Crystallization and Stone Formation Inside the Nephron

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Abstract

A model is presented visualizing the events leading to calcium-salt, crystal- and stone-formation inside the nephron. For each nephron segment, handling of urine components relevant to stone formation is considered and urine composition determined. This information was applied to nucleation experiments simulating passage of urine through a nephron. The model and *in vitro* experiments suggest that within normal transit times for the respective nephron segments, particles of a hydroxyapatite-like material first form near the bend in the Loop of Henle of juxtamedullary nephrons. From there on, calcium oxalate particles start to appear: first dihydrate, then monohydrate. In the collecting duct system, particle size increases primarily due to crystal agglomeration. Several conclusions with clinical and experimental relevance can be drawn. An increase in urinary volume does not decrease the chance of crystal formation in the Loop of Henle, but does decrease passage time through the collecting ducts, and thus, the time allowed for large particle formation. A calcium load does not increase the risk for nucleation up to the distal tubule, but does increase the risk of large particle formation in the collecting ducts. An oxalate load increases the chance for nucleation throughout the nephron. For experiments simulating crystallization processes occurring inside the nephron, diluted urines should be used. They should be diluted 16 to 50 times for testing nucleation, 2 to 30 times for testing crystal growth, and 2 to 20 times for testing crystal agglomeration. Undiluted urines may be used to mimic conditions in the pelvis and the bladder.

Key Words: Crystallization, calcium oxalate, urolithiasis, nephron.

Introduction

Crystalluria, the formation of particles which flow freely with the urine, is common for most people. However, formation of a particle which is not excreted freely, urolithiasis, occurs only in 5 to 9% of the population. Theoretically, and as seen in animal experiments, the step from a free particle to a stone probably involves particle retention [20, 31, 35, 36]. This may be a fixed particle mechanism, fixation of particles to the nephron wall or trapping in sites with poor flow-conditions, a free particle mechanism, formation of particles too large to be excreted freely, or a combination. Ideally, the fixed particle mechanism is preferred up to the late distal tubule. However, under hyperoxaluric conditions, a free particle mechanism seems to occur in the proximal tubule [31]. In the collecting ducts and the duct of Bellini, both a fixed and a free particle mechanism are likely [35]. The chances for the latter are especially increased by the size increasing process known as crystal agglomeration [35]. A role for crystal agglomeration in stone formation has been subscribed by experimental data. Urine contains numerous compounds capable of effecting crystal agglomeration [17, 25, 38, 60]. The ability of urine to inhibit crystal agglomeration is decreased in stone formers [19, 37, 40]. This may be due to a low citrate excretion [19, 27, 37, 40], or aberrant action of urinary proteins like crystal matrix protein [60], Tamm-Horsfall protein (THP) and nephrocalcin (NC) [22, 26, 58].

A problem with these *in vitro* data is that the test conditions may not reflect the conditions at the site of stone formation. But, what are those conditions? It appears obvious that crystallization experiments should be performed using whole, undiluted urine. However, whole undiluted urine is such a strong inhibitor of crystallization processes that for crystallization to take place, usually extra calcium or oxalate has to be added [50, 54]. This makes one wonder how any crystals could ever form in urine. Then, there is the phenomenon of patients who comply very well to their drinking advice and produce high volume urines. Often, these urines

have saturation levels so low that nucleation occurs only after very prolonged times or not at all. Still, these patients may continue forming stones.

The answer to these questions may lie in the kidney function. A kidney daily concentrates a large pool of filtered plasma to a relatively small volume of urine, in the process, removing and adding compounds which play a role in stone formation. During its passage through the nephron, the composition of the urine continuously changes and differs greatly from that of the pelvic urine. Further addition of compounds and cellular debris in the pelvis and bladder complete the final composition. In this paper, I try to model the changing conditions along the nephron and relate these to the chance of particle and stone formation inside the nephron. For most parameters relevant to urolithiasis, these changes under normal physiologic conditions per nephron segment are well described or can be assessed reasonably. The parameters included here are calcium, oxalate, citrate, phosphate, uric acid, sodium, potassium, chloride, magnesium, NC, pH, THP and volume. The following questions are addressed: where is crystallization most likely to start; what are the roles of calcium phosphate and calcium oxalate crystallization and of kidney anatomy; where and how do alkali-therapy, protein-, calcium- or oxalate-restriction, and drinking advice influence the risk for stone formation; what is the most realistic way for testing urine samples: *in vitro*, undiluted or diluted?

Abbreviations Used and Nephron Segmentation

The nephron is subdivided into proximal tubule; Loop of Henle (LH): descending (DLH) and ascending (ALH), short and long (SDLH, LDLH, SALH and LALH); distal tubule (without connecting tubule segment, CNT); outer and inner medullary collecting duct (OMCD and IMCD); duct of Bellini, and collecting duct system (CD, consisting of CNT + OMCD + IMCD + DOB). Abbreviations used are: TF/P and TF/UF, concentration ratios of tubular fluid to plasma and plasma ultrafiltrate respectively; THP, Tamm-Horsfall protein; NC, nephrocalcin; COM, calcium oxalate monohydrate; HAP, hydroxyapatite; UA, uric acid. The distal tubule referred to in micropuncture studies includes the CNT, which differs in properties from the true distal tubule, especially in its capacity to resorb water. Here, the CNT is included in the CD.

Data Used

Concentration limits and conditions per nephron-segment are calculated using limit-values for 24-hour excretion, plasma and ultrafiltrate concentrations and percent resorption/excretion per segment, mostly from ref. [9],

Table 1. A distinction is made between superficial and juxtamedullar nephrons. The latter have a larger glomerulus diameter, longer LH with a thin segment, different resorption characteristics, and in humans, constitute 14% of the nephrons. The values are compared to micropuncture data [54], which usually describe the proximal tubule and distal tubule of superficial nephrons and the LH of juxtamedullary nephrons. Conditions in the proximal and distal tubule of juxtamedullary nephrons and the LH of superficial nephrons must be assessed by extrapolation.

Volume

Per minute, 125 ml serum is filtered, yielding 58 ml/min filtrate per kidney and a total of 167 l/24 hr. Volume is reduced by reabsorption of water in three compartments of the nephron: the proximal tubule, the DLH and the CD to the daily urine volume of 0.6 to 7.5 liters (0.2 to 2.6 ml/min per kidney). During short time periods, flows up to 5.8 ml/min can be sustained. The fine regulation related to dietary load occurs mainly in the CD. Micropuncture studies in superficial nephrons show that 35% of the filtered load leaves the proximal tubule and 20% enters the distal tubule [9, 54]. Thus, 15% is resorbed in the SDLH. In juxtamedullary nephrons, the TF/P inulin ratio at the bend of the LH at the papillary tip averages 1:11.3, with extremes up to 1:18.1. Thus, volume is reduced to between 9 and 5% [21, 29, 42]. When the proximal tubule of juxtamedullary and superficial nephrons act alike, 65% resorption, then the LDLH resorbs 26 to 30% of the filtered load. These numbers probably are underestimates of the true concentrating capacity. In micropuncture experiments, exposure of the papilla results in a loss in concentrating capacity of up to 57% [44, 53, 57], probably because urea can no longer diffuse from the pelvic urine to the medullary interstitium, diminishing the effectivity of the countercurrent mechanism. Resorption up to the CD is fairly independent of the diuresis state, depending mainly on glomerular filtration rate. Regulation from the 5 to 20%, leaving the distal tubule to the daily end volume, occurs in the CD (Table 1).

