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OXALATE CRYSTALLIZATION IN THE KIDNEY IN THE PRESENCE OF HYPERURICEMIA

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Abstract

It has been a long time since uric acid was suggested to be a promoting factor in calcium oxalate stones, and a number of in vitro studies have been carried out on the relationship between uric acid or urate and calcium oxalate.

Concerning in vivo studies, urate or calcium oxalate stone-forming diets were given alone in most cases, and diets that induce formation of stones with different composition have not been given in combinations. We administered a low-concentration oxalemic diet, and a mixed diet containing oxalic acid and uric acid, and biochemically and histologically studied the effects of oxalate and uric acid on kidney stone formation.

In the kidney of the animals given the mixed diet, formation of crystalloids of uric acid or urate was evident when no crystallization was noted in the kidney of those given the low concentration oxalemic diet alone. The morphological differences in the uric acid and urate crystalloids in the kidney and the process leading to crystallization of calcium oxalate were examined under transmission and scanning electron microscopy.

Histological examination indicated that these uric acid crystals and urate crystals serve as seeds and induce formation and epitaxial growth of calcium oxalate crystals. Our in vivo study provides additional evidence that uric acid is a promoting factor in calcium oxalate stone formation.

Key Words: Nephrolithiasis in rat, Urate and Uric acid stone, Calcium oxalate stone, Epitaxial growth, crystal nucleation.

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Introduction

The effects of changes in the lifestyle, especially diet, on the pathogenesis of urinary stones are undeniable. These effects are observed in changes in the incidence of urinary stones and their composition with time and region. The most notable changes in the diet of the Japanese people are increases in the intake of animal protein, fat and oil, purine and alcohol.

On the other hand, despite the development of new treatments for urinary stones such as extracorporeal shock wave lithotripsy and percutaneous litholapaxy, the problems of residual stones and recurrence remain to be solved. These problems are caused by the diversity, and therefore, the difficulty in elucidation, of the pathogenesis of urinary stones. Hyperuricuria is a risk factor of urolithiasis and has been suggested to be related not only to uric acid and urate stones but also to calcium stones [14]. However, the manner in which uric acid is involved in the calcium oxalate stone formation is still ambiguous. We have performed animal studies on the pathogenic mechanisms of calcium oxalate stones and uric acid stones [4, 11].

In this study, we prepared a low concentration oxalemic diet and a mixed diet containing oxalic acid and uric acid, and administered them in rats to examine the relation between the two diets as well as the mechanism of precipitation of oxalate crystals in the presence of chronic mild hyperuricuria.

Materials and Methods

Male Wister rats weighing about 200 g were divided into: (1) oxalate diet group (OXD group); (2) oxalate-uric acid diet group (OXD-UA group); and (3) control group; according to the composition of the diets shown below:

OXD Group Animals in this group were given a low concentration oxalemic diet containing 1.0% oxalate, 0.3% calcium, and 16.01% protein [6].

OXD-UA Group This group was given a diet consisting of 98% of the oxalate diet given to the OXD group with 1.5% oxonic acid and 0.5% uric acid.

Control Group A standard food for rats (JAPAN-CLEA-CE-2) was given alone.

Five animals from each group were evaluated. They were given tap water (pH: 7.24-7.30) ad libitum.

In the present study, the animals were fed the above experimental diet for 20 days.

