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Calcium Oxalate Crystal Formation in the Kidneys of Rats Injected with 4-Hydroxy-L-Proline

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Summary. Calcium oxalate crystal formation induced in rat kidneys by intraperitoneal injection of 4-hydroxy-L-proline were studied by chemical, microscopic and size analysis techniques. Crystal growth rates were determined at different intervals of time from the size distribution curves. Calcium oxalate dihydrate (weddelite), formed in the first 2 hour after injection, undergoes gradual phase transformation to the more stable monohydrate (whewellite). This finding emphasizes the significance of crystal transformation in the development of experimental nephrolithiasis.

Key words: Experimental nephrolithiasis - Calcium oxalate - Growth kinetics - Phase transformation.

The development of a refined animal model to quantify the possible methods of treating calculus diseases has become indispensable. There have been some significant advances in our understanding of experimental urolithiasis since ethylene glycol (7), oxalic acid (10) and glyoxylic acid (8), were used to provoke intratubular crystallisation of calcium oxalate. Calcium oxalate deposition in rat kidneys was observed after intraperitoneal injection of 4-hydroxy-L-proline by Thomas et al. (11). More recently, Jordan et al. (6) using the modified techniques of Van't Reit, studied the kinetics of calcium deposition in rat kidneys after an intraperitoneal injection of sodium oxalate.

More insight in to the process of nephrolithiasis could be gained if one could characterise the nature of calcium oxalate crystals formed experimentally and examine the growth kinetics of these crystals. It is the purpose of this work to evaluate the growth kinetics of calcium oxalate after intraperitoneal injection of 4-hydroxy-L-proline in rats and to monitor by size analysis technique, the rate of growth and to characterise the nature of calcium oxalate crystals formed in experimental nephrolithiasis.

MATERIALS AND METHODS

Sprague-Dawley male rats of 300-350 g were used. The reagents used were as follows: 4-hydroxy-L-proline (crystalline) (Sigma); calcium analysis reagents (Corning Scientific); Soluene-100 (0.5 N Quaternary Ammonium Hydroxide in Toluene) (Packard); Benzene.

Induction of Crystal Growth in Rats. This was achieved by the administration of 4-hydroxy-L-proline 2.5 g/kg intraperitoneally (10 ml/kg); control animals received 10 ml/kg saline intraperitoneally. Animals were then placed in metabolic cages for urine collection. At different time intervals after drug treatment, groups of animals were sacrificed and both kidneys removed. One kidney was used for calcium analysis and the other kidney was kept overnight at -12°C and was used for crystal growth studies. Urine and, in some cases, blood were collected for examination and analysis.

Calcium analysis. One kidney from each animal was incinerated at a temperature of 1100°C. The residue was left to cool to room temperature and later, dissolved in 5 ml of 1 N HCl, and the Ca^{++} concentration was determined. The collected urine was centrifuged at 5000 rpm for 15 min. the supernatant was analysed directly and the sediment was treated in the same way as the incinerated kidney before calcium determination. All calcium determinations were carried out

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using the Corning calcium analyser. The principle is a complexometric titration of calcium with a fluorescent derivative. The analytical procedure is based upon the quenching of this fluorescence by chelating the calcium ions with the titrant ethylene glycol N, N, N', N', tetra-acetic acid (EGTA). The functioning principle is explained in detail by Holtkamp et al. (5)

Crystal Growth Studies. Frozen kidneys were sliced and digested for 2-4 hours in soluene-100 until complete solubilization of the tissue, using 1 ml of solubilizer per 100 mg of tissue at a temperature of 50°C. The crystals were examined by a polarized light microscope to verify that soluene-100 did not affect the crystal morphology nor the crystal size during the separation process. This was done by comparing the morphology and size of the separated crystals with those present in kidney slices. Very slight etching of the surface only was noticed when the crystals were left in contact with soluene-100 for a prolonged period of time (more than 12 hours).