Calcium, sodium, magnesium, chloride

The normal values for UF_{Na}, UF_{Cl}, UF_{Ca} and UF_{Mg} are 145 mM, 122 mM, 1.45 mM and 0.53 mM. The 24-hr excretion ranges (mmoles) I used are 50 to 400 Na, 50 to 400 Cl, 0.5 to 22 Ca and 0.5 to 10 Mg. Dietary loading with these compounds does not affect filtered load, but is regulated in the distal tubule and first part of the CD. In superficial nephrons in rodents, at the end of the proximal tubule, TF/UF_{Na} averages 1.03, TF/UF_{Ca} 1.2, TF/UF_{Mg} 1.8 [1, 15, 43, 45, 48, 62]. In the SDLH, no resorption of Na and Ca occurs. In the LDLH, volume is reduced 4 to 7 times compared

Table 1. Concentration and excretion ranges in plasma (mM), ultrafiltrate (mM) and 24-hr urine (mmoles, low and high end of the ranges), own data and [9, 54].

	Vol,l	Ca	OX.10^3	PO$_4$	Na	K	Mg	UA	cit
Plasma	---	1.58-5	0.5-50	0.4-2	136-145	3.6-4.8	0.6-1.2	0.17-0.30	0.087-0.147
Ultrafilt.	---	1.02 - < 3[*1]	0.5-50[*2]	0.48-2.4	136-145	3.6-4.8	0.45-1.00	0.17-0.30	0.087-0.147
24-hr, low	0.6	0.5	100	5	45	20	0.5	2	0.1
24-hr, high	7.5	7.5	2000	75	582	260	12.5	10	7.5

[*1]At high plasma calcium concentrations the ultrafiltrability decreases due to the formation of calcium phosphate nuclei in the plasma.

[*2]With normal renal function a plasma oxalate concentration of 10 μM is the maximum, with impaired renal function in hereditary hyperoxaluria, values up to 50 μM can be found.

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to the end proximal tubule. Thus, TF/UF$_{Na}$ should reach 4.12 to 7.21. Measured TF/UF$_{Na}$ values at the bend of long LH, however, average 2.2 to 2.6 [13, 29], with extremes up to 6 [13]. It appears that 35% of the NaCl load entering the LDLH (12% of the filtered load) is reabsorbed in the upper portion of the DLH [61].

Micropunctures of LDLH before the bend showed an average calcium concentration of 3 mM [28]. In light of the accompanying TF/P inulin values of 6.63, a calcium concentration of 4.1 mM would be expected. Thus ± 1 mM was reabsorbed, presumably in the upper portion of the LDLH. The maximum calcium concentration, when no further reabsorption occurs in the thin segment, will be 5.1 to 8.1 mM in loops where the TF/P inulin reaches 11.3 to 18. Comparable estimates of calcium concentrations are obtained when I assume that calcium and sodium are handled similarly. When 35% of the load entering the LDLH is reabsorbed, concentrations reach 4.6 to 8.05 mM. Reabsorption of Na, K, Mg, Ca and Cl in the ALH occur with equal efficiency, mainly in the thick part. In superficial nephrons, TF/P values for Na, K, Mg, and Ca reach 0.5, 5, 0.6 and 0.5 respectively at the early distal tubule and 0.2, 0.2, 0.3 and 0.2 at the late distal tubule [2, 54]. For juxtamedullary nephrons, these numbers are assumed here to be the same or higher.

Potassium

UF$_K$ is 3.6 mM. The regulation of K and Na differs especially in the LDLH. There, K is secreted, restoring it to approximately the filtered load. The K concentration in the LDLH is assumed to approach that in SALH as the urine enters the distal tubule. The amount of potassium entering the distal tubule is increased to the final pelvic urine value by active secretion. The resorption occurring in juxtamedullary LALH is not known. It is assumed here that the resorption process itself is equally efficient in short and LALH. The resorption in

LALH is thus calculated as (length short loop) / (length long loop) times resorption in short loops: (18/6) * 27 = 81%.

Phosphate

UF$_{PO4}$ averages 3.4 mM, ranging up to 4.7 mM. Below the reabsorption threshold, TF/P$_{PO4}$ falls to 0.7 in the proximal tubule [54], 75% reabsorption, 10% is reabsorbed in the distal tubule and 3 to 7% in the CD [23, 24, 52]. Above the threshold, increases in filtered load are excreted quantitatively.

Oxalate

Plasma oxalate concentration in humans averages 2 μM and varies with loading. In hyperoxaluric patients with normal renal function, a basal value of 5 μM is found. With impaired renal function, levels may reach 50 μM [65]. Oxalate reabsorption and secretion seems to occur in the proximal tubule. No clear quantitation is available for this. I assume all oxalate enters the nephron at the glomerulus. This affects the validity of the model for extreme situations where nucleation occurs in the proximal tubule. Upon dietary loads, the excretion in controls increases up to 290% over fasting values, from 0.011 to 0.033 mmole/hr [5]. When 167/24 = 6.96 liters of plasma is filtered per hour, this corresponds to plasma oxalate concentrations of 1.58 and 4.6 μM. Stone formers reportedly reacted twice as strongly to acute loads, plasma oxalate values reaching 5 times the reported fasting value of 1.6 μM [5].

Uric acid

Uric acid is reabsorbed and secreted in the proximal tubule; 7 to 12% reaches the final urine, determined by the daily purine load [9].

pH

Micropuncture data from superficial nephrons shows that pH is lowered from 7.4 in the plasma to 6.7 in the

Table 2. Conditions employed in nucleation experiment to simulate parameters at the end of a given nephron segment. The end values for DOB are based on high normal values for calcium and oxalate and a urine volume of one liter.

	added ml	total ml	time sec	pH	Ca mM	OX mM	PO$_4$ mM	Na mM	Cl mM	Mg mM	CIT mM
PROX	0.5	0.5	0	6.7	1.35	0.009	1.35	151.5	154.7	0.99	0.03
LDLH	0.5	1	40	7.26	7.9	0.05	8	408.7	423.8	4	0.2
LALH	5.85	6.85	100	6.65	1.35	0.05	8	158.7	153.9	0.59	0.2
DISTAL	16	22.85	31	6.38	0.75	0.05	3.2	48.9	47.1	0.3	0.2
Duct of Bellini	7.15	30	50	6.16	7.5	0.5	42	199	175	5	2

proximal tubule, and is 6.7 in the early distal tubule. When the urine is concentrated by water resorption in the DLH, the concentrations of CO_2 and bicarbonate increase. Some CO_2 diffuses to the interstitium, resulting in an increased pH. In the LDLH, pH is increased to 7.4. For the SDLH, no data is available. However, when I assume the increase is related to the concentrating efficiency, which is two times lower in the SDLH, pH will reach 7.1 to 7.2. The pH is brought to its end-value in the distal tubule and CD.

Relative supersaturation (RS)

The RS for COM, UA, brushite and HAP is calculated using EQUIL [63], adapted with values for activity constants and COM-solubility determined in our laboratory [7, 34].

Nephrocalcin (NC), Tamm-Horsfall protein (THP)

The production site for NC is unknown. Immuno-histochemically, it can be found in the proximal and distal tubule. I assume the average daily excretion, 1.9 μmoles, originates half from each site and that NC is not reabsorbed in the nephron. THP is localized in the ALH and early distal tubule. The total amount present is assumed to increase from zero entering the ALH to the average 24-hr value of 0.5 μmoles at the middle of the distal tubule.

