figs. 1 to 4). In this, only-aldehyde-fixed specimen, free crystals are not observed.

(2) Micrometer-sized domes protruding into the calycine space are present after a five days recovery phase (Figs. 5 to 8). The protrusions consist of large crystalline masses covered by a thin layer of the original epithelium plus a layer of undefined epithelial/macrophagic cells. In this, osmium plus ferrocyanide post-fixed SEM specimen, free crystals are not observed (Figs. 19 and 20).

(3) After ten days post-induction recovery (Figs. 9 to 12), μm-sized domes also protrude into the calycine

space. A thin epithelium covers large crystalline masses (Fig. 21). In this cover, cracks are present but bare crystals are not observed in this only-aldehyde-fixed SEM specimen.

(**4**) The papillae of rats, that after a three day post-induction recovery are re-challenged with an additional oxalate load from 0.1 vol% EG for twelve or forty-two days, do show some minor changes at the papillary tip. However, a re-challenge with 0.3 vol% EG for thirty-seven days, shows severe papillary damage and mm-sized stones.

(**5**) The presence of calcium is detected by SEM/EDX in some elevations. The COM nature of the stored bi-refringent sub-epithelial interstitial crystal masses has been previously established by electron diffraction [9]. Randall's plaques are not observed by a Von Kossa's stain at the basis of the crystalline masses.

Hence, after a CID plus one-ten days recovery, large interstitial crystalline masses are present at the periphery of the papilla, that bulge into the calycine space. These elevations are covered by a very delicate outer surface consisting of a sheet of epithelial-cells or a thin veil. At places, this sheet has rather large cracks and holes; the possibility that these may communicate with the calycine space during life, though a preparation artefact, cannot be completely excluded. Some dome-shaped elevations look like μm-sized stones (Figs. 7 and 12). After a re-challenge with 0.3 vol% EG, mm-sized papillary stones are present (Figs. 17 and 18).

Several concepts have been proposed about the very first steps in intra-tubular crystal formation leading eventually to stone formation [4, 11, 12]. Although it is dangerous to draw conclusions about dynamic processes from a series of static images, it might be speculated that there are two pathways for the etiology of these dome-shaped structures: (1) from a tubular towards an interstitial origin by exotubulosis and eventually opening up to the calycine space either directly or through small holes in the cover, or (2) in the opposite direction caused by free crystals provoking a cellular reaction of mobile cuboidal epithelial/macrophagic cells, leading subsequently to an action of overgrowing the free crystals in a process identical to the exotubulosis process observed from the tubular epithelial cells [10, Figs. 5, and 13 to 18]; or alternatively, both processes occur independently. We do not have an explanation about the origin of the free crystals in the calycine space as demonstrated recently [10], but crystals might be discharged through the widened ducts of Bellini (Fig. 19).

As a consequence of the design of our experiments, in which one-ten days are included for recovery, we might have missed the observation of the initial changes as described by Khan *et al.* [15] following oxalate injections. An alternative explanation for the absence of any free crystals might be that during the removal of the papilla from its surroundings and SEM specimen preparation, such loosely attached crystals are lost, whereas the crystals previously observed at this region were kept in place by the surrounding tissue. However, sub-epithelial crystal-containing elevations are already present after CID plus one day recovery, which leads to a conclusion that tissue changes acquired by a nine-days CID differ from those acquired after oxalate injections. Moreover, a second process is noticed after CID plus five days recovery, viz., the described crystal enrobing process by unidentified epithelial/macrophagic cells in the calycine space where a large population is seen on the outer papillary surface now (Fig. 6) and before [10].

Because the sub-epithelial crystalline masses have a size far beyond the size of the initial interstitial crystals, and because in these masses growth rings are seen, it can be assumed that during the one to ten days for recovery, these crystals do grow. This growth occurs either by a process generated from the relatively higher interstitial concentrations of the compounds involved or by the fact that the small holes observed in the epithelial cover might provide the entrance of calycine fluids into the crystal-containing sub-epithelial spaces. However, there are no calycine calcium and oxalate concentration data during this ten day period to support this idea.

A comparison between the original description of a Randall's plaque [18] and the present observations is hampered by several difficulties: (a) the difference in species {human versus (vs) rat} and approach (deductive vs inductive); (b) the difference in electrolytes involved (calcium oxalate vs various calcium phosphates, carbonates); and (c) differences in detection and detection limits/resolution (Von Kossa vs bi-refringence and LM vs LM/SEM/TEM/EDX/electron diffraction).

