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ETIOLOGY OF EXPERIMENTAL CALCIUM OXALATE MONOHYDRATE NEPHROLITHIASIS IN RATS

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Abstract

In a rat-model system, tubular crystal retention as a possible mechanism for the etiology of nephrolithiasis in man, was studied by conventional transmission electron microscopy. The animals were supplied for nine days with a crystal-inducing diet, with ethylene glycol plus NH_4Cl in their drinking-water. After this induction period, a two day regime with fresh drinking-water was included, to allow crystals to be removed by spontaneous crystalluria. After aldehyde fixation of the rat kidneys, large crystals were seen inside the tubular lumen. The crystals were attached to cell surfaces and covered by neighboring epithelial cells. Some crystals were overgrown by several epithelial cells and underwent a process of so-called exotubulosis, resulting in free or cell-surrounded crystals in the interstitium, and possibly in crystals in Giant cells. To investigate the fate of the retained crystals, some animals were additionally exposed to a low-oxalate challenge from drinking water containing 0.1 volume per cent of ethylene glycol for 12 or 30 days, respectively. It was assumed that this would interfere with the retained intratubular or interstitial crystals, and allow the crystals to grow into mini-stones. This was not observed. After the oxalate challenge, no crystals were found to be retained in the tubules (free or covered by cells). Interstitial crystals were observed, but it remains to be demonstrated whether such crystals actually grow into mini-stones or that they are removed by the sterile inflammation process observed.

Key Words: Calcium oxalate, stone etiology, crystal-inducing diet, rat model, transmission electron microscopy.

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Introduction

The presence of crystals in the intra-tubular space along the nephron plays an important role in the etiology of nephrolithiasis, irrespective of the chemical composition of the crystal. When crystals are present in the lumen, crystal growth and agglomeration may take place. Crystal growth/agglomeration can be promoted or inhibited by endogenous or exogenous chemical compounds. Endogenous inhibiting components, present in, or secreted into the lumen, by nephron-lining cells, contribute to the prevention of nephrolithiasis.

With respect to the lithogenic process, questions can be raised whether:

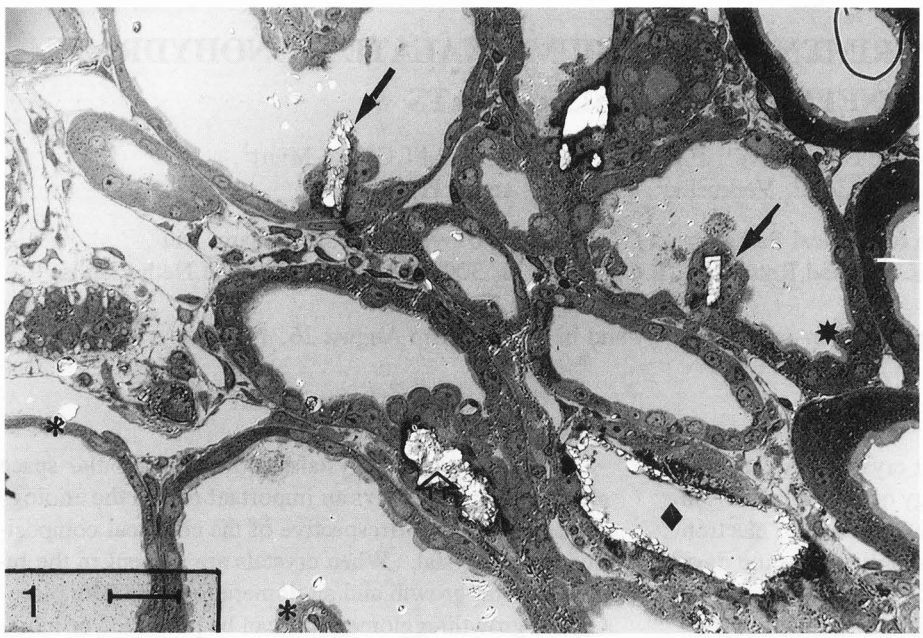
(**a**) inorganic chemical components related to crystal nucleation, growth and agglomeration are the sole source of nephrolithiasis, or whether it is a mixed process, in which organic components play a prominent role. The presence of a stone matrix has been reported, and recent information about the association of e.g., Tamm-Horsfall glycoproteins [10, 14], prothrombin [26], and nephrocalcins [25] with crystals, supports the idea of a mixed process.

(**b**) there is an active role for nephron-lining cells in stone-etiology [9], other than secretion of substances inhibiting crystal growth/agglomeration. When there is an active role for the lining cells, the position of the active cells along the nephron has to be taken into account, which may lead to the use of model systems.

Among the experimental model systems that allow *in situ* observation of the interaction with cells, two models can be used to elucidate a possible role of lining cells in the etiology of the nephrolithiatic process: (**a**) animal model systems, and (**b**) cultured cell systems of primary or immortalized cells on (porous) supports.

Experiments with animal model systems can be performed under the assumption of an analogy or homology of the urolithiatic process between cells of animals and humans. A combination with *in situ* analytical techniques is a prerequisite.

