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Citrate and urease-induced crystallization in synthetic and human urine

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Summary. The effects of citrate on the different phases of urease-induced crystallization were studied using Coulter counter techniques and optical microscopy. Citrate increased urine pH and markedly delayed the initiation of the crystallization (nucleation) in both human and synthetic urine. In synthetic urine, particle aggregation and espicially particle growth were delayed and inhibited by citrate. In human urine, aggregation was distinctly inhibited by citrate. It appears that the susceptibility of urine to form crystals in the precense of urease activity is influenced by its citrate concentration.

Key words: Citrate – Crystallization – Struvite – Urease

Most infection urolithiasis conditions result from a urinary tract infection (UTI) by urease-producing bacteria. Infection urolithiasis can be a serious health problem due to its rapid development and high rate of recurrence. In some individuals urinary colonization by urease-producing microorganisms does not lead to the formation of any concrements whereas in others there is rapid formation of large staghorn stones. Diversity in urinary citrate composition may explain the differing susceptibility of patients to forming infection stones in the presence of UTI with urease-producing microorganisms [10, 11, 13, 14].

Calcium oxalate urine crystallization is inhibited by glycosaminoglycans, citrate, magnesium, pyrophosphate, and proteins [2, 3, 4, 6, 8, 9]. Some reports claim that citrate is one of the most potent inhibitors [2, 6, 8]. The role of crystallization inhibitors in urease-induced crystallization has been less intensively studied [12, 17–20, 22]. Several studies in our respective laboratories have demonstrated struvite inhibition by zinc, citrate, pyrophosphate, and urinary proteins such as albumin [13, 17, 19, 20]. In contrast, the glycosaminoglycans, heparin sulfate and chondroitin sulfate, show no inhibitory effects [19].

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As a part of our continuing studies on urease-induced crystallization we have developed a method based on the Coulter counter technique [22]. This technique allows the determination of the number and size of particles in a solution, thereby making it possible to follow the different phases (nucleation, growth, and aggregation) of the crystallization process. The influence of potential inhibitors on each phase can also be estimated. This paper presents the results of studies on the effects of citrate on urease-induced crystallization in synthetic and whole human urine using the above mentioned technique. Light microscopy [18] was used to complement and verify the observations made using the Coulter counter technique.

Materials and methods

Urine collection and processing

Morning urines were collected from 3 men and 2 women, 28 to 42 years old, with no history of UTI or stone disease and with negative urine cultures. The urine samples were collected in sterile bottles without added preservatives, immediately chilled to 4°C and used within 4 h. Urine pH was measured immediately after collection with a surface pH-electrode (Orion 9–35, Orion Research). The citrate concentration in each sample was measured enzymatically [21].

In *experiment 1,* the urine samples were pooled as one and then divided into five portions. Each portion was incubated with citrate-lyase (Boehringer Mannheim, FRG) for 20 min until all citrate was decomposed. Citrate decomposition was experimentally verified. One of the portions was used as a control. Tri-sodium citrate dihydrate ($C_6H_5Na_3O_4 \cdot 2H_2O$) (Merck, Darmstadt, FRG) was added to the other four samples to achieve a final concentration of 1, 2, 3, and 4 mM citrate respectively. The synthetic urine used in this experiment had a composition as previously described, except for the omission of sodium citrate [17], and was adjusted to pH 5.7. It was sterilized by filtration through a 0.22-μm Millipore filter and stored in glass bottles at 4°C. Prior to use, citrate was added to give a final concentration of 0, 1, 2, 3, and 4 mM. These artifical urine samples and patient urine samples were then incubated with urease as described below.

In *experiment 2,* one urine sample from the individual with the lowest urinary citrate and one sample from the individual with the highest urinary citrate concentration were each divided into three

Table 1. The effects of citrate on urease-induced crystallization in *synthetic urine*

	Citrate concentation (mM)				
	0 (control)	1	2	3	4
Initial particle number[a]	139	135	140	146	167
Initial median size (μm)	4	4	4	4	4
Nucleation started at (min)	30	75	120	135	150
pH at this time	6.52	7.74	8.33	8.42	8.51
Maximum particle number[a]	37000	36000	37000	37000	38000
Growth started at (min)	45	90	135	150	180
Growth index	0.23	0.16	0.14	0.13	0.11
Aggregation main process at (min)	90	135	150	180	210
Final particle number	23000	27000	28000	29000	32000
Final median size (μm)	21	20	19	16	9

[a] All particle numbers are per 500 μl

portions. Two portions of each sample were treated with citrate lyase as described above. Citrate was added to one of these portions to give a final concentration of 3 mM. Each patient urine sample therefore had one portion with the initial (native) citrate concentration, one portion lacking citrate, and one portion with an adjusted (3 mM) citrate concentration. These portions were then incubated with urease as described below. Every experiment was carried out in triplicate.