Crystallization experiments

The effects of urine on COM-crystallization kinetics are determined with a well-established seeded crystal growth system in which uptake of ^{45}Ca by aged and pre-grown crystals is measured [6, 8, 64]. Two types of experiments are performed. In the first, the starting supersaturation is constant but growth time is varied. In the second, growth time is constant, while the starting calcium oxalate concentration product is varied around the expected solubility. Solubility is then measured as the lowest concentration product after which uptake of ^{45}Ca due to crystal growth occurs. From the kinetic experi-

ment, the crystal growth and crystal agglomeration parameters are calculated using a complete description of COM-crystallization kinetics as described extensively previously [6, 8, 64]. The solubility {($\sqrt{}$Lc in mM), where Lc is the solubility product based on concentrations}, growth inhibition (% G.I.), and agglomeration inhibition {(tm)/(tm)$_c$} of COM-crystals are measured as three separate, system-independent parameters. The latter two are relative to a control experiment. The urines were tested undiluted.

Undiluted urines

Undiluted urines were made under-saturated, required for measuring solubility, by dialyzing overnight against chelex, a cation chelating column material. This removes all Ca and Mg. Magnesium is restored to its original concentration and the solubility is measured. The removal of tracer ions has no effect on the solubility [34]. For the kinetic experiment, untreated undiluted urine was used directly or with 1.2 mM oxalate added.

Measurement of the nucleation lag-time

The nucleation lag-time (τ) was measured according to [3]. Supersaturated solutions were prepared by mixing a cation and an anion buffer into a polystyrene cuvette. The cation buffer contains calcium, magnesium, sodium, potassium and chloride. The anion buffer contains urate, chloride, phosphate, citrate, oxalate, sodium and optionally bicarbonate. The compositions of the buffers are chosen such that in the mixture concentrations, ionic strength and pH resemble those around the bend of a LH. For a short loop, the concentrations were as follows: 2.99 mM Ca, 4.5 mM or 16 mM PO$_4$, 0.01 mM oxalate, 252 mM Na, 21 mM K, 2.26 mM Mg, 0.132 mM urate, 0.08 mM citrate, pH 7.19, 7.20 and 7.21 and Cl as rest ion. For a long LH, the concentrations used were: 7.9 mM Ca, 8 mM phosphate, 0.05 mM oxalate, 408.7 mM Na, 80 mM K, 4 mM Mg, 0.432 mM urate, 0.331 mM citrate, pH 7.29 and Cl as
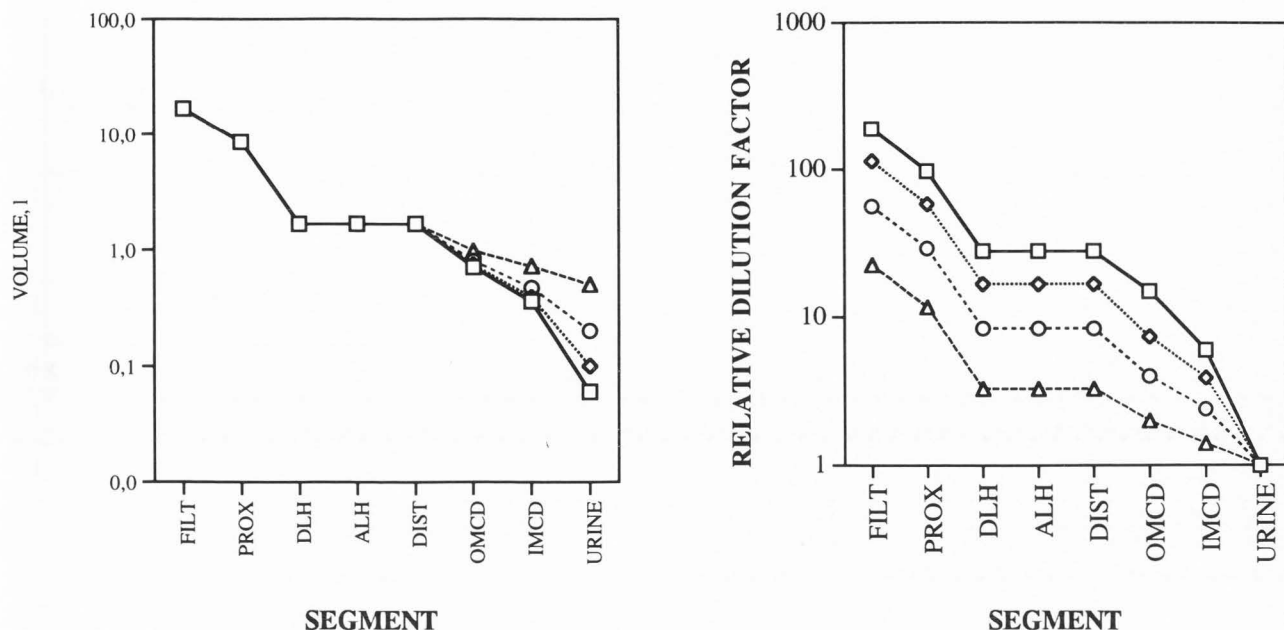
Figures 1 (at left) and 2 (at right). Changes in total volume per nephron segment for four 24-hr urine volumes (**Fig. 1**) and the relative dilution per segment as compared to the final total volume (**Fig. 2**): 0.6 (squares), 1 (diamonds), 2 (circles) and 7.5 liters (triangles).

rest ion. In some experiments, 47 mM bicarbonate was added. There, urate had to be left out. When simulating the conditions around the bend of the LH, it is assumed that the changes occurring in the descending and ascending limb are linear. Thus, the change per unit length is obtained by dividing the total change by the length of the limb. For instance, when the amount of water resorbed in the DLH is put at 100%, 75% is assumed to be resorbed, when 75% of the distance to the bend has been traversed.

The OD_{590} (optical density measured at 590 nm) is measured during ten minutes in a spectrophotometer. When particles are formed, an apparent absorption occurs due to particle-induced light scattering. The onset of this absorption is taken to be τ. Actually, this will slightly overestimate the real value. After 5 or 10 minutes, a sample was filtered over 0.05 μm Nucleopore filters. The filters were then gold-coated and analyzed using a 525 M Philips (Eindhoven, Netherlands) scanning electron microscope (SEM) operated at 15 kV.

Nephron nucleation system

The conditions in the nephron change continuously. The precipitation processes occurring under these changing conditions were tested in the dynamic nucleation system. In this nucleation system, the composition of the experiment is changed continuously (Table 2) by adding a series of different buffers. At time zero, 0.25 ml of an anionic and cationic buffer are combined under rapid mixing. The starting conditions in the mixture

represent what is present in the proximal tubule. A 0.01 ml sample is taken at time zero and filtered immediately over 0.05 μm nucleopore filters. Then, buffers are added gradually until at time = 40 seconds the conditions resemble those present at the bend of a long LH. A sample is taken at 40 seconds, and new buffers are added, working towards the conditions present at the end of the ALH. This process takes 100 seconds. Then, a sample is taken at 140 seconds (100 + 40) and new buffers are added, etc. Total addition time is 221 seconds. After this, no more buffers were added. The solution was kept mixed and samples were taken at 240, 300 and 600 seconds. The solution is kept at a fixed stirring rate in a double-walled round-bottom vessel at 37°C. The buffers are added at opposite vessel-sides, using multi-channel peristaltic U202 Whatson Marlow (Falmouth, Cornwall, U.K.) pumps. The filters were gold-coated and analyzed using SEM.

Results

Total volume and dilution factor

The total volume per nephron segment decreases, moving from the glomerulus to the pelvic area (Fig. 1). Four examples were used representing low, 0.6 l; average, 1 and 2 l; and high 24-hr volumes, 7.5 l. For *in vitro* experiments, it can be seen that relative to the 24-hr volume, urine needs to be diluted 2 to 20 times to simulate conditions in the CD, where agglomeration may be most important [35], and 16 to 50 times to simulate

Table 3. Standard dilution for *in vitro* experiments.

| 24-hr vol.	Dilution OMCD	Dilution IMCD
0.5	21.4	5.4
1	10.9	3.2
1.5	7.4	2.4
2	5.6	2.0
3	3.9	1.6
5	2.5	1.3

Figures 3, 4 and 5 at right.