It has previously been described [1, 5, 16-20, 27] that calcium oxalate mono- or dihydrate (COM or COD)



Figures 1 and 2. Light micrographs of rat kidney tissue after 9 days CID and at the end of a two-day crystalluria period. Epon, Toluidine Blue, crossed Nicol prisms, bars = 25 μm. **Figure 1.** Small retained crystals are seen at the cell surface (*), inside the cells (★), overgrown by adjacent cells (↓), at the basis of the tubule (⇑), in the interstitium (▾), or completely stuck in the lumen (◆). **Figure 2 (on the facing page 543).** Detail of completely cell-surrounded crystals (★), just under the epithelium (↓) and in the interstitium (⇑).

crystals can be created acutely (within minutes) inside rat kidney tubules after intraperitoneal (i.p.) or intravenous (i.v.) injections of oxalate salts. Scanning electron microscopic (SEM) observations confirmed that single calcium oxalate crystals (COM and COD) and crystal agglomerates appear attached to, or are in close vicinity of, various apparently damaged rat tubular epithelial cells one hour after i.p. injection. Three days after injection, crystals are observed to be retained in the outer medulla and papillary tip, and after a period of seven days they are completely eliminated [19]. A more chronic nephrolithiatic process, generated by application of crystal-inducing diets (CID) with ethylene glycol (EG) plus either NH_4Cl or vitamin D to rats, has shown similar structural observations in their kidneys. Orally applied EG is converted to endogenous oxalic acid by the liver; NH_4Cl is supposed to upset the enzyme-sorting mechanisms in the tubular cells, and vitamin D the calcium balance. Recently, we have shown the presence and crystalline character of intracellular COM crystals in proximal and distal tubule cells after a 8 days CID with EG plus NH_4Cl [2, 3]. In addition, these studies showed the presence of intratubular crystals. In the present study, the same EG plus NH_4Cl-treated rat-model system, as previously described, was used. The main objective of the present contribution is not to investigate the way intratubular or intracellular crystals are created, but to investigate:

(a) whether, why and how crystals are retained or are removed by crystalluria;

(b) the nature of the interaction of (existing) crystals with urinary substances (which might be called crystal opsonization) as a possible role in this retention process;

(c) the tubular or cellular aspects in relation to the fate of the retained crystals;

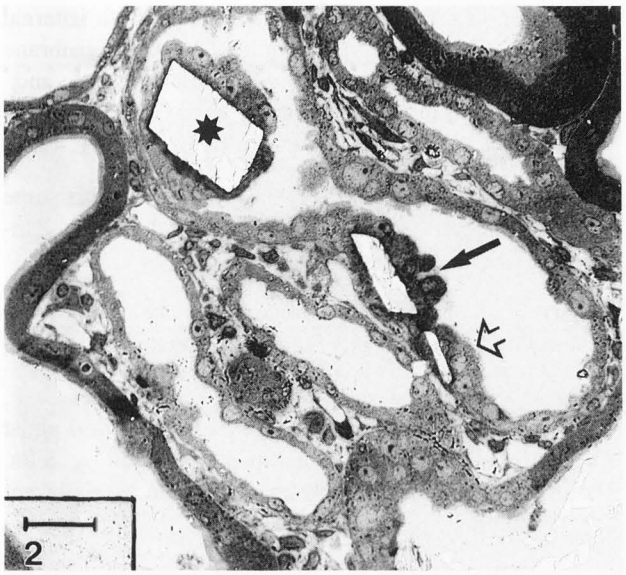
The protocol to obtain the desired objectives includes, after a CID regime of 9 days, a deliberate introduction of a two day period of crystalluria on normal drinking water. Subsequently, after that period, the (COM/COD) crystals assumed to be retained, are challenged by offering the animals an additional regime with drinking water containing 0.1 volume (vol.) per cent EG, for 12 or 30 days, respectively. The aim of this experiment is to determine the fate of the crystals on a chronic low-oxalate challenge and/or to allow the crystals to be converted into mini-stones.

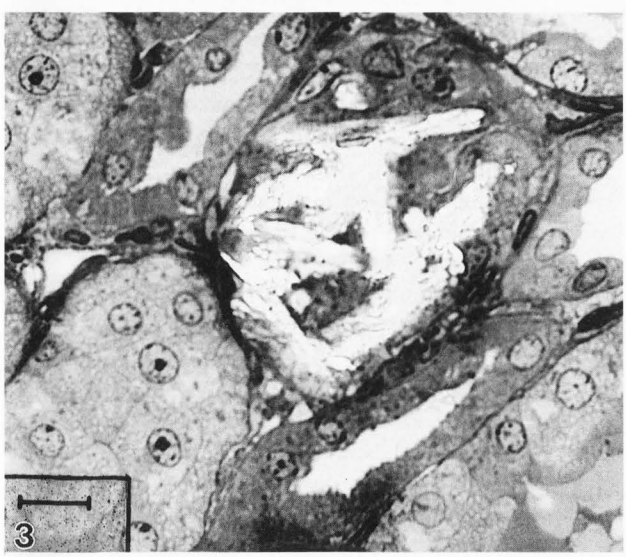
Materials and Methods

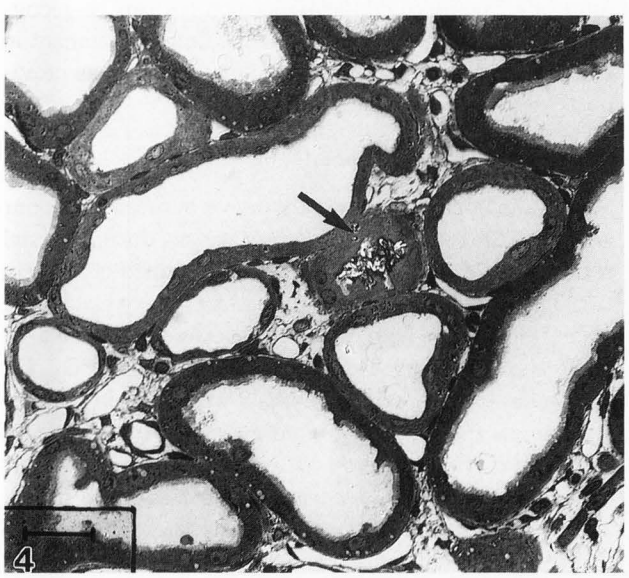
Male Wistar rats (weighing 200 g) from the Erasmus Animal Science Center were acclimatized and kept on a normal rat chow. At the start of the experiment, the drinking water was supplemented with 0.8 vol.% ethylene glycol plus 1 weight (wt.) per cent NH_4Cl for 9 days. Control rats were sacrificed to confirm the presence of the intratubular crystals as previously described in detail [2].