Analyzing the images from Randall's partially decalcified human material [in 18, Figs. 7 and 8, and 9 to 12], we believe that a very thin epithelial(?) veil has also covered the phosphate and oxalate calculi respectively, which were in close contact with the Von Kossa positive plaque material in the papillar sub-epithelial basal layers. However, in his showing the "liberation" of a stone [18, Fig. 10], such outer membranous material is not seen.

In our specimens, such a veil is also seen, but the plaque material at their basal site is lacking (Figs. 20 and 17 in [10]). Moreover, Posey [17] showed images in which small human calculi remained covered by an epithelial layer. Unfortunately, such thin delicate veils seen in the SEM images are vulnerable during the re-embedding process and are only partially present in LM images where sometimes sub-epithelial supporting cells are seen.

Comparing the presence of bi-refringence in our material in relation to Von Kossa's stain showed that the

area original filled with crystals becomes Von Kossa positive simultaneously loosing its bi-refringent nature, but a massive peri-tubular or peri-vascular localization, as described by Randall [18] and Rosenow [19], was not observed in our material. Moreover, ducts of Bellini choked with crystalline material, justifying the presence of nephrocalcinosis, were not observed either in any of the presently studied specimens. The exotubulosis process might be the way such ducts are cleared, but this remains to be clarified by future investigations.

Anderson and McDonald [1] emphasized the interstitial localization of the calculus forming large coalescent lakes of calcium and specified the role of interstitial macrophages. They showed images in which calculi are present just beneath the tubular epithelium; these images, described by them as a process in which the calculus is pressing against the lining cells, were similar to those attributed by us to exotubulosis. They [1] also described an amorphous stage with large calcium containing lakes present in the papilla interior but which, when located at its periphery, showed a locally "eroded" epithelial cover. Our observation in Figure 3 is suggestive of such a phase of material piercing through the epithelial cover, but our SEM images actually show an outer cover, though sometimes with rather large cracks and holes.

Comparing subjectively the amount of sub-epithelial crystal masses, there is a tendency of a relative increase from one day towards a ten days of normal drinking water regime. From investigations beyond ten days recovery, a conclusion about the fate of these crystal-containing domes might be expected. However, the image from the twelve days re-challenge rat's papilla (Fig. 15) does not "fit" in this sequence of events. Because urinary calcium and oxalate measurements during this phase of recovery are absent, the degree at which this crystalline mass grows is unknown and requires further investigation. The comparison of results from experiments which included, after a three day post-induction recovery phase, a re-challenge with 0.3 vol.% EG (Fig. 17 and 18) with those obtained with 0.1 vol% EG shown here and before [9, 10], might provide information about the process of formation of μm-sized stones out of the covered crystal-containing domes. These aspects will be studied further in the near future.

In conclusion, our CID rat-model results show the presence of large papillary sub-epithelial crystal (COM) masses and μm-sized stones, which might form the basis for the etiology of COM nephrolithiasis. However, the LM images differ from those described by Randall [18], Rosenow [19] and Vermooten [22] in human autopsies and those of Wright and Hodgkinson [23] in rats but are similar to those reported by Anderson and McDonald [1].

Acknowledgements

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Discussion with Reviewers

S.R. Khan: I have an argument with the title of the first of these papers which is suggestive of the fact that it is a human kidney stone model. It is not. Actually, no such model is currently available. It is a good model to study certain aspects of stone formation and provide information about the possibility of similar things happening in human stone formation. I suggest that the authors point it out and discuss the similarities as well as the dissimilarities between this model and human stone formation. For example, calcium oxalate crystals are not deposited in renal cortex during human nephrolithiasis.

H.G Tisselius: The observations are of great importance in as much as they provide a possible mechanism of crystal retention in the renal collecting system. The only major problem, that is encountered in experiments of this type, is the unphysiological supersaturation that the animals are subjected to as a result of their diet. This might make comparisons with human stone formation difficult, a problem that the authors are well aware of. The data, nevertheless, point to a most interesting physiological mechanism.

Authors: Both referees point to the vital question about the value of observations on an animal model system in predicting aspects of human nephrolithiatic processes. The question mark in the title of the first paper (I. A Model for Human Stone Formation?) indicates sufficiently that we also feel that a resemblance between the rat model and the human disease constantly needs to be substantiated further. Moreover, in the **Discussion** of the second paper, we indicated that cellular (ultra)structural dissimilarities exist, in spite of a gross morphological homology, between the Randall's plaques studied (deductively) in human post-mortem cases and the rat papillary structures in our study. However, in the mean time, we have collected arguments that the results of the recovery processes we have studied with our (inductive) rat model cast doubts about the possibilities to study early processes in human kidney (biopsies?) because the recovery process might be over at the end of fourteen days.