To facilitate separation of the calcium oxalate crystals, the digested material was diluted with 20 ml of benzene. After the complete sedimentation of the calcium oxalate crystals, the benzene was aspirated by a Pasteur pipette. The process was repeated several times to eliminate solubilized organic material. The time required for complete separation of the calcium oxalate crystal is 24 hours. Having collected the calcium oxalate crystals from the kidneys for each time interval, crystals were suspended in 5 ml of physiological saline saturated with calcium oxalate. The crystal size characteristics and size distribution were determined by a Coulter Counter Model TA II, with a 16 channel analyser using a 100 μ aperture tube. Particle size parameters including distribution based on number, volume, both differential and cumulative, were monitored. The overall growth rate of calcium oxalate crystals, was determined in cm/sec from the change in the cumulative crystal size distribution curves, at different periods of time. This technique has been previously reported from our laboratory (2). Later, the crystals were examined by scanning electron microscope (Cambridge S-4).

Because of the possible effect of temperature and time on the size characteristics of the calcium oxalate crystals formed in the kidney, a series of experiment were conducted to verify the effects of these variables. It was found that storage of kidneys at -12°C overnight did not affect the size characteristics of the crystals separated. This can be seen in Figure 4.

RESULTS

Morphology

Intraperitoneal injection of 4-hydroxy-L-proline (Hy P) 24 hr before sacrifice caused a definite change in volume and weight of the kidney. In some cases, the change was remarkable and in 4-Hy P-treated animals, the kidney doubled in volume and weight when compared with the controls. This can be attributed to the formation and deposition of a massive quantity of calcium oxalate crystals, the subsequent blocking of the renal tubules and the development of oedema. Figures 1 and 2 show the morphological changes and the massive amount of calcium oxalate crystals found after 24 hours of 4-hydroxy-L-proline administration.

Calcium Content

The urine collected was subjected to different tests. The volume did not vary significantly between control and treated animals but the pH was slightly higher for the treated (7.3) compared to the control animals (6.6). The calcium was analysed both in the supernatant (Ca^{++} in solution) and in the sediment (Ca^{++} crystalline form) separated by centrifugation. The total concentration of the calcium in urine collected during 24 hours was significantly higher in treated animals (2.55 vs 7.24 mg %). No significant differences were observed between the Ca^{++} plasma level in both control and treated rats. The analysis of calcium in the kidney after incineration is shown in Table 1 which summarise the results in these determinations. A significant difference ($p < 0.01$) was observed between the calcium concentration in the control and treated animals after 24 hours.

The kinetics of calcium oxalate formation are presented in Figure 3 where the Ca^{++} concentration was determined in the kidney as a function of time after 1, 2, 4, 8, 12, 18 and 24 hours respectively.

It is of interest to note that the increase in the renal Ca^{++} level was evident 2 hours after Hy P injection, a period which could account for biotransformation and a transport barrier effect in the animal model. The concentration increased gradually and tended to stabilise after 12-24 hours. The calcium ion concentrations in the kidney (Table 1) agree with Thomas' findings (11). The time course of the calcium concentration (Fig. 3) is consistent with recent data reported by Finlayson's group (6) using sodium oxalate to induce calcium oxalate deposition in rat kidneys.

Growth Rate Studies

Since the measurement of Ca^{++} concentration in the kidney cannot distinguish between free Ca^{++}, tissue Ca^{++} and calcium deposited as calcium oxalate as mineral phase, the evaluation of growth kinetics of the calcium oxalate crystals, seems to be more appropriate in the study of experimen-