Urease incubation

Sterile glass vessels were filled with 100 ml urine and incubated simultaneously with 0.1 ml urease solution. The urease used was a high-purity preparation of Jackbean urease dissolved in 0.1 M TRIS (pH 7.0) giving an activity of 109.09 mmol NH_3 from urea/min per milliliter at 37°C (Sigma, St. Louis, Mo.). The vessels were placed in the same water bath and incubated at 37°C. During incubation, continuous slow stirring was provided by a Teflon-coated stirring bar.

Analytical procedures

Every 15 min for 4 h after the addition of urease, the urine samples were analyzed for particle number and particle size using a Coulter multisizer (Coulter Electronics, Luton, UK) fitted with a 140-μm orifice tube. Particles from 2.8 to 84.0 μm could be registered. From particle number and size (diameter in micrometers) the total particle volume and the total surface area could be calculated using a specially developed program in a Macintosh SE/30 computer and further analyzed using the Statview program. Particle formation (nucleation) was considered to start when two consecutive measurements showed an increase in particle number. Particle growth was considered to have started when median particle size began to increase. Particle aggregation was considered to have substituted growth when surface area and particle number started to decline along with a stabilization in particle volume. An index of particle growth was calculated from the slope of the linear function satisfied by the increase in median particle size with time until the surface area started to decrease [22].

At the end of the experiment each urine sample was filtered through a 0.4-μm Millipore filter to collect the precipitated material. This material was then examined with phase-contrast light microscopy at a magnification of ×400 using an Olympus BH-2 microscope. Microscopical examination was performed to assess the degree of crystallization and the form (crystal habit) of any crystals present. This material was also assessed by X-ray diffraction and Fourier transform infrared spectroscopy (FTIR) [18] to identify the minerals present. FTIR analysis consisted of mixing desiccated material with potassium bromide to a final concentration of 0.5%–1.0% (w/w). For each specimen, 20 spectra were obtained from 4000 cm^{-1} to 400 cm^{-1} using a Bomem model MB 120 Fourier transform infrared spectrophotometer (Bomem Canada, Quebec, QC) interfaced with a personal computer. The effective resolution was 4 cm^{-1}. In addition an FTIR spectrum was obtained from the sodium citrate used in the crystal inhibition experiments. All results obtained were compared with published spectra [15].

Results

pH Increase

The addition of citrate to both human and synthetic urine gave a concentration-related pH increase (Table 2). After the urease incubation a steady increase in pH was noted in all urine samples (Fig. 1). The addition of citrate did not appear to stimulate the rate of pH increase and the final pH obtained was not related to the citrate concentration.

Coulter counter particle analysis

Experiment 1. The results of the Coulter counter measurements are presented in Figs. 2 and 3 and Tables 1 and 2. In both human and synthetic urine, crystallization (nucleation) started considerably later and at a higher pH when citrate was present than in its absence. In the absence of citrate crystallization started at pH 6.54 in synthetic urine and pH 6.68 in human urine. In contrast, in the presence of 4 mM citrate these values were pH 8.52 and pH 8.64 respectively.