After this induction period, all other rats were allowed a crystalluria phase of two days on normal drinking water. Subsequently, some rats were given a low-oxalate challenge by re-supplementing their drinking water with 0.1 vol.% EG without NH_4Cl for 12 or 30 days. At the end of that challenge, again two days normal drinking water were included in the regime to remove eventually all non-retainable crystals.

All rat kidneys were fixed by retrograde perfusion with glutaraldehyde in cacodylate/HCl buffer as described before [2]. All removed kidneys were examined







Figures 3 and 4. Light micrographs of rat kidney tissue after 9 days CID plus two days crystalluria plus 30 days low-oxalate challenge plus two days crystalluria. Epon, Toluidine Blue, crossed Nicol prisms. **Figure 3.** Large interstitial crystal conglomerate; bar = 10 μm. **Figure 4.** Possibly a crystal-containing Giant cell in the interstitium (↓); bar = 25 μm.

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by X-rays *in toto* for possible crystal/stone locations, though without any success.

Millimeter-thick slices were embedded in glycol methacrylate for light microscopic inspection of stained sections under crossed Nicol prisms. One hundred micrometer slices were obtained by a Vibratome (Oxford Instruments, Oxford, UK) for *in situ* enzyme cytochemistry, alternatively post-fixed with osmium tetroxide plus ferrocyanide for ultrastructural observations. This fixation step partially results in the appearance of crystal ghosts, as described previously [4]. An alkaline phosphatase reaction was performed according to Halbhuber *et al.* [13] with cerium chloride as capture ion.

Unstained, or uranyl acetate/lead citrate stained ultrathin sections on 400 mesh grids without plastic film, were observed in a Zeiss EM902 transmission electron microscope (Zeiss, Oberkochen, Germany) and the micrographs were routinely recorded in an analogue way on sheet film [2].

Results

Light microscopy

In parallel to the earlier results [2, 3], intratubular and intracellular crystals were observed in the kidneys of rats treated with a CID for 9 days (not shown). Crystal retention in the lumen of the kidneys from the rats allowed a two days crystalluria (Fig. 1) was shown by:

(**a**) agglomerates larger than the lumen diameter, surrounded by desquamated or necrotic cells or by cellular debris;

(**b**) attachment of single crystals to the cell apex;

(**c**) overgrowth of retained crystals by adjacent epithelial cells. To a minor degree, crystal retention is also seen inside tubular lining cells;

(**d**) at the apex of the cells; either as single crystals or as crystal complexes; and

(**e**) at the basal side of the cells as single crystals or crystal complexes.

Crystal retention was observed extracellularly (Fig. 2) in the:

(**f**) intercellular space; and

(**g**) the interstitium, as free crystals (inducing a sterile inflammation reaction), in Giant cells and possibly in necrotic tubules.

In the medulla of the same kidney, a similar type of crystal retention distribution was observed as in the cortex.

In the kidney tissue of the rats exposed to the 12 or 30 day low-oxalate challenge:

(**h**) large crystal complexes were seen in the interstitium (Fig. 3).

Intratubular and intracellular crystals were not seen. Ballooning of cell villi into the lumen was observed. The presence of possible Giant cells with crystals in the interstitium was noted (Fig. 4).

Conventional transmission electron microscopy

Figure 5 shows the ultrastructural details observed in a single crystal ghost attached to the cell surface. In the crystal ghost, mucopolysaccharide material is seen surrounding an electron translucent central core, along with some small filamentous structures at its periphery. Small globular particles (about 40 nm in diameter) are present, some free in the space surrounding the crystal ghost, and between the crystal ghost and the cell membrane, and others within membrane-bound vesicles. One vesicle is seen in close contact with the ghost, another incorporated in the ghost. In between the mucopolysaccharide mass, 20 x 70 nm rectangular spaces are present, resembling rows of tiny crystals, which may characterize the structure as a crystal agglomerate on top of a large single crystal in the center.

In Figure 6, a large crystal is seen, virtually without any internal structure of mucopolysaccharide matrix material. At the far left side, the apex of a proximal cell surface is seen. In the center, cells without a prominent villous apical structure cover the ghost of this large crystalline mass. Their intact basal membrane is facing the crystal surface and follows the small dimple in the crystal aspect.

In Figure 7, the apex of the crystal is covered by a thin cellular sheet with rather prominent villi. At places, the basal membrane is penetrating between smaller crystals within the larger crystal mass.

Figure 8 shows the basal portion of a large crystal ghost where more internal structures are visible. In this area, the cells facing the crystal surface are rounded and have no apical villi. In the interstitium, several granulated cells with a monocytic character can be observed. In the extra-cellular spaces between cells and crystals, basement membrane-like material and small bundles of collagen fibers are present.

In Figure 9, similarly a crystal complex composed of several 2-4 μm long, radially arranged crystals is seen at the tubular basis near the basement membrane. In one of the surrounding cells, smaller crystal complexes are seen inside the cytoplasm and at the apical cell border.

Figure 5. Large retained crystal-ghost with internal structure. Proteinaceous globules (↓) and membrane fragments (⇑). Membrane bound vesicles outside and inside the crystal (*). Small crystals inside the periphery (▾). Uranyl acetate/lead citrate, bar = 1.4 μm.

Figure 6. Large retained crystal ghost (◆) with some internal structure, covered by epithelial cells. Unstained, bar = 1.7 μm.