Immediately after collection of urine and blood

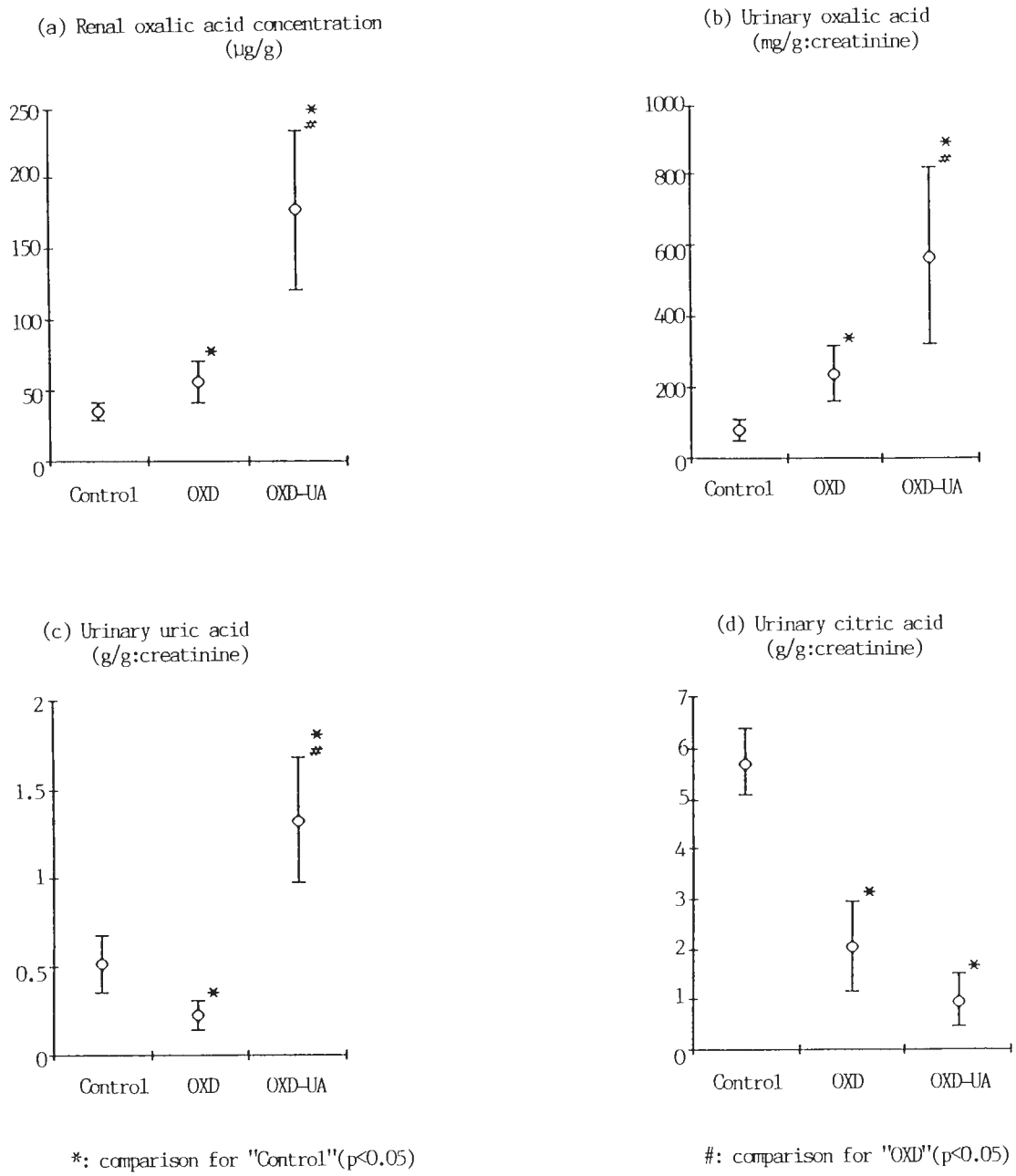

Figure 1. Comparison of **(a)** renal oxalic acid (μg/g), **(b)** urinary oxalic acid (mg/g:creatinine), **(c)** urinary uric acid (g/g:creatinine), and **(d)** urinary citric acid (g/g:creatinine).

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samples, the animals were given thoracolaparotomy under ether anesthesia, and perfused with physiologic saline via the thoracic aorta. The animals were then prefixed by perfusion of 2.5% glutaraldehyde. The kidneys were excised, minced, and fixed with the same fixative. They were fixed further with 2% osmic acid at 0-4°C for 60-90 minutes, dehydrated against an acetone gradient, and embedded in Epon 812. The embedded samples were cut into ultra-thin sections, stained with alcohol uranium and lead citrate, and observed under a transmission electron microscope (TEM). Specimens for scanning electron microscopy (SEM) were prepared by the frozen epoxy resin sectioning method [16]. Hematoxylin-eosin, von Kossa's, and De Galantha's staining were performed for light microscopy.