Figure 3. Seeded crystal growth experiment using whole undiluted urine directly (squares) or with 1.2 mM oxalate added (circles). Hollow and filled symbols represent two consecutive experiments with the same urine. Ut is the fractional uptake of calcium into the crystal mass.

Figure 4. Nucleation lag-times (squares) in solutions representing the conditions around the bend of a long Loop of Henle. On the x-axis is given how far the point is removed from the bend, depicted as 0. The units are % of the DLH on the left side of the bend and % of the ALH on the right side of the bend. The diamonds represent the surplus time, the transit time from the point of measurement to the end of the LALH minus the lag-time at that point. When the surplus time is positive, there is sufficient time for nucleation to occur.

Figure 5. Increasing doses of phosphocitrate were added to solutions representing the situation at the tip of a long Loop of Henle (Table 2, but with 10 mM PO_4, diamonds), phosphocitrate was added in increasing doses at different pH-values: 0.5 μM (circles), 2.5 μM (triangles), 5 μM (crossed squares), 25 μM (crossed circle).

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conditions in the DLH and ALH, where nucleation may occur first (Fig. 2). The dilution factor in the CD depends on the end volume (Table 3). In the distal tubule, urine flow is fairly constant: 5.8 ml/min per kidney or 16.7 liters per day [35]. Concentration to the final volume takes place in the OMCD and IMCD. The following formulae are obtained assuming resorption occurs evenly over the total length of both segments 16 mm (OMCD) and 6 mm (IMCD) [35]: volume removed equals: 16.7 - 24-hr volume; volume at midpoint OMCD equals: 16.7 - (16.7 - x)(22/8); volume at junction OMCD/IMCD equals: 16.7 - (16.7 - x)(22/16); volume at midpoint IMCD equals: 16.7 - (16.7 - x)(19/22). Of this concentration, approximately two-thirds occurs in the OMCD and one-third in the IMCD. Thus, the urine should be diluted in the above-mentioned manner.
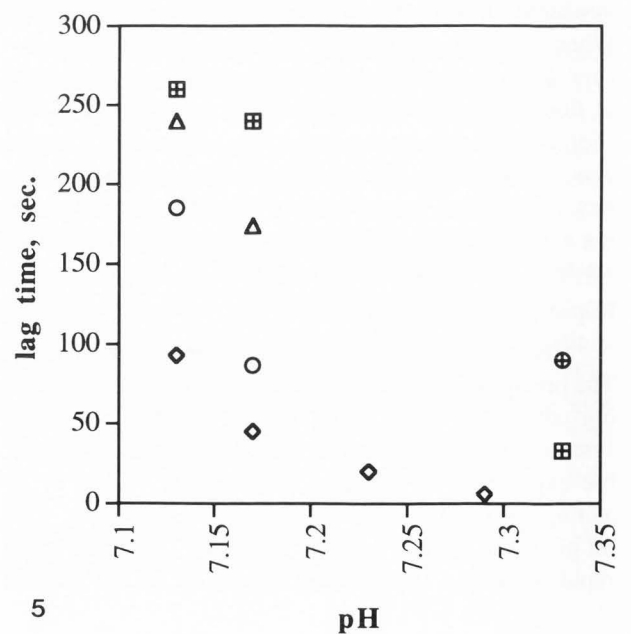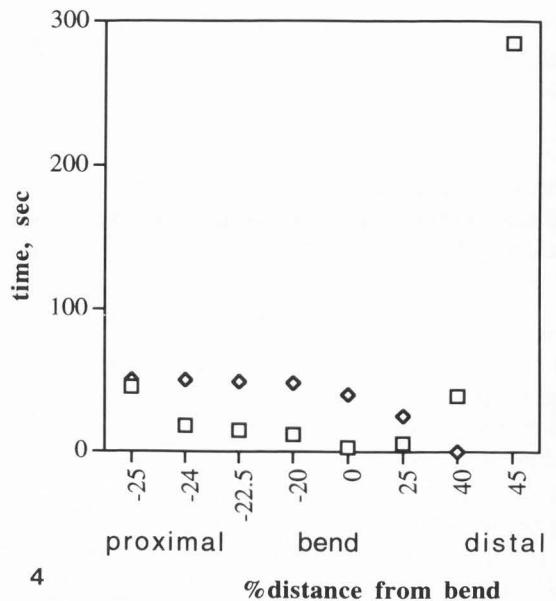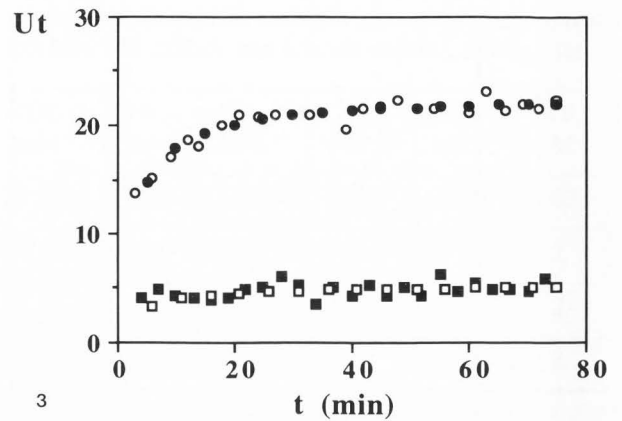
Table 4. TF/UF-values at end of each segment.

.UF	Proximal	SDLH	LDLH	SALH	LALH	sup. distal	juxt. distal	CD	
1	0.35	0.20	0.05-0.09	0.20	0.05-0.09	0.20	0.05-0.09	0.004-0.045	VOL
1	1.22	2.1	3.2-5.6	0.52	0.52	0.21-0.52	0.21-0.52	0.07-21	Ca
1	3.5	5	11-20	5	11-20	5	11-20	250	OX
1	0.75-1.2	1.3-2.1	2.9-8.4	0.75-2.1	0.75-8.4	0.5-2.1	0.5-8.4	0.5-90	PO_4
1	1.03	1.8	2.2-6	0.5	0.5	0.2	0.2	0.04-6.7	Na
1	1.1	5	10-20	5	10-20	0.2	0.2	0.6-100	K
1	1.8	3.1	7-12.6	0.55	0.55	0.21-0.55	0.21-0.55	0.07-21	Mg
1	0.2-0.4	0.35-0.7	0.8-2.8	0.35-0.7	0.8-2.8	0.35-0.7	0.8-2.8	0.8-40	UA
1	0.06-0.85	0.1-1.4	0.2-6	0.1-1.4	0.2-1.4	0.1-1.4	0.2-6	0.4-83	CIT
7.25	6.7	7.1	7.4	6.7	6.7	*	*	-	pH
0	0	0	0	50	50	0	0	0	THP
0	50	0	0	50	50	0	0	0	NC

*The regulation of pH to its end-value occurs in the distal tubule [9].

Concentrations

Table 4 shows the changes along the nephron for Ca, Na, Mg, oxalate, PO_4, K, UA, citrate, pH, NC and THP. High normal values for Ca- and oxalate 24-hr excretion and a 24-hr volume of 2 liters were used.

Crystal growth and agglomeration

In undiluted urine, measurable ^{45}Ca-uptake occurred only after addition of 1.2 mmoles/liter oxalate (Fig. 3).