Figure 7. Apex of a large retained crystal ghost (◆), covered by epithelial cells facing the lumen. Unstained, bar = 1.7 μm.

Figure 8. Basal portion of a large retained crystal ghost (◆) with some internal structure surrounded by cells. Basement membrane is absent, monocytes are present (*). Unstained, bar = 2.5 μm.

In Figure 10, large intratubular crystals are shown in the vicinity of the brush border of a cell. At the crystal surface, the presence of unidentified material is evident.

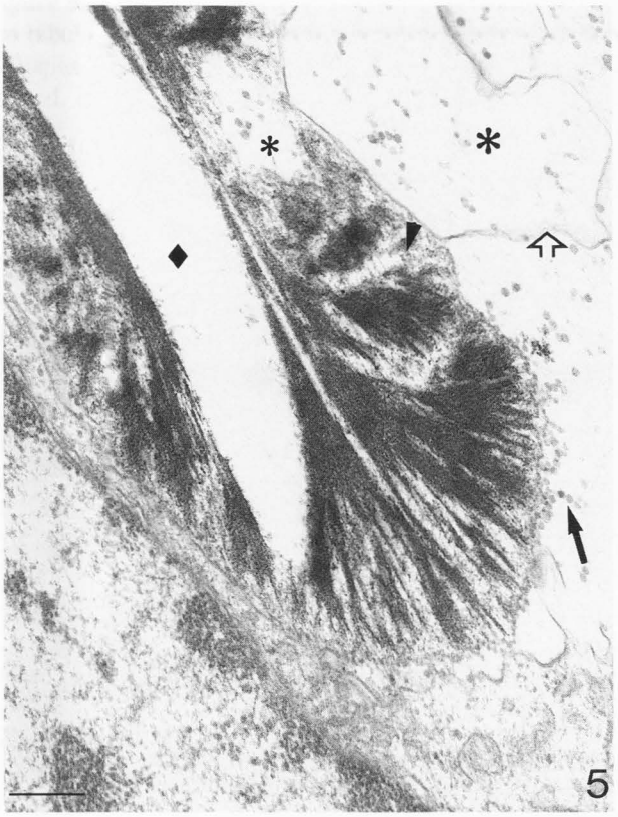
In Figure 11, there is a rather close contact between material on the crystal surface and the altered villi of the cell apex. Membranous debris is seen around this area and on the crystal surface.

Figure 12 shows a detail of ballooning villi seen in a Vibratome section treated for the detection of alkaline phosphatase (Alc.Pase). The black cerium-containing precipitate is distributed irregularly over the cell surface and the swollen apical part, in which cell organelles, cytoplasmic vesicles, and cytosol are present.

In Figure 13, the center of a very large crystal agglomerate is seen, surrounded by large, rather unstructured, crystals. In the cellular debris, (altered) mitochondria and cytoplasmic structures can be recognized. A vacuole can be seen inside this cell remnant in which a small intracellular crystal primordium, as previously described [2, 3], is present.

Discussion

In the present investigation, we succeeded in our aims: (**a**) to study the sequence of events during crystal retention, and (**b**) to study the fate of retained crystals in the kidneys which were submitted to a low-oxalate challenge, assumed to be induced by feeding the rats on a 12 or 30 days drinking water regime with 0.1% EG. By light microscopy, retained crystals were seen in the cortex and in the medulla, at either side of the loop of Henle, in close connection with the cell apex. However, this localization should be investigated in more detail, and longer intervals of the crystalluria phases chosen, so that true retention can be ascertained.



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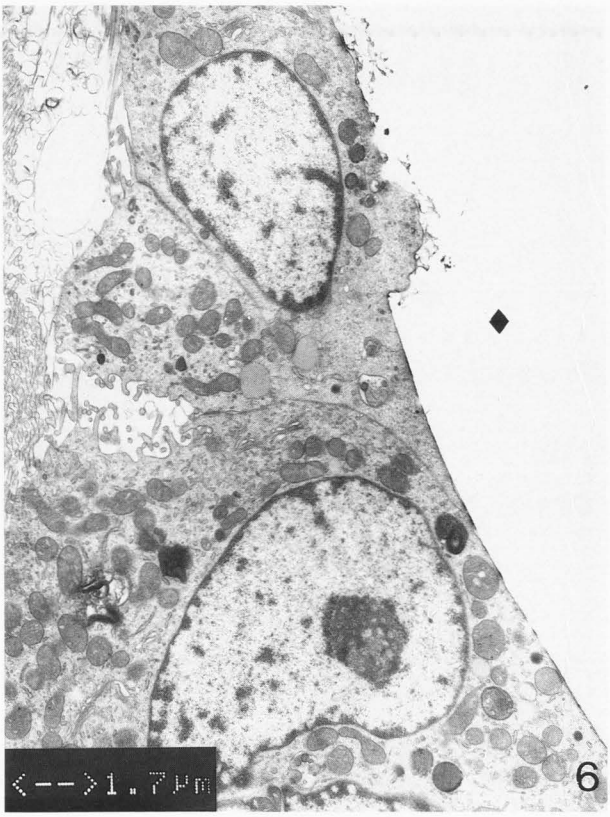