Results

The blood, urine, and kidney tissue were examined biochemically and histologically. The serum uric acid level was significantly higher ($p < 0.05$) in the OXD and OXD-UA groups than in the control group. Urinary uric acid and oxalic acid levels showed significant differences ($p < 0.05$) among the three groups, with both values being highest in the OXD-UA group.

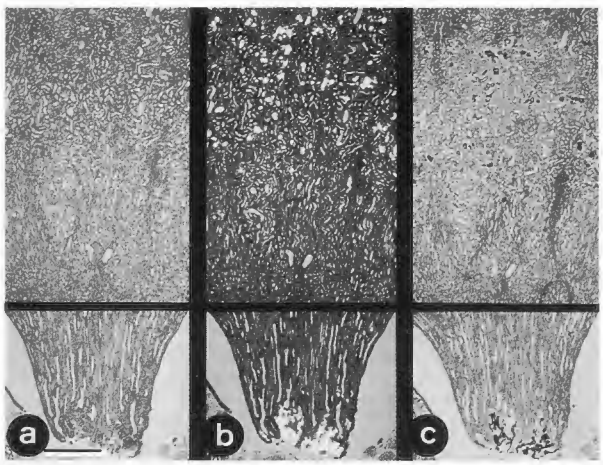

Figure 2 (at left). Light microscopic findings in the kidney of the OXD-UA group. **(a)** Hematoxylin-eosin staining. **(b)** Polarizing micrograph of the section shown in (a). **(c)** De Galantha's staining. **Above**: The corticomedullary junction. **Below**: Papillary region. Polarizing crystals and De Galantha-positive crystals are observed in large numbers in corticomedullary junction and papillary region. **Bar** = 200 μm.

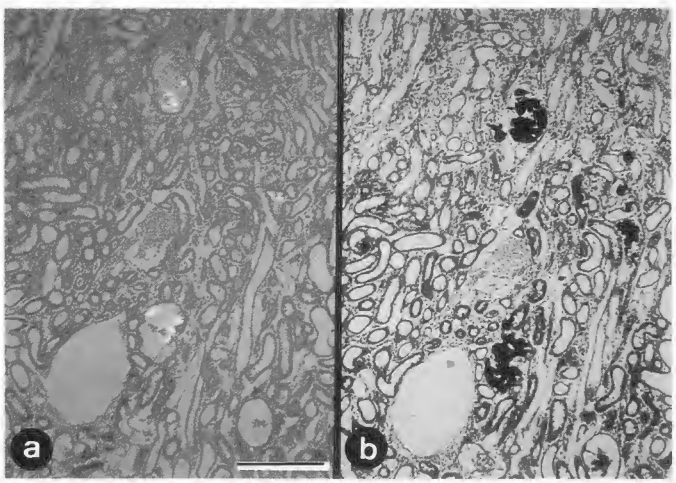

Figure 3 (at right). Enlargement of Fig. 2. **(a)** Polarizing micrograph of a specimen stained with hematoxylin-eosin. **(b)** serial section shown in (a) after De Galantha's staining. Polarizing refractive crystals and De Galantha-positive crystals are observed simultaneously in the same renal tubules. **Bar** = 100 μm.

The oxalic acid level in the kidney tissue was significantly different ($p < 0.05$) among the three groups, and was highest in the OXD-UA group, followed by the OXD group and the control group in this order. The urinary citric acid concentration showed no significant difference between the OXD group and OXD-UA group, but it was significantly lower ($p < 0.05$) in these groups than in the control group (Fig. 1) [5].

Light microscopic findings

No crystals emitting refractive polarized light or dark brown De Galantha-positive deposits were observed in the OXD group.