In *synthetic urine*, citrate significantly affected the crystallization process (Table 1). The nucleation was markedly delayed but the maximum number of particles (ca. 37000 per 500 μl) was independent of the citrate concentration. Citrate distincly inhibited the particle growth rate (Table 1). The growth index in urine without

Table 2. The effects of citrate on urease-induced crystallization in whole *human urine*

	Citrate concentration (mM)				
	0 (control)	1	2	3	4
Initial pH	6.06	6.14	6.35	6.48	6.75
Initial particle number[a]	21000	21000	21000	22000	22000
Initial median size (μm)	6	6	6	6	6
Nucleation started at (min)	45	90	135	150	165
pH at this time	6.85	7.95	8.43	8.57	8.61
Maximum particle number[a]	25000	25000	25000	26000	26000
Growth started at (min)	60	105	150	175	195
Growth index	0.17	0.16	0.15	0.15	0.13
Aggregation main process at (min)	105	150	175	195	210
Final particle number	9000	13000	15000	18000	24000
Final median size (μm)	19	20	21	22	20

[a] All particle numbers are per 500 μl

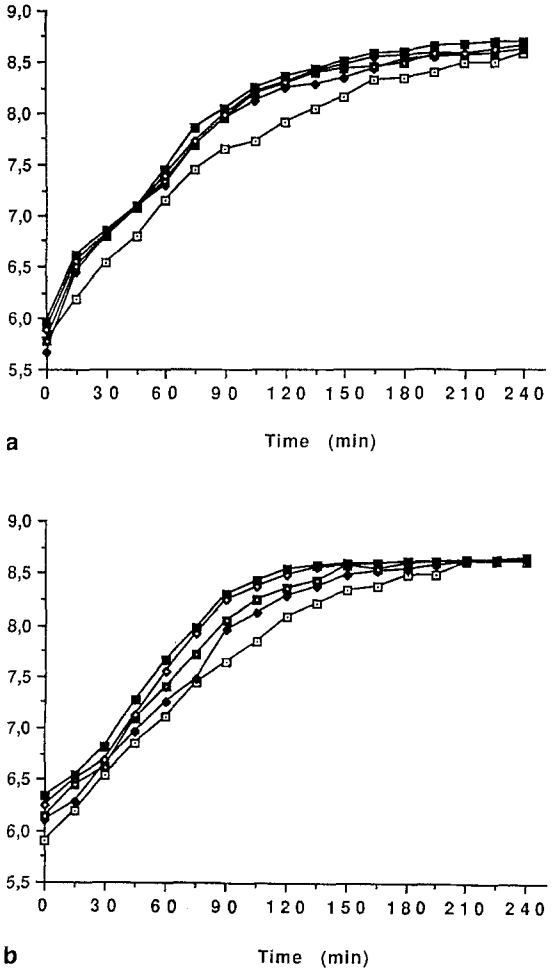


Fig. 1 a, b. The increase in pH in synthetic urine **(a)** and pooled human urine **(b)** after urease incubation; —⊡— control; —◆— citrate 1mmol/l; —□— citrate 2mmol/l; —◇— citrate 3mmol/l; —■— citrate 4mmol/l

citrate was 0.23 compared with 0.16 in urine with 1 mM citrate and 0.11 in urine with 4 mM citrate. The median particle size was also affected. In urine without citrate it was 21 μm compared with 9 μm in the presence of 4 mM citrate. This may be partially attributable to a pH effect in that crystal growth tends to cease at very high pH [17]. When the pH reached 8.75–8.82, the rate of increase in particle size declined. The difference in particle growth between urines containing 3 and 4 mM citrate appeared, however, to be due mainly to true inhibition. At high particle concentration, aggregation was also inhibited by citrate (Table 1). As seen in Fig. 3, very little variation was seen in triplicate experiments.

The number of particles in *human urine* was initially high (ca. 21000 per 500 μl). As was the case with synthetic urine, citrate delayed the nucleation process, but the increase in particle number was much less pronounced. The maximum number of particles was only ca. 25000 per 500 μl. It is possible that, with the higher basal background counts in human urine, the counter could cope less well with the high counts which the background and crystals would produce. A more pronounced increase could thus have been missed. Citrate did not have the strong effect on particle growth that it did in synthetic urine. The growth rate index was little affected and the maximum particle size was virtually identical in all portions regardless of citrate concentration. On the other hand, citrate profoundly influenced aggregation in a concentration-dependent manner. Both the onset and rate of aggregation were affected (Fig. 2b1, Table 2).

Experiment 2. In urine from both individuals the elimination of citrate accelerated the start of both nucleation and growth. Subsequent addition of citrate reversed these effects. In the urine sample from the patient with low urinary citrate, nucleation started earlier and at a lower pH than it did in the sample from the individual with high urinary citrate. Growth and aggregation were also inhibited by citrate in both urines.