Nucleation lag-times

In Figure 4, τ is shown for solutions simulating the conditions around the bend of a long LH (Table 2). Coming from the end proximal tubule conditions, where concentrations approach 75% of those at the bend of the LH, τ falls within the remaining passage time of urine through the loop, 140 seconds for the total loop [35]. When the solution is diluted from the bend-conditions to early distal tubule conditions, τ increases strongly after 40% of the ALH has been traversed. The precipitate is a HAP-like material. Addition of bicarbonate further decreases τ, suggesting a carbonate-containing type of HAP. When conditions in a short LH are simulated (2.99 mM Ca, 4.5 mM PO_4, pH 7.2), τ exceeds 300 seconds. With 2.99 mM Ca, 16 mM PO4, pH 7.2 (high phosphate load), τ is 40 seconds. The lag-time is very dependent on the pH, decreasing 8-fold when pH in-creases from 7.19 to 7.22. During the lag-time period, pH did not change. When low doses of phosphocitrate were added to solutions representing the bend of a long LH (Table 2, LDLH with 10 mM PO_4), τ increased greatly (Fig. 5).

Nephron nucleation

Samples were taken at 40, 140, 171, 221, 240, 300 and 600 seconds and analyzed using a SEM. At 40 seconds, rounded particles, that are 0.7 μm in diameter, are found, mainly as aggregates up to 6 μm large (Fig. 6). By infrared analysis, the material was characterized as HAP. After 140 seconds, representing the end of the LALH, single COD-crystals, 2 μm, and agglomerates, up to 4.4 μm, start to appear. At 171 seconds, leaving the distal tubule, both COD and COM can be found. At 221 seconds, agglomerates up to 7 μm, and after 600 seconds, large, 15-30 μm, COM-agglomerates and COM rosettes are found (Fig. 7).

Discussion

Crystal formation is an essential first step in stone formation. However, while crystalluria is common in both healthy subjects and stone formers [18, 46, 55], the step from a harmless free flowing crystal to formation of a stone occurs in only 5 to 9% of the population. A
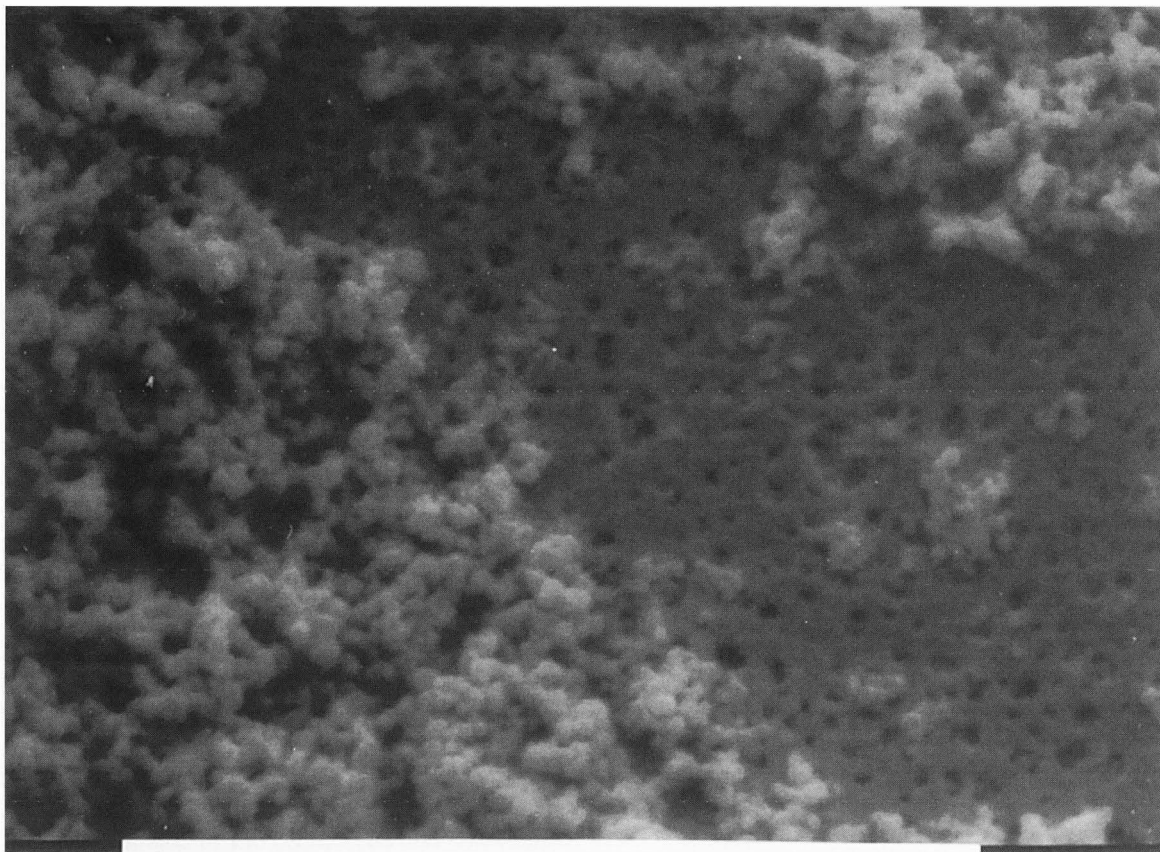
Figure 6. A scanning electron micrograph of the material which precipitates in the conditions existing at the tip of a long Loop of Henle as described in Table 2. By infrared analysis, the material was shown to be HAP. Bar = 10 μm.

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particle causes harm when it's size approaches the dimensions of the urinary tract it is passing through or when it adheres to the cell linings, free and fixed particle mechanism respectively [20, 35]. Important factors in this respect are: processes increasing particle size (crystal growth and agglomeration), stickiness of particles and cell lining, architecture of the kidney, the site of nucleation, and transit time through the kidney. Previously, we have shown which retention mechanisms play a role in specific parts of the nephron [35] and how crystal agglomeration poses a risk factor in urolithiasis [19, 35, 36, 37, 40]. Remaining questions were: do 24-hr urine samples mimic the actual situation at the site of stone formation; where does nucleation start in the nephron; where do dietary factors and commonly used therapies intervene? Here, I modeled the chances of crystal formation per nephron segment and their dependence on factors such as diet and medical therapy. The literature data used for this model are obtained from micropuncture studies using dog, rat and rabbit. These data cannot be extrapolated directly to the human situation, which should be kept in mind while evaluating the model. To

increase the validity of the model, ranges of concentrations etc. were used which may comprise those occurring in humans.