In the kidneys of the OXD-UA group, microcrystals emitting refractive polarized light were noted in large numbers in the corticomedullary junction and renal papillae. After De Galantha's staining of the same section, dark brown deposits were noted at corresponding sites (Fig. 2).

At a higher magnification of the renal tubules of the corticomedullary junction, crystals were found to disrupt epithelial cells and grow into the interstitial tissue. Comparison of polarizing microscopic findings of these crystals with findings after De Galantha's staining showed that crystals emitting polarized light were localized in the center of the peripheries of the dark brown deposits (Fig. 3).

Electron microscopic findings

Little changes were observed in the glomeruli, but various characteristic features were noted in the tubules of the OXD-UA group. The body and tip of the brush border on epithelial cells of the proximal tubule were enlarged in saccular forms of varying sizes. The lumen of the tubule was filled by the saccular enlargement of the brush border and the glomerular filtrate (Fig. 4). The irregularity in the electron density observed in the lumen of the renal tubules was considered to be due to differences in the concentration and chemical actions of urate or oxalate in the glomerular filtrate and the cytoplasmic matrix released from disrupted epithelial cells (Fig. 5).

In addition, electron dense gel-like materials and acicular crystalloids emerging from inside this material were observed in the lumen of proximal tubules. These crystalloids, about 10 nm in width and 0.85 μm in length, showed a minute and dense structure (Fig. 6). Laminar structures, probably clusters of these acicular crystalloids, measuring about 3.8 μm in width and about 5.3 μm in length, were also noted (Fig. 7).

The presence of these acicular crystalloids could be confirmed by SEM also, which revealed structures 10-15 nm in width with a morphology very similar to the crystalloids observed by TEM. Columnar crystals 0.2-0.27 μm in width were also noted around these crystals (Fig. 8).

TEM also disclosed laminated circular structures 1-5 μm in diameter surrounding the core in the lumen of distal renal tubules. The core was composed of irregularly shaped highly dense components, and microgranular material was distributed sparsely around it. This was further surrounded alternately in a laminated fashion by dense and loose layers of granular material. At a higher magnification, layers of granular material appeared to have the same shape and structure and were considered to grow by repeated agglutination and fusion (Figs. 9 and 10).

Fig. 11 shows a SEM image of a section of a spherical structure found in the lumen of the distant tubule. In the center of the section, crystals of irregular shapes and crystallizing substances were noted in the microgranular material.

Discussions and Conclusions

Many of the pathogenic factors in urolithiasis are those of systematic nature such as endocrine disorders and disturbances of electrolyte metabolism in the blood and urine. For this reason, the importance of the pathophysiological effects of the diet on urolithiasis is widely recognized. In this study, we evaluated the effects of an oxalate diet in the presence of chronic mild hyperuricemia in rats. Such an in vivo study using combination administration of experimental diets is unprecedented [7]. In in vitro studies, Coe et al. [2], and Pak and Arnold [12] observed that seeds of urate induce crystallization of calcium oxalate in supersaturated calcium oxalate solutions, pH 5.7-6.7. Meyer [9] added seeds of urate to supersaturated solutions of calcium oxalate of various concentrations and observed a progression of crystallization under SEM. They observed the coexistence of acicular crystals of urate and calcium oxalate and suggested interrelationship among urate, calcium oxalate, and pH in this process. In the present study, we noted marked crystal deposition in the kidney after administration of combination diets containing oxalic acid and uric acid at concentrations that cause no crystallization in the kidney for about 1 month when given alone. The significant increases in urinary oxalic acid excretion and kidney tissue oxalic acid concentration in the OXD-UA group as compared to those in the OXD group, despite the lower acid content in the diet in the OXD-UA group, were characteristic in vivo biochemical changes. These findings suggest that hyperuricuria induced salting out in the lumen of renal tubules, promoting crystallization of poorly soluble calcium oxalate and, thus, inhibiting oxalic acid absorption in the renal tubules by passive diffusion.