Table 3. The effects of citrate on urease-induced crystallization in *different human* urines

	Individual 1			Individual 2		
Citrate concentration (mM)	0	2.4	3.0	0	0.7	3.0
Initial particle number[a]	20400	22406	20300	19000	20100	19000
Initial median size (μm)	6	6	6	6	6	6
Nucleation started at (min)	45	75	120	30	45	105
pH at this time	6.94	7.95	8.63	6.24	7.08	7.96
Maximum particle number[a]	29000	30000	29000	29000	30000	30000
Growth started at (min)	75	105	135	45	60	120
Growth Index	0.20	0.18	0.12	0.21	0.19	0.17
Aggregation main process at (min)	120	150	180	90	105	165
Final particle number	12000	15000	17000	4700	6800	16000
Final median size (μm)	16	16	17	20	21	24

[a] All particle numbers are per 500 μl

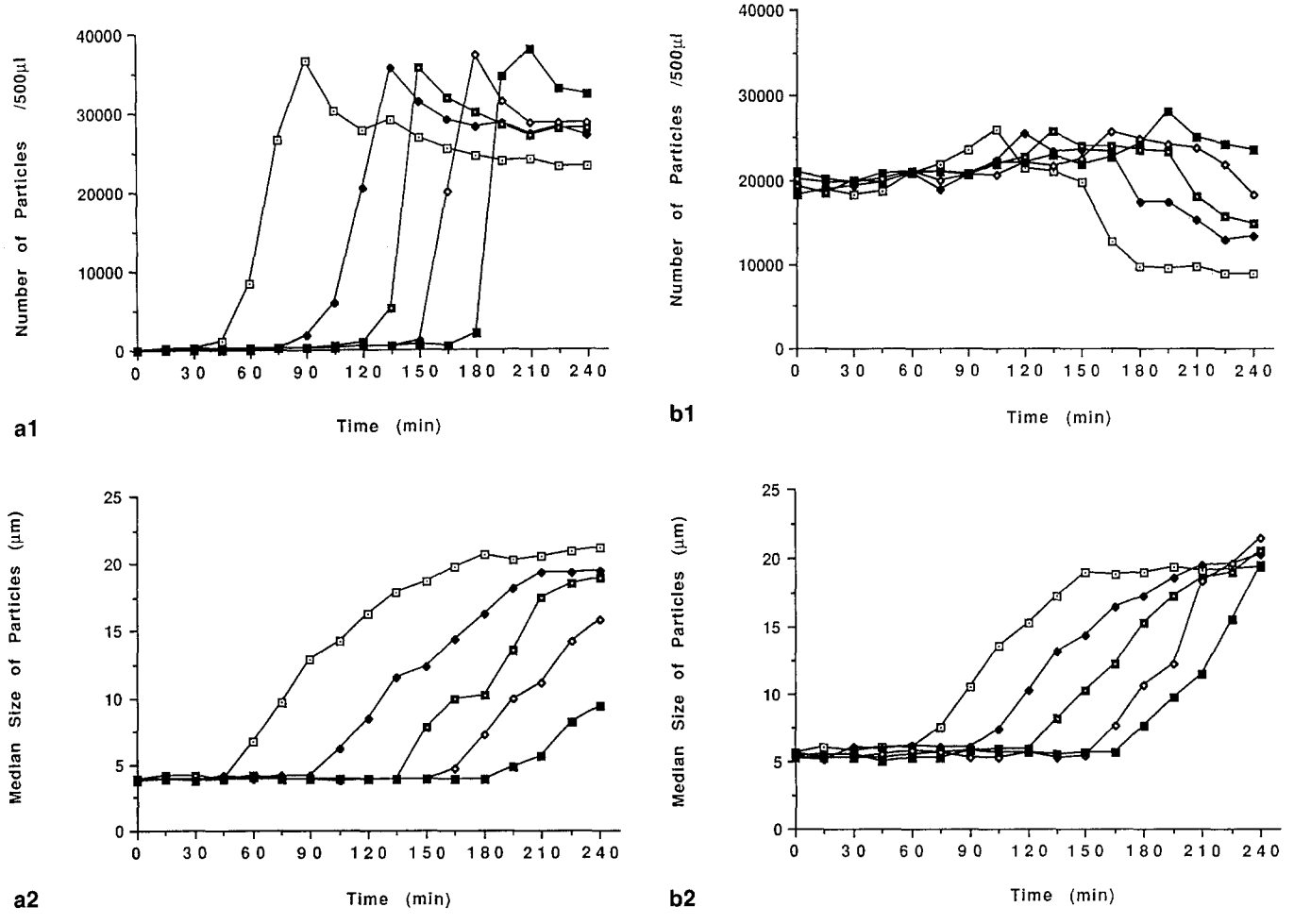


Fig. 2a, b. The increase in particle number and size related to time in synthetic urine **(a1, a2);** and pooled human urine **(b1, b2)** —⊙— citrate 0mmol/l; —◆— citrate 1mmol/l; —□— citrate 2mmol/l; —◇— citrate 3mmol/l; —■— citrate 4mmol/l;

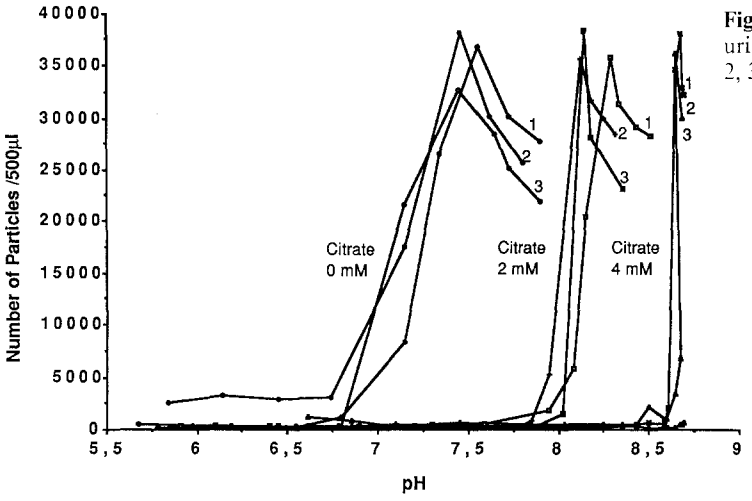
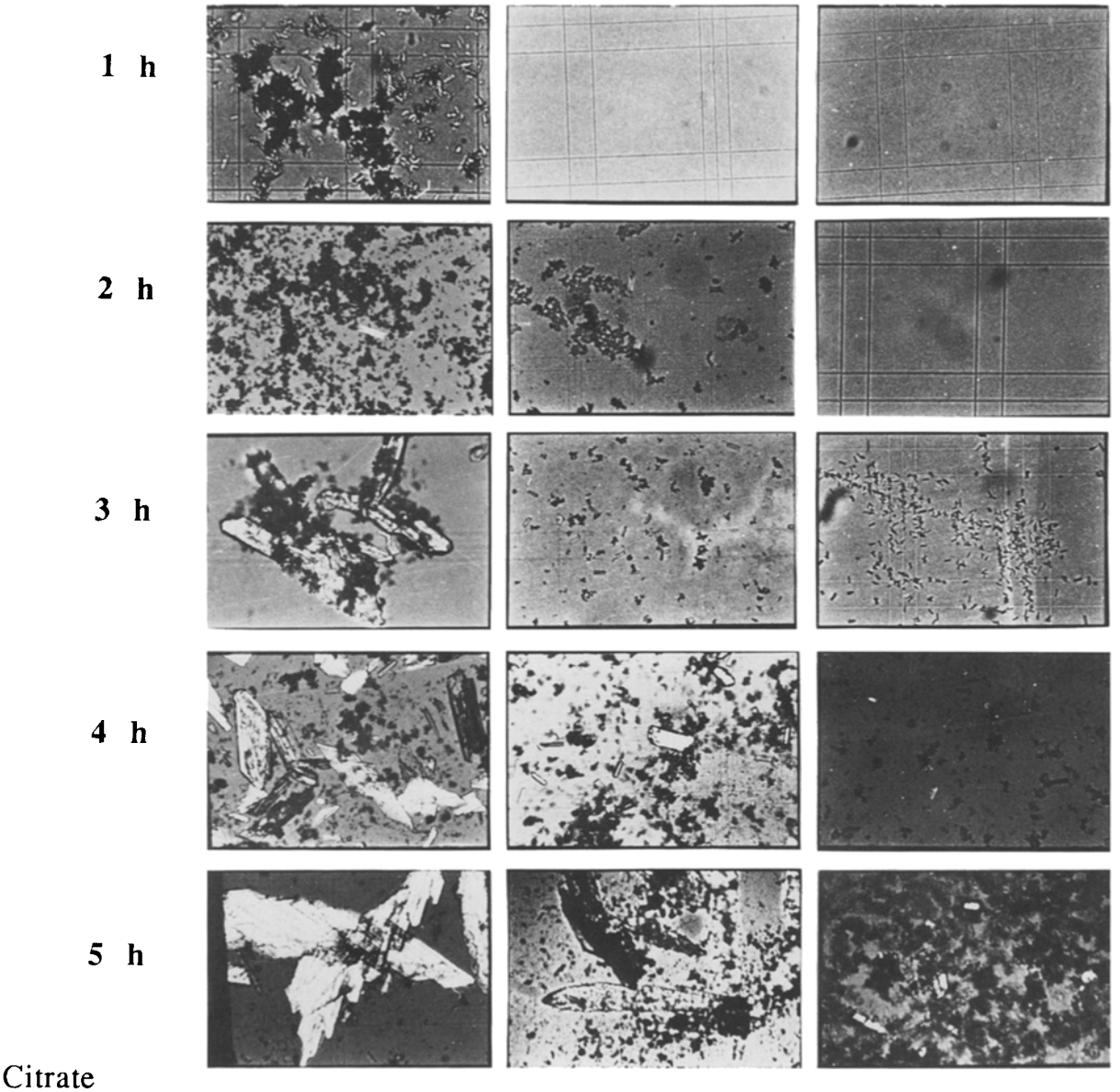