It is clear from the model that under normal conditions, no crystal formation occurs in the proximal tubule. Total volume is reduced by 65%, but stone components are removed too. As a result, the urine generally remains too dilute. Crystal formation in the proximal tubule occurs only with a high oxalate or uric acid filtered load, as witnessed in animal studies [31]. Moving into the LH, a distinction must be made between superficial and juxtamedullary nephrons. In the first, urine is concentrated to 20% of the filtered load. Formation of crystals occurs only with moderate increases in phosphate load or strong increases in oxalate or uric acid load, and at pH values over 7.20. At the bend of the LDLH, however, urines are concentrated much more, to 5-9% of the filtered load and pH reaches 7.4. It was shown previously that under the conditions existing at the bend of the LDLH, an apatite-like material is found after 1 hour [14]. I have confirmed here that a HAP-like material, probably containing carbonate, is
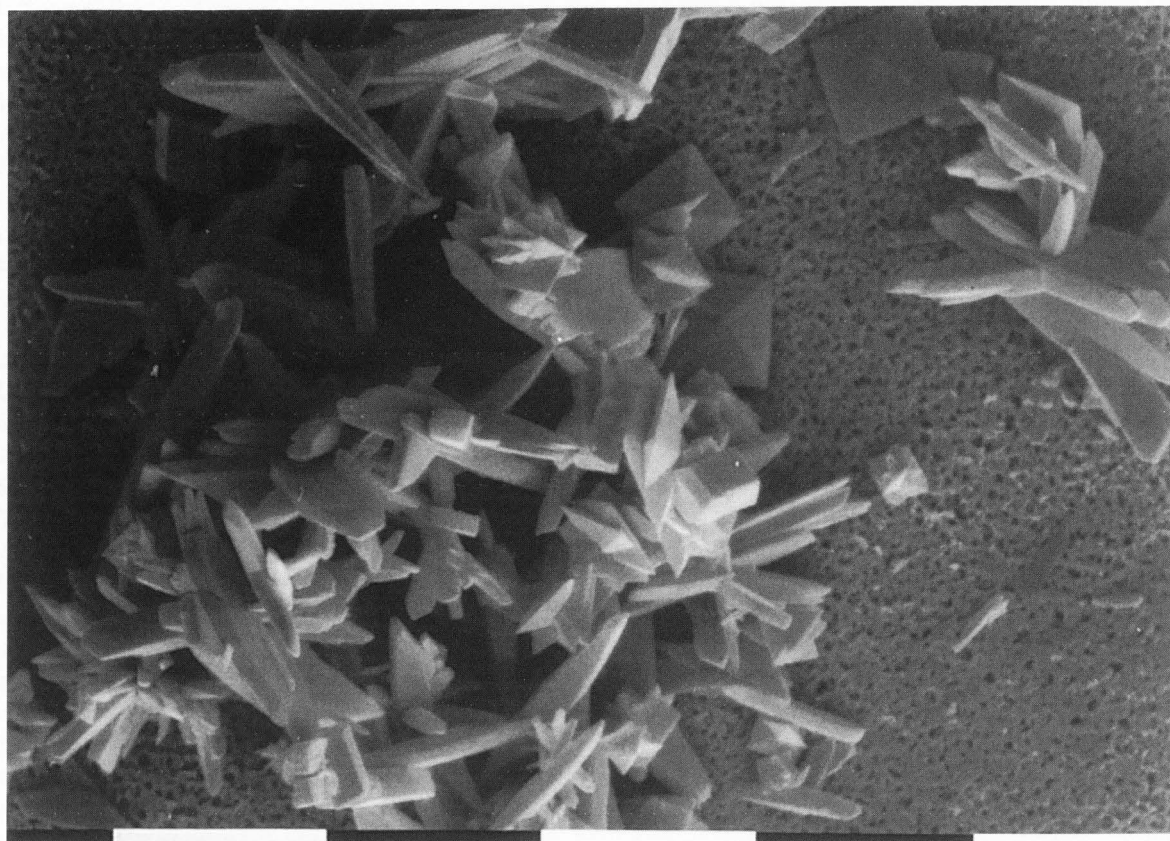
Figure 7. A scanning electron micrograph of the crystal material present in the nephron nucleation system after 600 seconds. Bar = 10 μm.

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formed under conditions routinely present in the LH of juxtamedullary nephrons. The nucleation lag-times fall well within the passage time through the long LH. The sizes of single particles (0.7 μm) and agglomerates (6 μm) formed are small relative to the inner diameter of the ALH (19-29 μm). However, the experiments were performed under relatively high shear forces. *In vivo*, these forces may be smaller, allowing for larger agglomerates to be formed. It was concluded before [47] that while calcium-phosphate is nucleated in the LH, calcium oxalate nucleation is more likely to occur in the later parts of the nephron. For calcium oxalate nucleation to occur within the time-frame of the loop passage (140 seconds), a relative supersaturation of 28 is required [3]. The calcium concentration in a specific loop is relatively constant in time, depending mainly on the resorption-efficiency of that loop. When, in my calculations, I take a range of possible calcium concentrations in different loops (2, 4, 6 or 8 mM), the oxalate values needed for a RS of 28 are 0.39, 0.195, 0.13 or 0.097 mM. For a short loop, concentrating 5 times, 390 and 195 μM oxalate loop-concentrations correspond to plasma values of

78 and 39 μM. For long loops concentrating 11.1 times, loop concentrations of 130 and 97 μM correspond to plasma oxalate values of 11.7 and 8.7 μM. For long loops concentrating 20 times, a loop concentration of 97 μM corresponds to a plasma value of 4.9 μM.

Thus, in juxtamedullary nephrons, HAP nucleation may occur routinely. This is also seen in *in vivo* experiments in rat models of nephrolithiasis, where calcium phosphate crystals preferentially deposit at the LH/proximal tubule junction [30, 49] and calcium oxalate nucleation occurs when plasma oxalate exceeds 5 μM. Normal fasting plasma oxalate concentrations (2 μM) are insufficient. Upon acute oxalate dietary loads, the urinary oxalate excretion rate in normal controls increases up to 290%, from 0.011 to 0.033 mmole per hour [5]. Assuming that 167/24 = 6.96 liters of plasma is filtered per hour, this corresponds to plasma values of 1.58 and 4.6 μM. Stone formers are reported to react stronger to acute loads, with maximum plasma oxalate values reaching 5 times the fasting value [11]. With a fasting plasma oxalate of 2, this would imply a peak plasma value of 10 μM. In hereditary hyperoxaluria, fasting

plasma oxalate values approach 5 μM. Nucleation requirements may be met under acute oxalate loading in normals and more likely in stone formers. In hyperoxaluria, calcium oxalate nucleation in the LH or earlier seems inevitable. In the most severe conditions, hereditary hyperoxaluria with renal impairment, plasma oxalate values may reach 50 μM and calcium oxalate crystals are found at the end proximal tubule already. The same is seen in rat studies where oxalate excretion is increased 6 times by administration of oxalate or ethylene glycol [35]. In these severe cases, calcium oxalate crystals are found also in other organs like the skeleton. This would imply that calcium oxalate nuclei circulate in the plasma. However, since the crystals are not found in the early proximal tubule, it is more likely that the nucleation is locally triggered.

Thus, thermodynamically, nucleation is likely to occur in the LDLH within the required time-frame. However, nucleation kinetics may be affected by other means. Nucleation lag-time can be decreased by membranous material [33] or crystal matrix protein [59] or increased by inhibitors like citrate, magnesium and NC [4]. However, when all low molecular weight compounds were included at the concentrations occurring in the LDLH, nucleation lag-times were still within transit times. NC was not included. NC can decrease the rate of secondary calcium oxalate nucleation by 42% at a concentration of 0.2 μM [4]. The concentration of NC in the LDLH will be between 0.016 and 0.097 μM, thus below its effective concentration range. The lag-time for HAP nucleation in the LDLH is below 3 seconds, compared to a transit time of 140 seconds for the total loop. As was shown here with phosphocitrate, low concentrations of effective inhibitors could delay nucleation long enough to allow safe passage of the LH. Theoretically, such compounds can postpone the nucleation risk to the late distal tubule and collecting duct system.

The sizes of particles formed up to the distal tubule seem too small to allow a free particle mechanism. Retention in the LH would require adherence to the cell lining. In this respect, it will be interesting to investigate if the presence of a compound like THP [41] or crystal matrix protein [60] in the coat of tubular cells in the ALH interferes with crystal-cell adhesion. Downstream through the ALH and further, conditions become less favorable for HAP crystallization and calcium oxalate crystals appear. Interestingly, in the in vitro experiments, the amount of HAP material seems to diminish. THP now enters the urine and may interfere with calcium oxalate nucleation together with compounds like NC. The HAP nuclei may thereby act as heterogeneous nucleators for calcium oxalate [16]. First, COD is found, then also rosettes and agglomerates of platelet-like COM. Up to the conditions simulating the CD, the single particles are approximately one-tenth the size of the inner diameter of the segments they are passing through. However, within the transit time from the nucleation-site, the bend of the LH, to the end of the Duct of Bellini, 181 seconds under low diuresis, larger COM-agglomerates are formed in the collecting duct conditions. Kidney architecture favors agglomeration to take place in the CD, where crystals originating from some 4000 nephrons meet in one collecting duct. The agglomerates will include organic material [32] and the process will be influenced by compounds not included here like THP and NC. These events occur already at high normal calcium, phosphate and oxalate excretion rates of 7.5, 42 and 0.5 mmoles/24 hr respectively and a total volume of one liter. Under more extreme acute loading conditions, concentration of oxalate may increase two-fold, and that of phosphate four-fold, in the LH. Precipitation then also may occur in the superficial nephrons, further enhancing the chance of big particle formation in the collecting ducts.