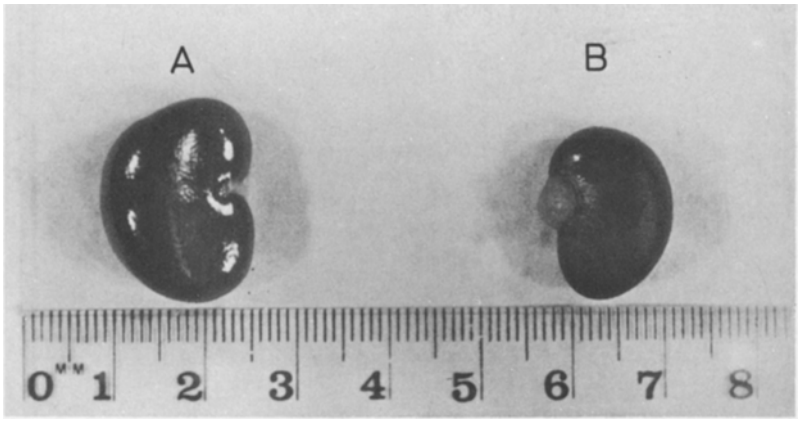

Fig. 1. Morphological change in rat kidney after 24 hrs, following the intraperitoneal injection of 4-HyP (2.5 g/kg); A 4-Hyp treated; B Control, untreated

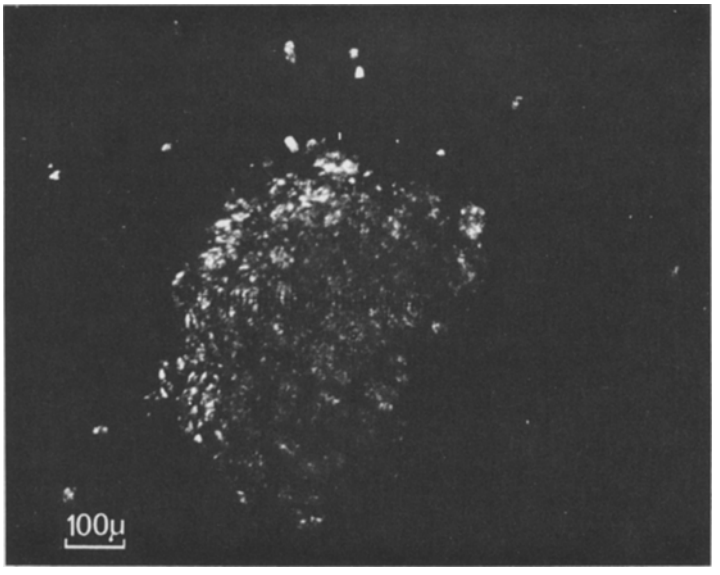

Fig. 2. Calcium oxalate crystals found in the renal pelvis 24 hours after treatment with 4-HyP X125

Table 1. Weight and concentration of calcium in the kidneys of Sprague-Dawley male rats after injection of: distilled water (10 ml/kg animal) for the controls; 2.5 g of 4-Hydroxy-L-Proline in 10 ml of distilled water per kg of weight. Calcium concentration is given in mg per g of kidney weight

Kidney	Controls		Experiment	
	Weight in g	Calcium mg/g	Weight in g	Calcium mg/g
1	1.359	0.697	1.424	1.919
2	1.251	0.472	1.442	1.944
3	1.211	0.797	1.735	1.239
4	1.187	0.747	1.780	1.242
5	1.079	1.019	1.387	1.871
6	1.222	0.937	2.158	2.097
7	1.010	1.140	1.103	1.030
8	1.211	1.045	1.061	0.989
9	1.188	0.916	1.923	1.118
10	1.206	0.602	1.544	1.647
$\bar{X}$	1.207	0.837	1.556	1.517
S.E.	0.028	0.063	0.104	0.126