Light microscopic observation of serial sections of the kidney showed a mixed presence of microcrystals emitting refractive polarized light and dark brown De Galantha-positive deposits everywhere in the tubular lumen. This finding, indicating the coexistence of calcium oxalate and urate crystals, is sufficiently suggestive of epitaxial growth in the renal tubules. The process leading to this epitaxial growth is speculated on the basis of electron microscopic findings. Little changes were noted in the glomeruli, but the lumen of the proximal renal tubules was filled by cytoplasmic matrix, organelles, and granular material released from cells due to disruption of the brush border by urate and oxalate. These became smaller in size depending on the conditions of the tubular lumen, advancing from the sol state to the next stage. Namely, gel-like material was noted in the lumen of proximal renal tubules with acicular crystalloids emerging from inside this gel-like material. Furthermore, these acicular crystalloids were occasionally found to cluster with one another. SEM also demonstrated acicular crystalloids of the same size and lamellar crystals with the same width as urate crystals. These structures were considered from their morphology and size to be urate crystalloids and crystals [8]. Therefore, urate crystals are considered to be formed in a process preceding from urate sol to lyogel, to xerogel, to needle-like urate crystalloids, and then to urate crystals.

The circular structures observed under TEM are considered three-dimensionally to be spherules. The highly electron dense cores were identical to the cytoplasmic matrix, organelles, and granular substances observed in the lumen of proximal tubules (Figs. 4 and 5). The spherules centering around these cores were formed by alternating agglutination and fusion of layers with identical structures. These observations suggest that the spherules at this stage were still highly fluid, being in the gel state. In the wide and loose layer of microgranules in enlarged spherules, granules were arranged in radial patterns but no evident crystals or crystal ghosts were demonstrated. However, the morphology and structure of these spherules indicated that they consisted of uric acid [1, 8, 15].

Urinary uric acid is reported to be saturated at pH 6.0 and supersaturated to 4 times the concentration of the saturation point at pH 5.0 [13]. Continuous aciduria induces formation of uric acid stones, and urate stone formation is promoted at pH 6.0-7.0 [17]. In our study, the urinary pH was significantly more acidic in the OXD-UA group than in the OXD group or the control group. However, these values were obtained in discharged urine and not in the urine in the lumen of renal tubules. The development of uric acid spherules and urate acicular crystalloids is considered to have been due to changes in the biochemical milleu and pH in the lumen of renal tubules. Urate crystals appeared to develop via the growth of acicular crystals around nidi of urate crystalloids themselves, but uric acid crystals were considered to be formed as fluid microspherules develop around nidi of cellular components and grow into spherulitic bodies, leading to crystallization depending on changes in the internal milleu. The deposition or precipitation of polarizing crystals and De Galantha-positive crystals in the same sites of renal tubules and renal papillary canals further indicated that these uric acid or urate crystals induce and promote epitaxial growth of calcium oxalate crystals by providing seeds for crystallization [3, 10]. Further studies on the in vivo interaction between uric acid and calcium oxalate are needed.

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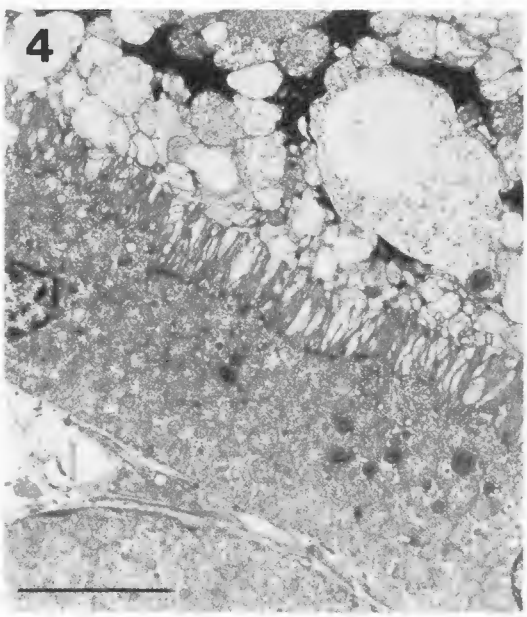
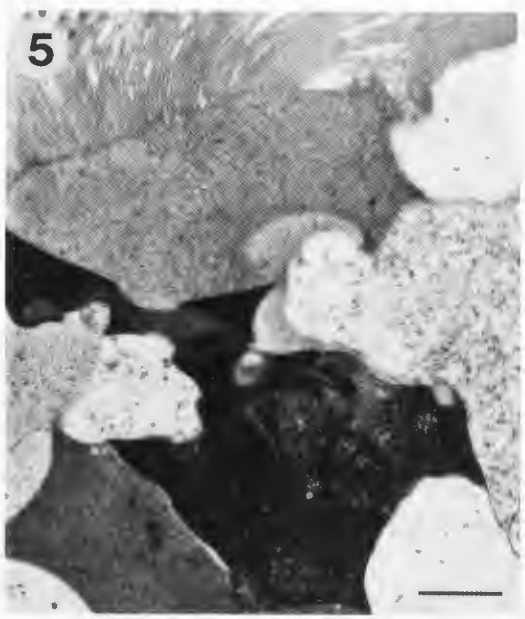