Fig. 3. The increase in particle number related to pH in synthetic urines with varying citrate concentrations performed in triplicate (1, 2, 3)

Fig. 4. Micrographs of material precipitated in synthetic urine with different citrate concentrations after urease incubation for 1–5 h. (×400 in all pictures)



Light microscopy

Visual examination by light microscopy (Fig. 4) showed that larger X-shaped or dendritic crystals and amorphous material (calcium phosphate) appeared much earlier in control urines lacking citrate. Aggregated crystals were also present to a greater extent in control urines. In contrast, urines containing citrate developed only tabular, coffin-shaped crystals with well-defined edges.

Crystal analysis

X-ray diffraction analysis identified crystals produced in the presence and absence of citrate as struvite. Slight differences in the crystal types were seen using FTIR in that low levels (5%–10%) of brushite ($CaHPO_4 \cdot H_2O$) and carbonate-apatite were consistently seen in all crystals grown in the presence of 0–3 mM citrate. Crystals grown in the presence of 4 mM citrate had greatly reduced quantities (<4%) of brushite.

Discussion

The addition of sodium citrate increased the initial pH both in synthetic and human urine. The initiation of the crystallization process (nucleation) was markedly delayed by citrate in both synthetic and human urine. In synthetic urine, particle growth and aggregation were delayed and inhibited by citrate. In human urine, only aggregation was distinctly influenced by citrate.

Urinary citrate excretion varies greatly between individuals: 2.67 ± 1.79 mM/24 h according to Burr et al. [5]. The citrate concentrations used in this investigation are thus within the normal range for human urine.

Previous studies concerning the role of citrate in urease-induced crystallization did not include a methodology which allowed a distinction of how the different phases of crystallization (nucleation, growth, and aggregation) are influenced by this compound [13, 19]. This information can be obtained with the Coulter counter technique which allows an estimation of particle number and particle size distribution in a solution [22]. The reproducibility of the technique is readily apparent in Fig. 3. Coulter counter techniques do not reveal the nature of the particles. To rectify this, light microscopy was used. In this manner we were able to estimate the distribution between crystalline and amorphous material, analyze crystal shape, and conduct a semiquantitative estimation of particle aggregation [18]. X-ray diffraction and FTIR enabled us to define the chemical composion of the precipitated materials [15].