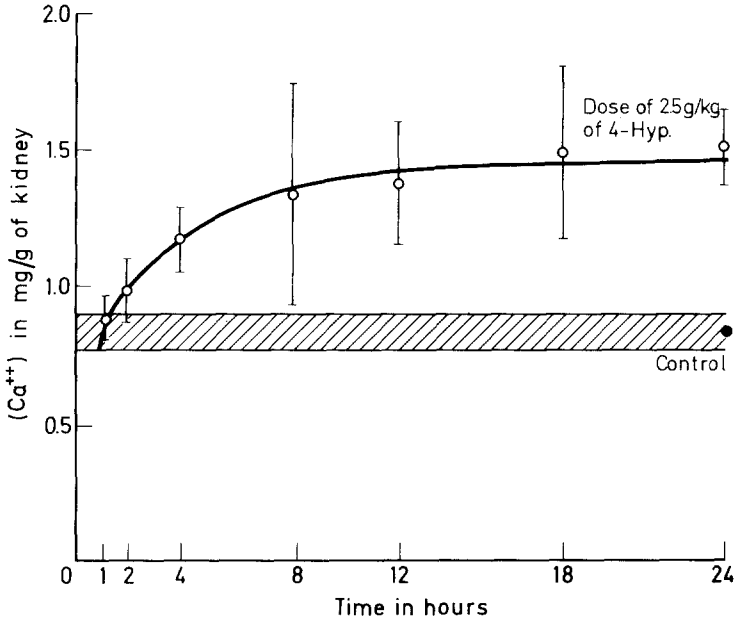

Fig. 3. Kinetics of Calcium oxalate formation and deposition as determined by the Calcium concentration in the kidneys. Each point is the mean of 3 to 10 rats ± the standard error

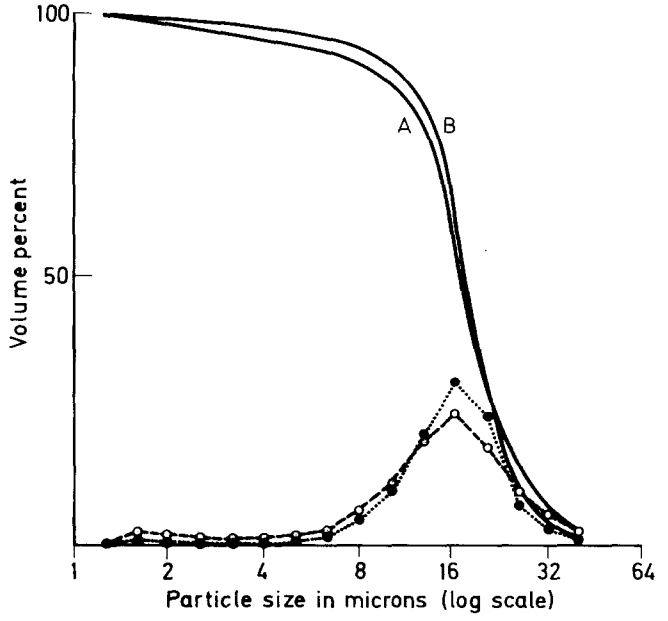

Fig. 4. Differential and cumulative size distribution curves of calcium oxalate crystals as measured by a Coulter counter; A) crystals obtained from kidneys kept overnight at -12°C B) crystals obtained from kidneys sliced and treated with Soluene-100 immediately after their removal from the animals. The animals were sacrificed 4 hours after treatment with HyP 2.5 g/kg (i.p.)

tal nephrolithiasis. The particle counting technique used in this study offered a valuable method for evaluating the crystal growth process and the change in crystal size distribution as a function of time.

Figs. 5 and 6 demonstrate the effects of time on the differential and cumulative distribution curves of calcium oxalate crystals. The shift in the mean volume diameter was used to calculate the growth rate in cm/sec. Table 2 shows the different rates of growth determined from the crystal size distribution in the 24 hours after the injection of HyP.