A distinction between two types of nephrons also occurs in animal studies where mild crystalluria is induced using 0.75% ethylene glycol and 2% ammonium chloride. There, crystals are found in a restricted number of collecting ducts [31, 35]. It is not known whether this stems from differences in the type of nephrons discharging into the collecting ducts.

The model and experimental data lead to several simple but surprising conclusions. The first concerns the question as to how urine should be tested in in vitro crystallization experiments. Usually, it is assumed that using untreated and undiluted urine mimics the situation at the site of stone formation. Surprisingly, when undiluted urines are tested, it is seen that crystallization proceeds at an extremely low rate. Only when urine was ultrafiltered, removing high molecular weight compounds, diluted, or when extra oxalate was added did appreciable crystallization occur. If undiluted whole urine is representative for the site of stone formation, it is hard to imagine how crystals, let alone even large particles, could ever form. From the model, it follows that using undiluted urine actually simulates events in the bladder and the pelvic area. The situation inside the nephron is much different. From the site where nucleation starts, the DLH, the following events (relevant for stone formation) occur: water is reabsorbed, causing concentration of compounds which are not reabsorbed, such as NC, oxalate and citrate; calcium may be reabsorbed and pH reduced, and proteins like THP enter the solution. Thus, if you want to mimic the situation inside the nephron, you need to add water and calcium, increase pH, etc. For testing crystal agglomeration and growth occurring in the OMCD, IMCD and duct of Bellini, the collected urine should be tested at 1:2 to 1:20

dilutions after addition of up to 5 times the 24-hr amount of calcium and at pH values higher than the 24-hr urine pH. For testing nucleation and crystal growth in the ALH and distal tubule, urine should be diluted to a value of 16.7 to 33.4 l. For testing nucleation occurring in the DLH, urine must be diluted to a volume of 16.7 to 58.5 l, after removal of THP and other compounds entering the urine after the proximal tubule.

The clinical use of the model lies in visualizing the effects of specific conditions and (therapeutic) actions. A high Ca-load, with relative constant serum Ca, does not influence the calcium concentrations nor the chances for nucleation up to the distal tubule. Conditions involving an increased serum-Ca do increase the chances of nucleation in the LH. A load of oxalate or PO_4 directly increases the chance for nucleation. An increase in filtered oxalate occurring after ingestion of food with a high oxalate content, like chocolate [5], almost triples the RS with COM. This will cause formation of calcium oxalate crystals to occur earlier in the nephron. In extreme cases, induced in rat studies, calcium oxalate crystals are found in the proximal tubule already [35]. A load with uric acid will increase the likelihood of sodium urate formation in the LH and of UA crystallization further in the nephron. The effects of an acid load are more complicated. Acid loading causes an increased citrate resorption proximally. Since citrate acts as an inhibitor of calcium salt nucleation, this decrease in concentration increases the chance of apatite and calcium oxalate nucleation in the LH. In the distal tubule and further on, calcium reabsorption is decreased and acidification increased. On one hand, this lowers the rate of HAP crystallization, counteracting the effect of a lower citrate. On the other hand, conditions of lower pH, lower citrate concentration and higher calcium concentration, increase the self-aggregation of THP [27]. This decreases the ability of the urine to inhibit crystal agglomeration [19, 27]. Obviously, alkali loading, as applied to the treatment of calcium oxalate urolithiasis [51], has the reverse effects. It decreases the likelihood of HAP crystallization in the LH, due to the increased citrate excretion. In the distal tubule and further, the effect on HAP crystallization of an increased citrate concentration is counteracted by an increased pH. The main beneficial effect of alkali therapy may be the changed conditions in the CD, higher citrate, and higher pH, which increase the inhibition of crystal agglomeration at that site [19, 27, 34].

Even more complicated are the effects of a high intake of animal protein. An animal protein load increases calcium excretion [10, 56] and acts as an acid load [39], with the same negative effects. In addition, it will cause an increase in uric acid excretion [12], which further increases the likelihood of nucleation and crystal agglom-

eration. Also, phosphate excretion is increased [37], causing increased supersaturation with HAP in the LH. When an animal protein load is combined with an increased sodium chloride intake, the increased ionic strength in the collecting ducts provides an additional factor for promoting THP self-aggregation and increasing crystal agglomeration. This may explain the exaggerated decrease in crystal agglomeration inhibition found in urines of persons receiving a combined protein salt load [39].

A special case of acidification effects is posed by renal tubular acidosis (RTA). Normally, with acid loading, the decrease in pH distally acts to decrease the propensity for HAP crystallization. With RTA, the decrease in citrate is not compensated by a decrease in pH; consequently, CaP crystallization is more likely to continue in the distal tubule and further on.

Thiazide treatment increases calcium reabsorption in the distal tubule. Thus, this will not affect the chances of nucleation in the LH. The reduced calcium in the distal tubule and CD does, however, decrease the rate of crystallization there and diminishes the propensity for THP self-aggregation.

Also interesting are the effects of water loading. The concentrations in the proximal tubule and DLH are relatively independent of the hydration state of the body. In the CD, however, a water load will strongly increase the urine flow and decrease the possibility of large particle retention by increased shear forces and decreased residence time. Additionally, the higher dilution decreases the propensity for THP self-aggregation and crystal agglomeration.

The model also points to what value urine parameters actually have. Citrate and oxalate values give information on chances for crystallization throughout the nephron. Measuring urine volume is relevant for what happens in the collecting ducts, pelvis and bladder. The interpretation of urine calcium values depends on the source of the calcium, hypercalcemia or dietary loading. Measuring crystal growth and crystal agglomeration effects in undiluted urine bears relevance to the pelvic and bladder situation. Measuring these parameters, using urines diluted between 1:2 to 1:9 times, is relevant for describing the events in the IMCD and duct of Bellini, where *in vivo* agglomeration probably plays its biggest role.

The model also points to the relevance of some structural kidney features. When retention occurs in the LH or distal tubule, the main mechanism seems to be fixation of crystals to the cells lining the segments. Interesting in this respect is that THP and NC, which are thought to play a role in stone formation, are produced by cells at these sites. Tamm-Horsfall protein is expressed on the membrane in the ALH and the early

parts of the distal tubule, and is thought to form a water impermeable layer. This property might also play a role in repelling crystals. Changes in the density or the structure of membrane-bound THP might contribute to increased adherence.

Another structural feature which plays a role is the natural-occurring variety in length of the LH. The length can vary from 33 to 140 mm. When the length of the loop increases, two risk factors increase as well. First, the urinary saturation with calcium salts increases with increasing loop-length. This is somewhat counter-acted by the increasing ionic strength. However, espe-cially in the LDLH, sodium chloride is also resorbed. Second, the passage time of the urine and the particles formed in it increases because of both the increased length and the reduced volume, thus reduced flow of urine. The presence of more, longer loops and condi-tions where preferentially more long loops are used might thus be considered risk factors for nephrolithiasis. In the development of human kidneys, growth continues until adulthood, mainly due to elongation of the proxi-mal tubule and LH. This continuous increase in long loops may be relevant to the finding that the incidence of stone formation before adulthood is low and reaches its peak in the second decade of life.