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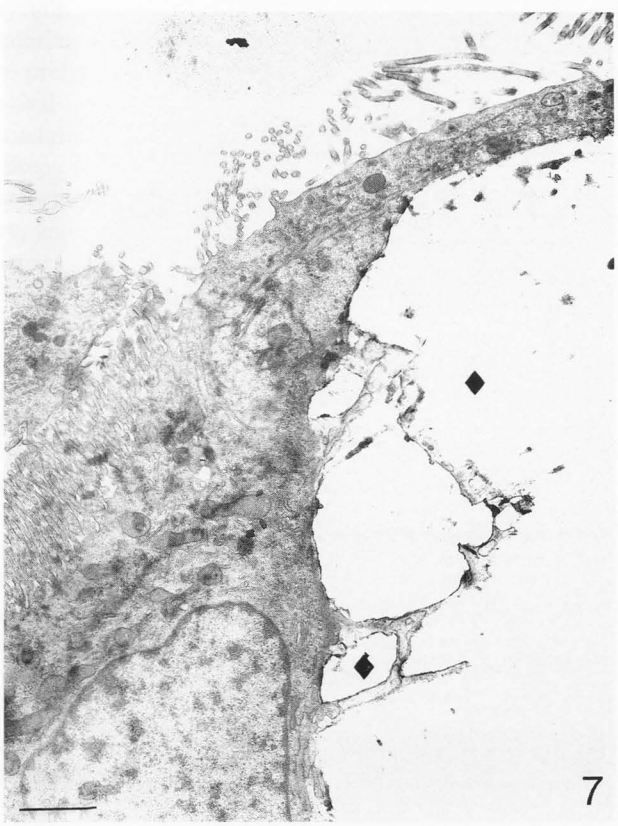

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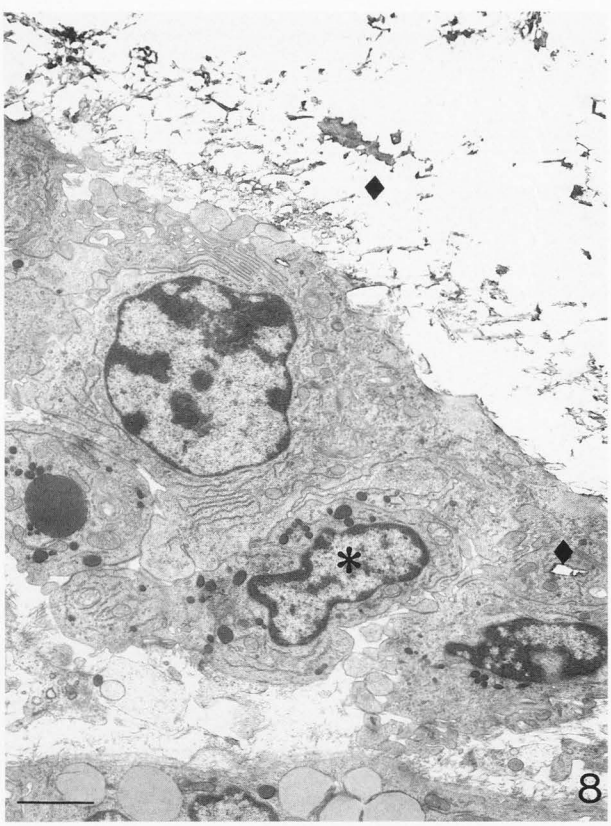