S. E. M. Studies

In addition to optical microscopy, the mineral phase, separated from the kidneys, was examined by the scanning electron microscope. The samples were deposited on a Aluminium Copper mount and coated with gold (approximately 200 Å thick). The improvement in resolution obtained by the scanning electron microscope has made it possible to observe clearly and to characterise 2 different forms of calcium oxalate crystals: The dipyramidal, octahedral calcium oxalate dihydrate (weddellite) and the calcium oxalate monohydrate (whewellite). The monohydrate was the predominant form found at all different periods of time. However, calcium oxalate dihydrate was present in appreciable quantity in the early hours after administration of 4-hydroxy-L-proline. This form tended to diminish gradually in number and was less frequently observed later. Figures 7, 8 and 9 represent the different forms of calcium oxalate crystals observed using the scanning electron microscope. Kidneys from control animals sliced and digested with soluene-100 yielded no calcium oxalate crystals.

DISCUSSION

To gain more insight into the process of urolithiasis, it is essential to have clear understanding of the kinetics of crystal growth and to

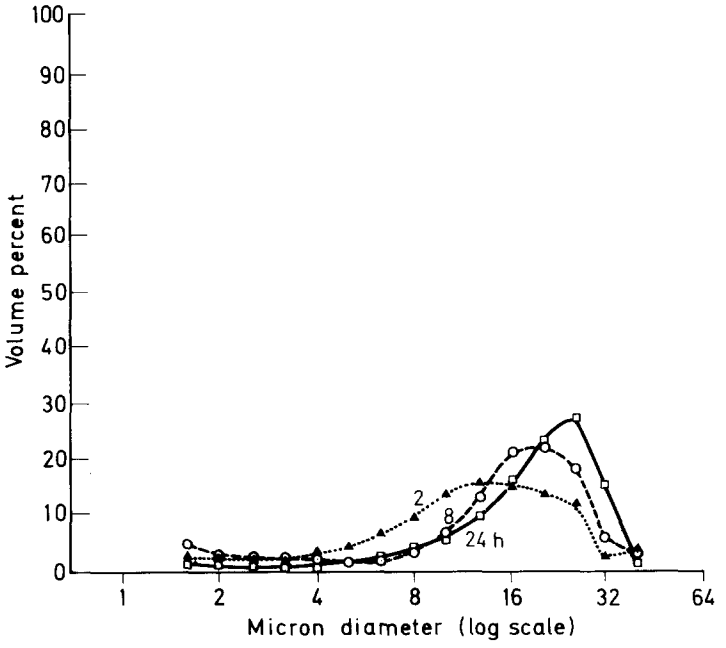

Fig. 5. Differential crystal size distribution curves of calcium oxalate as measured by a Coulter counter (model TA II) using a 100 μ aperture tube

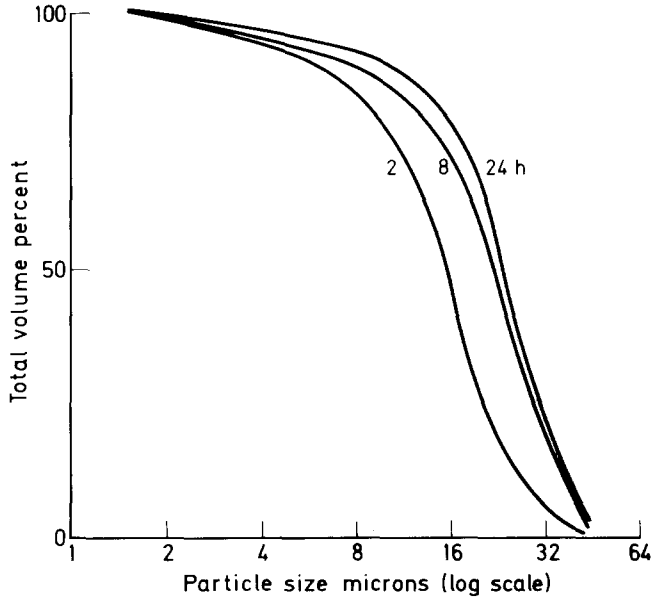

Fig. 6. Kinetics of crystal growth, cumulative size distribution of the crystals of calcium oxalate collected from the kidneys after different post-injection intervals

Table 2. Calcium oxalate crystal over-all growth rates as calculated from the cumulative distribution curves using the Coulter counter.
Δr: the change in crystal radius corresponding to 50% volume