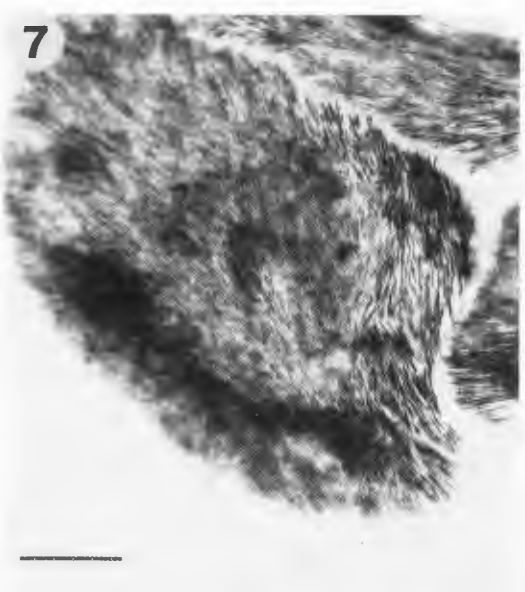
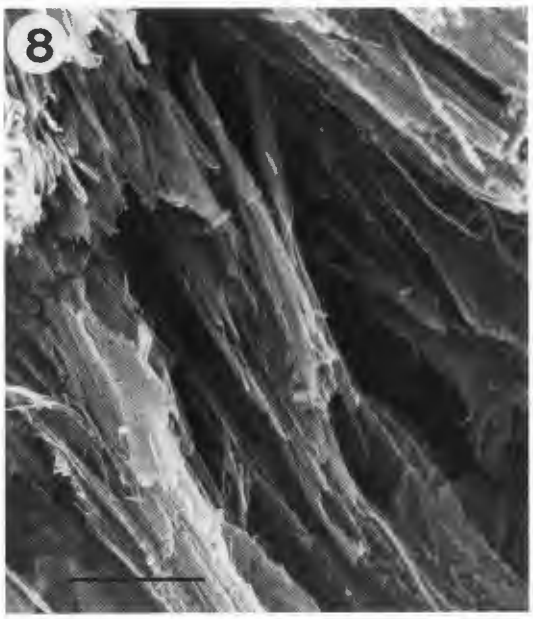


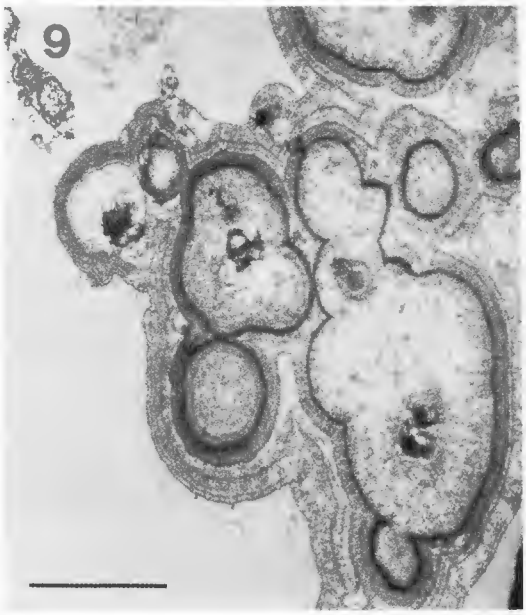
Figure 4. TEM image of the kidney of the OXD-UA group. The lumen of the proximal renal tubule was filled by the brush border showing saccular enlargements and glomerular filtrates. Bar = 2 μm.