Concluding, the model presented here may help in-vestigators by clearly defining the conditions occurring at the site of nephrolithiasis.

Acknowledgements

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Discussion with Reviewers

S.R. Khan: Nucleation experiments were carried out in inorganic solutions without the proteins which are generally present in the urine. Do you think calcium phosphate will crystallize in the presence of these proteins in the Loop of Henle?

Author: At the tip of the Loop of Henle, one may expect membranous material, crystal matrix protein (urinary prothrombin fragment 1), nephrocalcin and proteins I am not aware of. In the discussion, I have pointed out that the concentration of nephrocalcin in the Loop of Henle will be at least 2 to 10 times below the concentration needed to decrease the rate of nucleation by 42%. Actually, at the tip of the Loop of Henle, the induction time is less then 3 seconds compared to the transit time through the ascending limb of the Loop of Henle of 100 seconds. The nucleation rate would need to be decreased more then 95% to completely prevent nucleation. I have no assessment on the concentrations of crystal matrix protein and membranous material at the site, nor about how the balance of counteractive actions would be.

S.R. Khan: Renal papillary surface epithelium may also be involved in urine concentration. What will be its effect on urolithiasis?

Author: Given the high flow rate through the Duct of Bellini, I am not convinced that changes in urinary concentration can play a dominant role there. This may be different when you consider that the ducts may be closed at times at those sites. I do not know how to fit this into the model. Finally, I see a role for damaged epithelium, where the chance of particle adhesion may be increased.

J.P. Kavanagh: How well can one make quantitative estimates for average human nephrons from measurements using individual animal nephrons?

Author: As mentioned in the text, extrapolating the animal data to the human situation poses a potential error. For the sake of the model, however, I feel that when the precaution is taken to use ranges of values including the extreme low and high values, in most cases, the "human" situation will be included.

J.P. Kavanagh: Using average data may tell us something about the normal possibility of nucleation events in

different nephron segments, but it may be important to consider the nucleation behaviour at all extremes of anatomical and excretory patterns. Lack of nucleation in average nephrons does not necessarily mean its absence in all, and one burst of nucleation may be sufficient to initiate a stone.

Author: This is completely true. For this reason, I explicitly dealt with the situations of extreme high excretions of oxalate and phosphate and distinguished between types of nephrons.

J.P. Kavanagh: You note that a pH change of 0.03 can cause an 8-fold change in nucleation lag-time which might cause one to conclude that in one case, nucleation might occur in the Loop of Henle, while in another case, it would not. This emphasizes how critical the choice of experimental conditions is and, of course, the danger of trying to extrapolate the *in vitro* findings to *in vivo* interpretations. Can this be done with any confidence?

Author: At the calcium and phosphate concentrations probably existing at the bend of a short Loop of Henle, variations around the probable pH-value there, 7.20, are very critical for HAP-nucleation. At the higher concentrations existing at the bend of a long Loop of Henle and the higher pH there, 7.4, small variations in pH play no role for HAP-nucleation and absolutely no role for calcium oxalate nucleation. Thus, it seems safe to extrapolate these findings to the *in vivo* situation.

C.R. Scheid: The site of nephrocalcin production is not known with certainty, and local concentrations may vary. Could you explain how you get to the statement that NC levels in the LDLH will be too low to act as an effective inhibitor of nucleation?

Author: I described in the **Data Used** section that the production site of NC has been suggested to be in the proximal and/or distal tubule. If NC is produced only in the distal tubule, the concentration in the Loop of Henle will be zero. Since I do not know where it is produced, I assumed the production occurs 50% in the proximal tubule and 50% in the distal tubule. When all NC would be produced in the proximal tubule, the concentrations in the Loop of Henle would be twice those mentioned in the text and still below what would be needed to increase the lag-time from less then 3 seconds to the needed 100 seconds (> 95% reduction of the nucleation rate). Of course, it is also possible that NC is produced in only a few proximal tubules. This might bring the NC-concentration into the needed concentration range. One should also consider the counteractive action of membranous material deriving from the proximal tubule.

C.R. Scheid: Why does Figure 2 differ so markedly from Figure 1? Fluid remains isoosmotic in the early regions of the nephron. Variations in urine volume do not reflect differences in the relative dilution of urine in the ultrafiltrate (as inferred in Fig. 2). Will the use of these values not affect subsequent calculations as to where crystals are likely to form?

Author: In Figure 1, the lines represent absolute numbers for volume at specific sites. Figure 2 is drawn for the sake of experimental design and shows the dilution at a specific point relative to the 24-hr volume. Four different values of 24-hr volume were used. The aim of Figure 2 is to show how much the fluid at a given point is diluted compared to the end product, the 24-hr urine. The lines are not related to the ultrafiltrate conditions. Where relevant, I used the both the lower and upper ends of ultrafiltrate ranges to calculate which effects these variations have further on in the nephron. I agree that much more detail can be put into the model and I hope that this will be done in the ensuing discussions you predict.

W.C. de Bruijn: In the description of the crystallization experiments, urines were used undiluted and 5 times diluted. In **Results**, you showed in Figure 3 the results from undiluted urine but failed to show the 5 times diluted and mentioned dilutions from 2 to 50 times for the DHL and CD parts. Does this mean that beyond the Loop of Henle, calcium oxalate crystal formation can be ignored?

Author: I performed experiments with undiluted urines to show that crystallization proceeds exceedingly slow under those conditions. I did not include the experiments with 1:5 diluted urines (which have been part of many previous publications from our group) here, since they do not contribute to the message. From the model presented here, it follows that using undiluted urines in fact is representative for what happens in the bladder. Should one want to test what happens earlier, in the Loop of Henle or the collecting ducts, one should apply the dilutions mentioned in the text. I did not perform these experiments since it was not the aim of this work. I also do not conclude that beyond the Loop of Henle no calcium oxalate nucleation occurs. In fact, calcium oxalate nucleation is likely to occur in the collecting ducts.

W.C. de Bruijn: In the **Introduction**, you narrowed down stone formation to free particle retention of particles too large to be excreted and fixed particle retention to the epithelial cell surface coat materials at sites with poor flow conditions. Do you hold the view that the step from crystals to stones is a pure intra-tubular crystallization process or that crystal retention (either the former or the latter) is a prerequisite for stone formation either interstitially or at papillary sites?

Author: The fixed particle mechanism is an interplay of particle size, interactions between particles and cells and particle removal. The fixed particle mechanism may be enhanced by increased sticking of particles due to particle size, by increased adhesion of particles to cells (due to different coating of the particles and/or the cells) and by deficient removal of particles (by shear forces or actively by the kidney cells).

W.C. de Bruijn: In your proposed nephrolithiasis model, nephrons are differentiated in a longitudinal sense. In rat experiments, we found that not all nephrons were responding alike to a generalized chronic (calcium oxalate) challenge (de Water *et al.*, unpublished). Could you speculate about the chances that in human stone-formers there is also a lateral differentiation (that is to say, a complete renculus or even one of the two kidneys behaves abnormal)?

Author: Central to the model is the notion that local circumstances play a very important role in stone formation. Local variations in nephron properties, as you mention, may very well be important in that respect.

Reviewer V: Does Figure 5 show all concentrations of phosphocitrate at all pH-levels?

Author: No. I did measure them but at low pH-values, no nucleation occurs within 10 minutes at the high PC-levels (5 and 25 μM) and conversely, at pH values above 7.23, the low concentrations of phosphocitrate (0.5, 2.5 μM) do not increase the lag-time measurably.