Time interval in hours	Mean volume diameter in microns	Δ r in microns	Growth rate $= \frac{\Delta r}{t}$ cm/sec
0-2	16	8	$11.0\ 10^{-8}$
2-8	23	3.5	$1.62\ 10^{-8}$
8-24	26	1.5	$0.26\ 10^{-8}$

characterise the possibilities of phase transformation during the growth process. Like any crystal growth by chemical reaction, the formation of calcium oxalate in rat kidney involves a number of processes, one or more of which can establish the overall growth rate. Under these conditions, the bio-transformation of HyP, the transport processes and diffusion of reacting ions will be important in determining the growth rates. The crystallisation medium and kidney environment might control the crystal form, geometry and eventually habit modifications.

As seen in Table 2, the growth rate of calcium oxalate tended to decrease gradually as a function of time, to a zero growth rate after 24 hours. The decrease in the growth rate can be attributed to : i) the depletion of oxalate ion required to form the calcium oxalate. ii) phase transformation of the less stable dihydrate to the more thermodynamically stable, less soluble monohydrate. iii) the adsorption of impurities on the crystal surface which tends to inhibit the crystal growth process.

Recently, we examined in our laboratory, the crystal size and size distribution of the mineral phase in different renal stones. It was interesting to find that the mean size of the building unit lies between 21-28 μ. In this study, the size distribution curves (Fig. 6) at different periods of time indicated that the crystal size obtained after 24 hours of injection of HyP was about 25 μ.

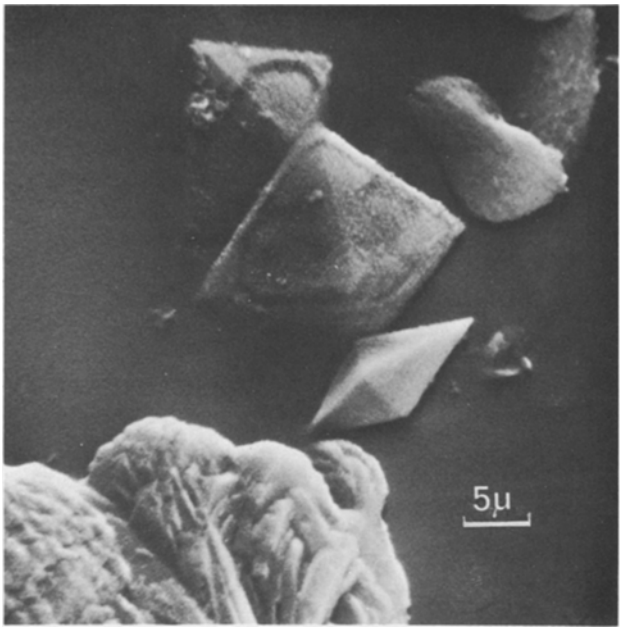

Fig. 7. Scanning electron micrograph of calcium oxalate dihydrate crystals (Weddellite), isolated from kidneys of rats 4 hours after treatment with 4 HyP. X2000