Figure 5. Enlargement of Fig. 4. The lumen is filled by saccular tips of the brush border, organelles, cytoplasmic matrix, and urine. Bar = 2 μm.

Figure 6. Electron dense gel-like material and precipitating acicular crystalloids observed in the lumen of the proximal renal tubule. Bar = 0.5 μm.

Figure 7. Lamellar structure consisting of aggregation of circular crystalloids shown in Fig. 6. Bar = 1 μm.





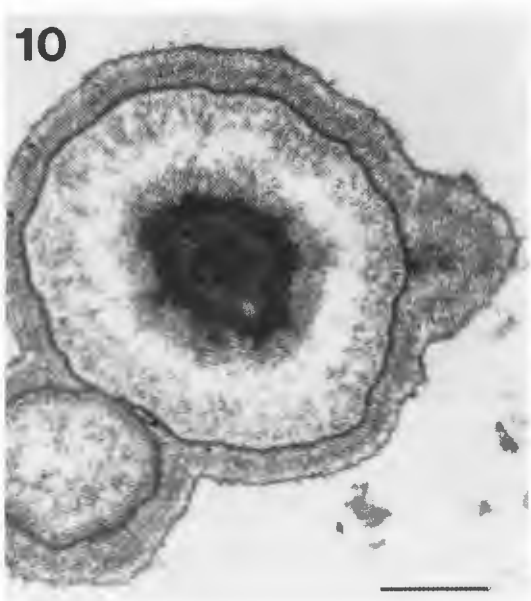


Figure 8. SEM image of acicular crystalloids with columnar crystals around them. Bar = 1 μm.

Figure 9. TEM image of the lumen of the distal renal tubule of the OXD-UA group. Laminar circular structures developing around a core and their clusters are observed in the lumen. Bar = 4 μm.

Figure 10. Enlargement of Fig. 9. The core of the laminar structure consists of highly dense cellular components. In the layer of loosely distributed material, the granules appear to be undergoing rearrangement into radial patterns. Bar = 1 μm.

Figure 11. Section of spherical structure observed in the lumen of the distal tubule of the OXD-UA group. Irregular crystals are emerging from the microgranular substance in the center. Bar = 10 μm.

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Discussion with Reviewers

W.G. Robertson: In the light of the fact that uric acid is not normally ingested in the diet, thereby removing the possibility of conversion to oxalate in the gut, what relevance does the study have to kidney stone disease in human?
Authors: As you pointed out, uric acid is not normally ingested in the diet. However, hyperuricemia and hyperuricuria are well known pathologic conditions. Moreover, urate colloid in urine is suggested to be a promoter of crystallization. Therefore, we think it is very important to clarify how hyperuricemia is involved in kidney stone formation in animal models with the above pathologies.

W.G. Robertson: What is the evidence for true epitaxy in your study?
Authors: The light photomicrographs display a large number of crystals of oxalate showing refractive polarization of light, and those of De Galantha-positive urate were observed as mixed crystals at the same sites in the renal tubules in serial sections of the kidney.

E.L. Prien: What methods were used to determine the uric acid, citrate, and especially oxalic acid (which is notoriously difficult)?
Authors: The uric acid level was measured by the uricase-peroxidase method, and citrate by an enzymatic assay using citrate lyase. Oxalic acid was measured using a commercial kit for oxalate assay (Kit No. 590 OXALATE, SIGMA).