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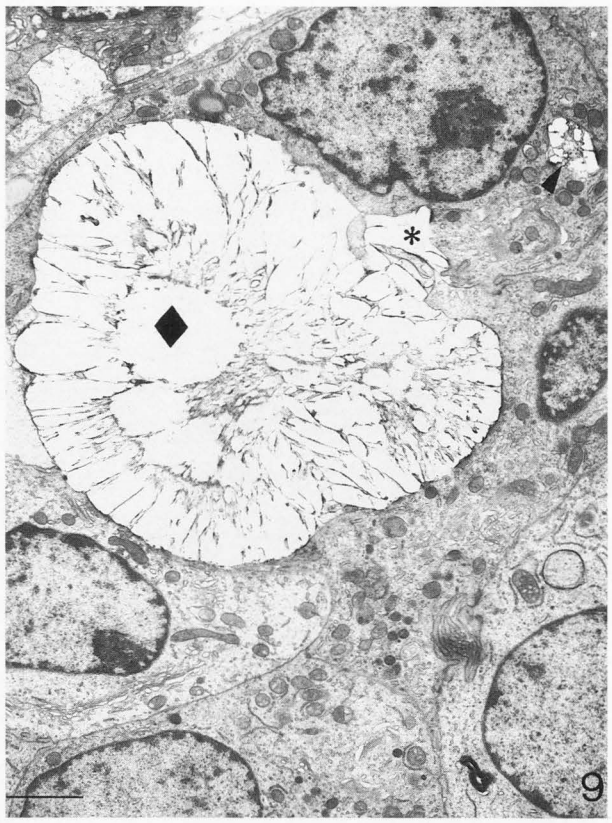
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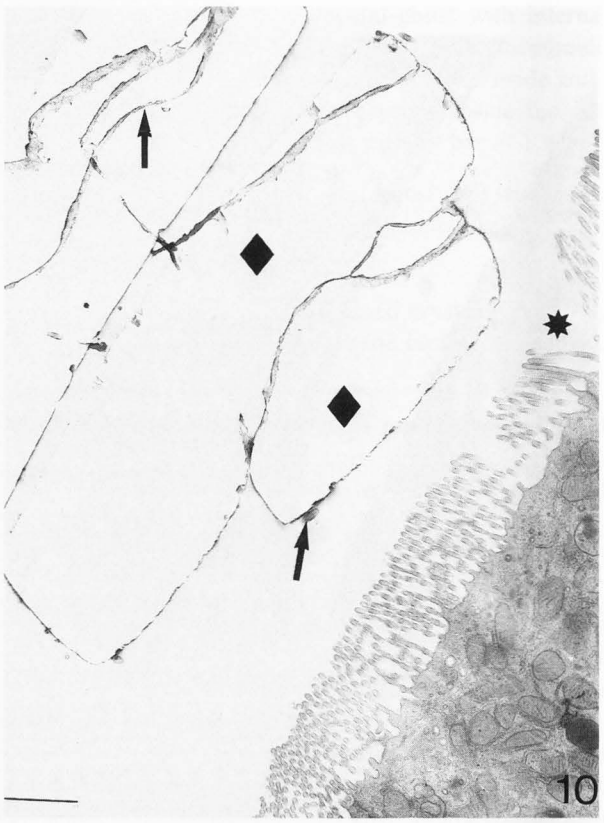
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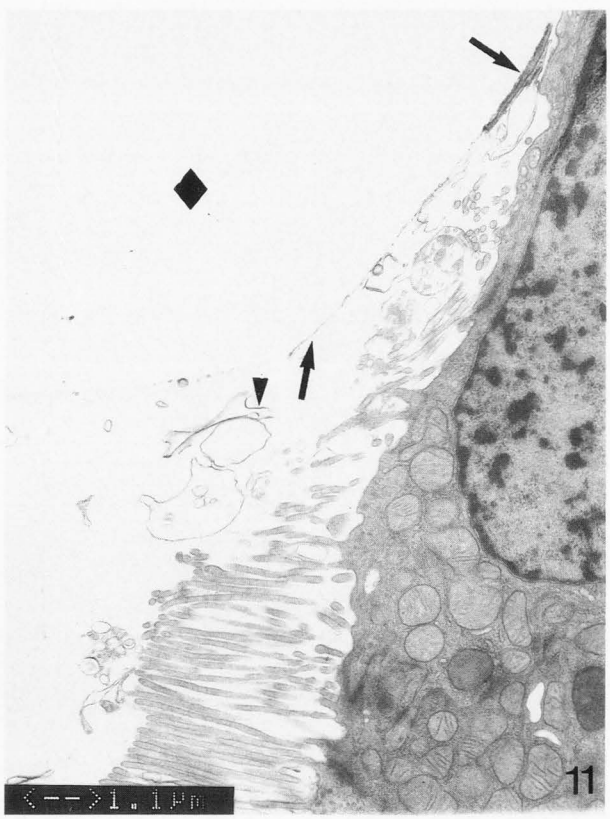
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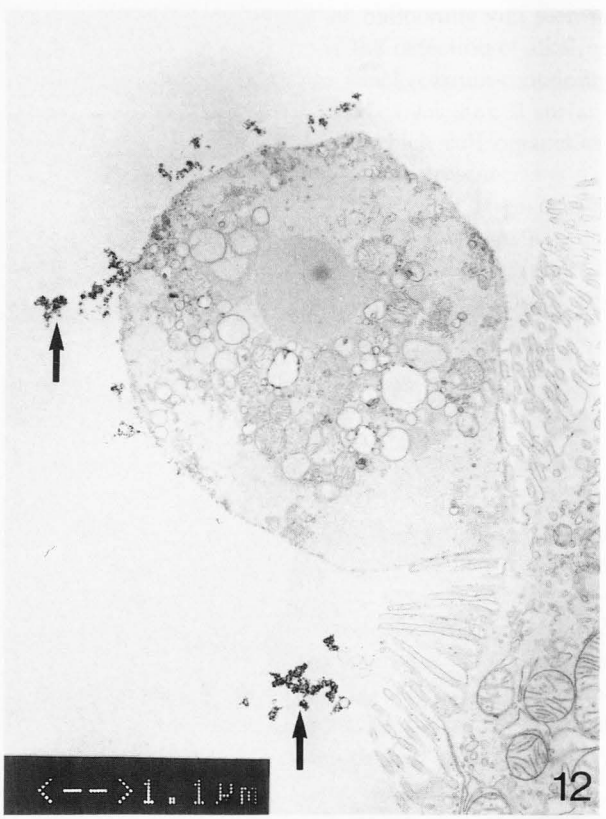
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Figure 9. Ghost of crystal complex (◆) at the basis of the tubule. Note the intracellular crystal ghosts in the cell apex (*) and at the basal part (▾) of the cell. Unstained, bar = 2.5 μm.

Figure 10. Large retained crystal ghost without internal structure with opsonization layer (↓). Reacting villi (★). Unstained, bar = 1.7 μm.

Figure 11. Large retained crystal ghost (◆) without internal structure with opsonization layer (↓) and membranous debris (▾) in close contact with the altered cell apex. Unstained, bar = 1.1 μm.

Figure 12. Swollen villi in the apex of a proximal tubule cell, containing cytoplasmic organelles. Alc.Pase-related cerium phosphate precipitate (↓). Alc.Pase, unstained, bar= 1.1 μm.

Ultrastructurally, two types of apparently retained crystal ghosts can be described: (a) large single crystals without any internal mucopolysaccharide-matrix structure, and (b) large single crystals with an internal mucopolysaccharide-matrix structure.

The presence of unidentified material on the crystal surface (for which we suggest the term crystal opsonization) has been demonstrated previously [2, 5, 17]. The crystals (a) above, are surrounded by a delicate layer of material, in which membranes are observed. Although, we prefer to think of the observed difference between crystal types (a) and (b) as real, the absence of internal structure, which is the criterion of distinction, might be a preparation artefact. In crystal type (b), also proteinaceous globular particles are present at the crystal periphery, and membranous material is included in the crystal matrix. For both types, the crystalline nature (COM or COD ?) has yet to be established by electron diffraction. For this, specimens not postfixed with OsO_4 plus ferrocyanide, are needed, in which more crystalline material is preserved. When large crystal ghosts were present in the vicinity of the cells, the latter seem to flatten their villi, but the cell membrane remains intact. Unfortunately, the cell-coat material is not visualized by the tissue-processing procedure applied in this study. The membranous remnants and proteinaceous globular particles are present between the cell membrane and the layer around the crystals. The presence of such a layer around crystals may call for a general reconsideration of (intratubular) crystal growth and agglomeration at the nanometer scale. Moreover, whether the crystal/crystal interaction and the crystal/cell interaction are identical processes, and the mechanism which determines the difference in crystal appearance, remain to be demonstrated.