The enzymatic removal of a crystal inhibitor such as citrate from human urine samples and its subsequent replacement provides a unique opportunity to study crystallization processes in human urine under chemically controlled conditions. It is important that such crystal growth studies be performed in human urine if the results obtained are to be relevant for renal stone formation in vivo. The results from both this and other studies demonstrate that crystal growth studies involving synthetic urine do not reliably mimic conditions in human urine [14].

That citrate causes an increase in urine pH is well known and is used therapeutically to prevent calcium stone formation [2, 4]. The main physiological effects of citrate addition are not only an increase in urinary pH but also a decrease in urinary calcium.

Previous studies have found citrate to stimulate the urease-induced pH increase in synthetic urine first in concentrations above 2.5–3.0 mM and to have no such effect in human urine [13]. This is not consistent with the result of the present study in that we found no such effect in either urine. We would suggest that any influence of citrate on urease activity would occur only at citrate concentrations above those found in normal urine.

Urease induces the precipitation of phosphates by increasing urinary pH which in turn reduces the solubility of these salts. As citrate increases urinary pH, it would therefore be expected that addition of citrate would stimulate crystal precipitation while citrate depletion inhibited it. In fact the opposite happened-nucleation started both later and at a higher pH in the presence of citrate. This effect was rather marked. In urine from the individual with low citrate (0.7 mM), nucleation started after 45 min of incubation; in the high citrate urine (2.4 mM) it started after 75 min. This inhibitory effect is probably due in part to the formation of a complex (chelation) between citrate and magnesium and/or calcium, but other mechanisms may also be involved. This inhibitory action of citrate may block struvite formation in situ such that the urine can be discharged without crystallization.

The crystal habit of struvite crystals has been shown to vary quite markedly depending on the rate of growth [1]. Very high growth rates induce the appearance of dendritic or X-shaped crystals because of preferential elongation along one or two crystal planes. When growth rates slow down, the more balanced growth along the several struvite crystal planes is reflected in the development of a more tabular, three-dimensional morphology or crystal habit [1, 18]. This agrees with our findings: the crystals in citrate-rich urines with a slow growth rate were tabular while the crystals in urine without citrate were dendritic of X-shaped (Fig. 4). The relevance of this to stone formation remains to be explained.

The influence of citrate on calcium oxalate crystallization is well established [2, 6, 8, 9]. Some workers have recognized calcium-citrate ion pairing as a factor, but there is also evidence that it inhibits not only crystal formation but also other phases of the crystallization process such as growth and aggregation. This may be accomplished by surface adsorption phenomena, explaining the strong inhibitory effect that citrate has on particle aggregation in human urine. Citrate also exerts an inhibitory effect on hydroxyapatite crystallization apart from its calcium-complex formation. Citrate is thought to represent about 50% of the toal inhibitory activity of human urine against hydroxyapatite crystallization [6].

Our results showing citrate inhibition of urease-induced crystallization in human urine are thus in line with

its effects on calcium oxalate and calcium phosphate crystal aggregation. Citrate delays both the onset of nucleation and the growth of struvite. This inhibitory effect differed between synthetic and human urines. That citrate did not have such a distinct effect on the growth index in human urine may be due to the fact that other growth inhibitors are also present. This assumption is reinforced by the observation that growth indices differed rather markedly between human and synthetic urines with the same citrate concentration.

An interesting aspect in the context of infection stone formation is that many bacteria can use citrate as a carbon source. Consequently urinary citrate is reduced in infected urine [7]. Bacteria can thus make urine more susceptible to the impact of urease by decreasing urinary citrate, thereby increasing the risk for stone formation. The risk factor for struvite stone formation resulting from UTI can be enhanced if any of the organisms present can metabolize and remove crystal growth inhibitors such as citrate. There is some evidence that citrate-depleting organisms enhance formation of other types of calculi notably calcium phosphate [16].

In summary there are several mechanisms by which citrate can be involved in urease-induced crystallization and stone formation in the urinary tract. We must stress, however, that citrate is only one of many complicated and interrelated factors which influence the formation of struvite urinary calculi. Despite this, it appears justified to assume that a high urinary citrate concentration reduces the risk for stone formation while a low urinary citrate content enhances the risk. While it is still too early to recommend citrate as a prophylactic agent for infection stone control, the potential is there.

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