S.R. Khan: What do you think is the role of citric acid in urolithiasis in your animals?
Authors: The rats given the mixed diet became acidotic because of the synergy between chronic hyperuricuria and chronic hyperoxaluria. This causes an increase in citric acid re-absorption by the renal tubules and relative decrease in urinary citric acid excretion. This reduction in urinary citric acid excretion is considered to have a stimulative effect on the formation of calcium oxalate crystals and urate crystals.

P.-T. Cheng: What is the mechanism for the synergistic elevations of both urinary oxalic acid and urinary uric acid and the concomitant reduction in urinary citric acid?
Authors: It is well known that both uric acid and oxalic acid are reabsorbed in proximal tubules. Therefore, chronic hyperuricuria and hyperoxaluria caused by the mixed diet synergistically disrupt the molecular structures of the epithelial cells of proximal tubules and their cytoplasm. This induces impairment of the mechanisms of re-absorption and secretion of uric acid and oxalic acid, resulting in increases in their urinary excretion.

The amount of citric acid re-absorption by proximal tubules is determined by the gradients in the citric acid concentration and pH between the cytoplasm and mitochondria of the tubular cells. The synergism between oxalic acid and uric acid widens these gradients, leading to an increase in citric acid re-absorption and a relative decrease in urinary citric acid excretion.

S.R. Khan: Since both calcium oxalate and urate crystals were found together in the kidneys of rats receiving mixed diets, why do you think that urate crystals promote calcium oxalate crystallization?
Authors: No oxalate crystal formation was observed throughout this study in the animals administered OXD alone. However, TEM showed stone embryos of uric acid or urate from early stages, and calcium oxalate crystals and mixed crystals of calcium oxalate and uric acid later, in the kidneys of rats in the mixed diet group. These findings suggest that urate or uric acid colloid is promoter of crystallization of these materials.

P.-T. Cheng: What evidence is there in this in vivo study that it is urate crystals which form first and not oxalate crystals?
Authors: Hyperuricuria would acidify the glomerular filtrate in the renal tubules and facilitate formation of urate colloid. Light microscopy suggested that the crystals that formed first were De Galantha-positive urate crystals, not polarized or refractive crystals.

S.R. Khan: Do rats on the oxalate diet that you gave, ever produce calcium oxalate crystals in the kidneys?
Authors: In our previous study (Kawada K. (1975). Study of mechanism of oxalate stone formation and prevention of recurrence of it. Nishinihon J. Urol. 37, 25-52), calcium oxalate crystals were observed in the kidneys of all rats given a diet containing 2% oxalic acid after 30 days. However, no crystal formation was noted in the group given a diet containing 1% oxalic acid during the same observation period.

P.-T. Cheng: At what stage (time and location) do oxalate crystals first appear?
Authors: Oxalate crystals appeared in distal tubules or collecting tubules after the development of uric acid crystals or urate crystals, i.e., about 3 weeks after the beginning of the study.

E.L. Prien: What does the De Galantha stain indicate?

Authors: De Galantha stain is a silver method described by Elena De Galantha (Am. J. Clin. Pathol., 5, 165-166, 1935). By this method urate crystals are stained dark brown to black, and connective tissue is stained yellow. The usefulness of identification of urate crystals in hyperuricemic nephropathy has beendemonstrated by Waisman [Waisman J, Bluestone MB, Klinenberg R. (1974). A preliminary report on nephropathy in hyperuricemic rats. Lab. Invest. 30, 716-722], Sommers [Sommers SC, Curg J. (1982). Kidney pathology in hyperuricemia and gout. In: The Kidney in Gout and Hyperuricemia, Yü TF, Berger L (eds.), Futura Publishing Inc.], and others.

S.R. Khan: Please provide the urinary pH of various animals.

Authors: The urinary pH of our animals were:

Control	7.12 $\pm$ 0.33
OXD group	6.32 $\pm$ 0.07
OXD-UA group	5.60 $\pm$ 0.08

The value for the OXD-UA group was significantly reduced ($p < 0.05$) as compared with other two groups.