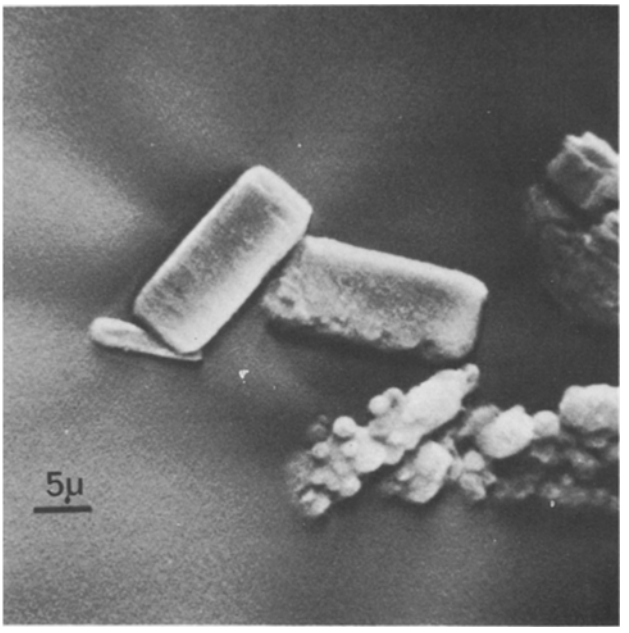

Fig. 9. Scanning electron micrograph of calcium oxalate monohydrate crystals (Whewellite), isolated from kidneys of rats 24 hours after treatment with 4 HyP. X1900

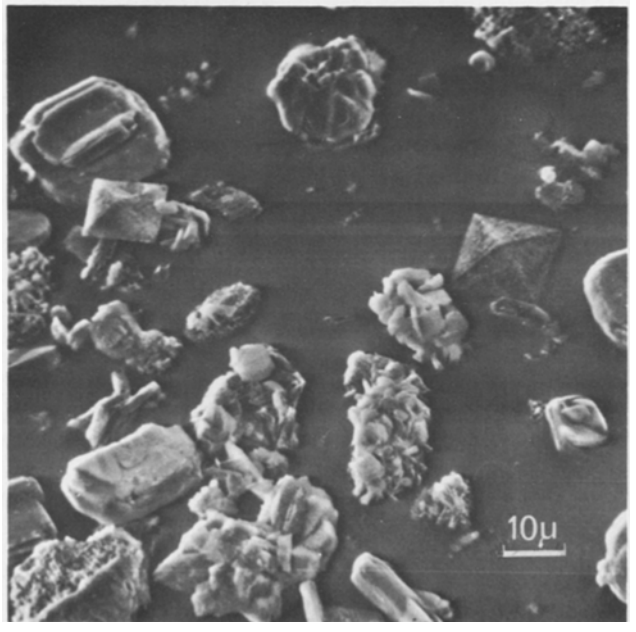

Fig. 8. Scanning electron micrograph of monohydrate and dihydrate crystals isolated from kidneys of rats, 12 hours after treatment with 4 HyP X1000

This size represents the mean diameter corresponding to 50% volume and it is in good agreement with the mean size of the building unit in most renal stones examined in our laboratory (9).

Hess et al. (4) investigated the formation of whewellite and weddellite in vitro. Using different precipitation models, the authors studied the influence of cationic minerals on the transformation of weddellite into whewellite. This transformation process required few days in the absence of stabilizers. In presence of Mg^{++} this conversion could be prevented. On the basis of these observations, and other earlier documentation (1, 3), the authors suggested the possible conversion of weddellite to whewellite in urinary calculi.

In this work, the study of the mineral phase by SEM revealed some new information about the nature of calcium oxalate crystals formed in vivo. It was interesting to find that solid-solid transformation took place in vivo confirming the in vitro work of Hess (4). The less stable calcium oxalate dihydrate undergoes rapid phase transformation into the more thermodynamically stable, less soluble calcium oxalate monohydrate. In the rat kidney both calcium oxalate monohydrate and dihydrate were present at the early time intervals studied, and we observed the gradual decrease in dihydrate crystals as a function of time. The transformation to monohydrate took a few hours in vivo, whereas similar transformation required several days to occur in vitro (4). It was also noticed that the calcium monohydrate crystals differed significantly from the dihydrate crystal. The monohydrate form was often covered with a thin layer of adsorbed organic matter, in spite of repeated washing with benzene. This organic matter is absent from the surface of the dihydrate crystals which always possess a well

defined geometrical shape and smooth crystal faces and sharp interfacial angles.

The characterization and quantification of the factors which control the dihydrate phase transformation and the utilization of new techniques for probing the crystal interface on the molecular scale could increase our understanding of the process of stone formation and could uncover some of the mechanisms involved in this process.

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