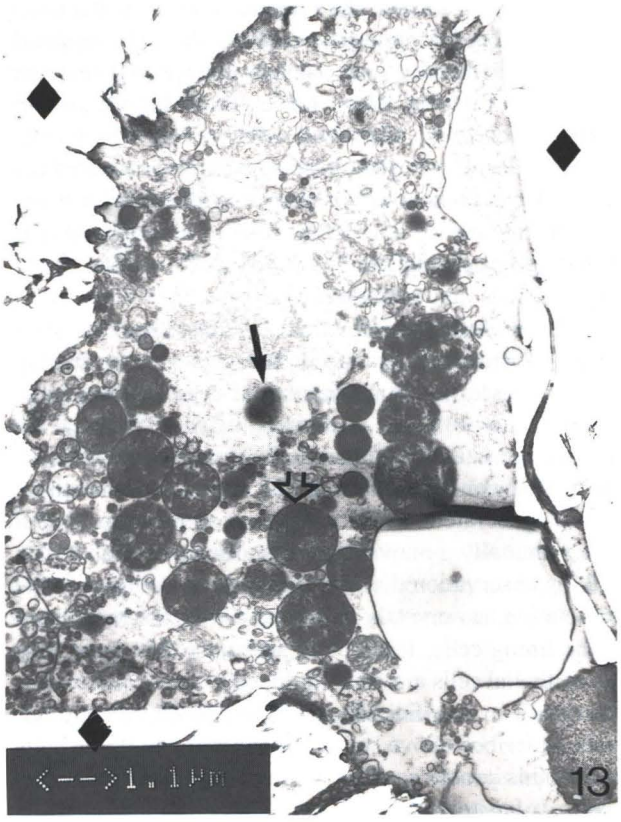

Figure 13. Cellular remnants in the center of a large crystal (◆), altered mitochondria (⇓) and small intracellular crystal primordium (↓). Unstained, bar = 1.1 μm.

Non-opsonized calcium oxalate crystals have been reported to induce cell lysis in erythrocytes, enzyme release from polymorphonuclear leukocytes, and damage to cultured cells [6, 7, 28]. Such injuries have not been observed in the present situation. Although, changes are seen both at the apical (Figs. 10 and 11) and basal side of the cells (Figs. 5-8) a limiting cell membrane is present. However, enzyme losses or charge changes in the cell coat cannot be demonstrated by the tissue processing procedure used in the present study. Alternatively, the observation of acute damage might have been missed, because tissue repair has already started during the two days on fresh drinking water.

The present study introduces a new aspect of epithelial cells in their *in vivo* reaction to large crystals, namely, when retained crystals are overgrown by adjacent cells. The cell polarity and some morphological characteristics of the original cell type are maintained during this event. The increased cellular activity shown before by Lieske *et al.* [23, 24] fits within this concept. At the basal cell part facing the crystal surface, the possibility of internalization of crystal fragments cannot be completely excluded.

Characterization of the original cell type at the basis of a large crystal aggregate is difficult. The original basement membrane material has disappeared and the boundaries between intra- and extratubular spaces are vague. Moreover, interstitial cells, among which cells recognized as monocytes, have apparently penetrated the area. It has been observed that re-synthesis of a basement membrane *de novo* at the base of a peripheral epithelial cell can complete this process of exotubulosis of large crystals and crystal-complexes.

The presence of interstitial crystal complexes after the long-lasting low-oxalate challenge is quite evident by light microscopy. Such interstitial crystals have been described before [21]. With additional cytochemical reactions, the fate of the interstitial crystals could be studied. This confirmed our initial impression that these crystals generate a sterile inflammation reaction, which will eventually remove the crystalline material. The striking observation that after thirty days, contrary to the expectation, no crystals were retained in the lumen and in the lining cells, leads us to speculate that apparently the epithelial cells are well equipped to cope with retained crystals in an efficient way. The process of exotubulosis, described above, might be the way the lining cells handle this problem. The process may lead to the formation of interstitial crystals, and possibly their removal.

Crystal "retention" has two aspects: retention in time and in place. Recently, Kok and Khan [22] have reconsidered previous calculations of Jordan *et al.* [15] about the speed of transfer of fluids through the nephron. Crystal agglomeration is considered to be the fastest way to collect crystal mass, to retard the passage through the nephron, eventually leading to crystal retention in the tubular lumen [19]. The present study indicates that in a later phase (due to the chronic hyperoxaluria in our model and the two days of restoration), or in addition to the fast crystal/crystal agglomeration reaction, also a (slower) crystal/cell aggregation reaction plays an important role in the retention of much smaller crystals, provided it can be established that both processes are the same.

When the (fixed particle [8]) retention process, in addition to lumen-blocking aspect in the thinnest part of the nephron (= free particle retention), is an independent process, that can take place at any location along the nephron, retention in the renal papillary, as described by Grases *et al.* [11], does not form an exceptional case. Cellular injury as previously described by Hackett *et al.* [12] has been seen to occur in the present material (Fig. 12), but, according to our observations, did not contribute to the crystal retention. At the sites where retention was observed, the tissue processing procedure applied in the present study did not show any local deficiency. However, in this case also, tissue restoration might have taken place in the mean time. On the other hand, in Figure 13, a situation is described in which the material discarded from the cell apex into the lumen might form the basis of large crystal agglomerates in the lumen [20].