Our experience with human and animal kidney pathology is restricted. Hence, we cannot oppose the certainty of the first referee's remarks on the dissimilarities (e.g., absence of crystals in the human cortical regions). Our uncertainty about the observation of the very first changes in human kidneys comes: (1) from our own observations about the speed of removal of cortical crystals during the recovery phase, and (2) from Randall's criteria about the diagnosis (virtually the absence of any detectable sign or complaint prior to the (post-mortem) detection of a mini-stone or an ureter obstruction *durante vitae*.

Still accepting the idea that intratubular supersaturation and crystal formation are prerequisites that precede stone formation, our present investigations are directed to answer the question whether urine chemistry can predict what happens in the kidney during the induction phase.

We agree with the second referee that our model in this respect is based upon the creation of a rather unphysiologically high supersaturation, which might exert various pathological processes (e.g., exotubulosis, crystal phagocytosis, sterile inflammation, sub-epithelial interstitial crystal growth in the papillary tip, calyceal crystal enrobing, etc.). However, it cannot be excluded that such transient episodes do occur in humans too, but remain unnoticed. The re-challenge experiments with controlled (?) milder (?) oxalate loads were performed to test the usefulness of the model to study the stone-recurrence process.

S.R. Khan: How would you define exotubulosis? Is it the migration of intraluminal crystals to interstitial

locations in the kidney?

H.-G. Tiselius: It is suggested in the first paper that crystals are formed intratubularly. Are you certain that crystals retained in the papilla are of tubular origin and not formed by a precipitation in the interstitial tissue as a result of the probably very high concentrations in this area. What is the possible physiologic role for exotubulosis?

Authors: Microscopists are the first to admit that it is not safe to give, what is called, "time and direction" to an isolated pictorial observation. Nevertheless, they invariably always do. There are two arguments that frequently can support them in doing so: (1) the total process points in that direction (= crystal retention), and (2) the single example-observation can be placed in a series of physically registered (or unregistered) observations that is **thought or can be argued** to represent the process "in time". In our previous papers about this process [9, and the first paper, reference 10], such images were obtained from the cortical region. In the present papers, we describe in a less extensive way, the process in the other parts of the nephron. In short: crystals are seen located free in the tubular lumen at the end of the induction phase; are seen attached to the apex of tubular lining cells, covered by neighbouring epithelial cells, completely isolated from the lumen and surrounded by epithelial cells after two-three days recovery, but at that moment not in the interstitium; an ectopic sub-epithelial basement membrane is observed on top of the crystals and a basement membrane at the tubular basis is absent. In addition, we noticed the presence of interstitial crystals after 5 days and the absence of cortical interstitial crystals after 10 days post-induction recovery. This series of multiple observations is considered to describe the actual process in that order, but can (though this appears unlikely to us) also be read in a reversed direction. It is, by definition (outside the tubule = interstitium), a relocation, not a migration phenomenon.

The physiological role of the process of exotubulosis can be seen as a natural defence mechanism to free the tubular lumen from crystalline obstructions or as a way for the cortical crystals to pass the loop of Henle. In the present papers, we collect arguments that in the papillary region, as a consequence of the interstitial crystal localization, and a presumably high (increased?) supersaturation, may induce additional interstitial crystal formation and/or enhance crystal growth.

S.R. Khan: The second paper is a little difficult to follow. It is not clear how many rats had calcium oxalate crystal deposits at the renal papillary tips. I think presentation of results in the form of a table will be helpful. Apparently, in this model, papillary tip stones of calcium oxalate are not common and changes are a result of nephrotoxicity of ethylene glycol or ammonium chloride or the combination? Did the authors look at rats with only ammonium chloride? Rat renal papilla is extremely sensitive to nephrotoxic challenges. These lesions have nothing to do with crystal deposition.

Authors: The experiment described had a character of an "in depth" investigation to study the fate of retained crystals. The experiment reported in our papers ([9] and these two) included a total of ten animals, each with a different "treatment". Wistar rats with long term regimes of only 0.5 and 0.8 vol% ethylene glycol were included but LM observation did not reveal any crystal deposits. Previously [3], as well as in this paper, we reported that a similar treatment with ammonium chloride also did not create crystals. In the meantime, we have observed that similar microstones can be created in some, but not in all rats (4 out of 25) following a less nephrotoxic(?) regime including (0.5%) EG plus Vitamin D_3. Also, we have seen similar changes in duplicate experiments. We do not understand the meaning of the last remark. The regimes described do deposit crystals/stones in the rat's papillary tip according to three processes (free crystals, sub-epithelially located crystals due to exotubulosis, and calyceal crystal enrobing) which we have described and illustrated. These results are compared to those reported in the literature.