It remains to be established whether there is a role for these interstitially located crystals as part of the nephrolithogenic process in man. When this role is absent, our observations just add another way in which the epithelial cells can remove intratubular crystals from the lumen, thus reducing the risk of stone formation.

Nevertheless, we think it is worthwhile to establish:

(**a**) whether the retained crystals form the basis of the stone primordia;

(**b**) which cell type in the nephron and/or from the interstitium is involved in the assumed nephrolithiatic process; and

(**c**) which (additional?) pathognostic event may convert the retained crystals into stones.

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Discussion with Reviewers

D.J. Kok: What calcium oxalate concentration product is maintained during 0.1% EG feeding, undersaturation, saturation or supersaturation and how constant is this during the day? Does a prolonged 0.1% EG feeding regimen ever lead to formation of stones in rats?

Authors: The amount of ethylene glycol consumed and the efficiency of conversion into oxalate is unknown. Moreover, we have not measured the oxalate concentration in the rat urine. So, the 0.1% EG was a blind guess and the oxalate challenge was not confirmed, but we deliberately have chosen this low concentration to prevent any confusion with the crystal induction process shown before. This aspect has to be investigated in detail in the future. Previously [2, 3], we have shown in a control experiment, that 30 day feeding with 0.8 vol.% EG alone did not create any crystals that could be observed by light microscopy.

D.J. Kok: If removal of the attached crystalline particles by nephron lining cells is a protective mechanism, could this indicate that a low, absent or overwhelmed regeneration capacity of the epithelial cells form the basis of stone formation? Can you speculate whether this would involve the presence of particles too large to remove, whether it needs the continuous addition of new (crystalline) material or whether it will only occur at specific sites, e.g., those sites where attached stones are mostly located?

F. Grases: Due to the high supersaturation degree attained during the treatment with ethylene glycol, forma-

tion of calcium oxalate crystals was highly favorable and as a consequence, practically impossible to avoid, even in the presence of crystallization inhibitors. In spite of this, the formed crystals were overgrown by neighboring cells (Figs. 1 and 2), which demonstrates a high regeneration capacity of the rat kidney. Could this indicate evidence that only when a low or null regeneration capacity of the injured or damaged kidney tissue exists, then the stones can develop?

Authors: When stone formation is considered solely as an intratubular event, it can be speculated that is indeed the case. Previously [2,3], we have emphasized that the observed presence of intratubular crystals frequently surrounded by patent-looking cells and (desquamated) cellular debris in the acute phase of nephrolithiasis interferes with the ideas about (purely inorganic) crystal growth and agglomeration. Our present observations add to this interpretation, that the process of exotubulosis starts right from the moment intratubular crystals are present. We do not know which signal (other than the presence of crystals) triggers the hyperplastic reaction of the nephron-lining cells and whether it is site-dependent. From the present observations, it seems to be partially crystal-size independent. However, the reaction of the lining cells towards large crystals completely blocking the lumen might differ from that at sites where crystals, which can pass the lumen freely, are retained (free and fixed particle retention [8]) or at sites with small-crystal endocytosis. The crystal removal/regeneration capacity of lining cells is unknown to us.

When stone formation includes a role for interstitial crystal agglomerates, it can be assumed that the (COM/COD) crystals have to be degraded by phagosomal-lysosomal reaction mechanisms in Giant cells and monocyte-derived macrophages. In that case, a lysosomal enzyme deficiency might cause such a type of nephrolithiasis.

F. Grases: What formation mechanisms do you suggest for the calcium oxalate aggregates (Fig. 9): by collision of different preformed crystals (secondary aggregation) or concretions formed by ingrown crystals with a complex crystal arrangement (primary aggregation)?

Authors: The main reason for including the description of the internal structural differences of the crystal ghosts in this paper (even though preparation artifacts cannot be excluded) was that the images may provide answers to the type of questions you have raised. We hold the (speculative) view that intratubular, pure inorganic crystal formation, including supersaturation and surpassing the formation product, leads to crystals of various μm-scale sizes. The crystal ghosts from such crystals are those which are internally unstructured and which, at a certain moment(?), acquire the observed unidentified pellicula (the so-called opsonization by organic intratubular material). Such crystals may undergo secondary aggregation (Fig. 9, and also Fig. 10), by collision and/or adhesion. However, the ratio between the amount of inorganic and organic material might fluctuate in this chronic model system. Hence, the pure inorganic crystals (e.g., the centrally located crystal in Fig. 5) may become surrounded by an increasing amount of organic material in which tiny nanometer scale crystals (Fig. 5, ▼) are incorporated, possibly by primary aggregation. This type of crystal ghosts has a highly structured internal aspect. It cannot be excluded that either type of crystal aggregation is not only time dependent but also place dependent, e.g., at sites of the loop of Henle where Tamm-Horsfall glycoproteins might interfere.

F. Grases: Please include data on the main urinary lithogenic biochemical parameters.

P. Messa: Please show data on the number and volumes of the crystals in the urine of the rats studied.

Authors: We did collect some urinary data but the information it too limited to be conclusive and is therefore not incorporated in this paper.

S.R. Khan: What active role do you envisage for the renal tubular cells and why?

Authors: Previously, it has been observed by various authors that nephron-lining cells are activated by the presence of intraluminal crystals in the form of cell proliferation, crystal endocytosis and/or intracellular crystal formation and cell desquamation. The present observations add to that: removal of intraluminal crystals by exotubulosis. This might be interpreted as a protective action diminishing the risk for stone formation, or, alternatively, as increasing the risk for interstitial stones. If the tubular cells do not play a role in the latter process, or if this role is restricted to the animal model and not to man, only the former role remains.