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A new approach to studying inhibitors of calcium oxalate crystal growth

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Summary. The nucleation and crystal growth of calcium oxalate (CaOx) were studied at pH 5.5 using turbidimetric measurements at 620 nm of suspensions produced by mixing calcium chloride and sodium oxalate (initial conditions: Ca, 3×10^{-3} M; Ox, 0.5×10^{-3} M). CaOx crystallization kinetics were defined first by the induction time t_i and then by the slope of turbidity as a function of time during the interval corresponding to a correlation coefficient $r^2>0.99$. The technique described requires only a small amount of material, is quick, convenient, and can be used to study inhibitors of CaOx crystallization by comparing t_i and the rate of crystal growth in the presence and absence of inhibitors. The effects on CaOx crystal growth of several low molecular weight compounds, i.e. di- and tricarboxylic acids, were examined. The majority of these compounds were inhibitors of crystal growth, the greatest effect being seen with citric acid (50% inhibition in the presence of 1.5×10^{-3} M citric acid), isocitric acid (50% inhibition in the presence of 0.75×10^{-3} M isocitric acid) and pyrophosphate (30% inhibition in presence of 0.15×10^{-3} M pyrophosphate). The inhibitors' behaviour regarding the medium was studied without any assumptions about their possible mechanisms of action. Measurements of ionized calcium before and after the reaction, as well as the observation of crystals by scanning electron microspopy, allowed us to formulate the hypothesis that the effect of citric acid and tartaric acid can be attributed mainly to ion pairing, in contrast to that of pyrophosphate and the other carboxylic acids.

Key words: Carboxylic acids – Citric acid – Inhibition Kinetics of calcium oxalate crystallization – Pyrophosphate – Turbidimetric method

Calcium oxalate (CaOx) is the most common constituent of urinary calculi, particularly in industrialized countries. Supersaturation is a necessary condition for the occurrence of crystallization. However, it does not of itself explain crystal growth and aggregation since it was shown that the urines of normal people and stone-formers had a similar level of supersaturation [4, 9, 24]. The effect of inhibitors of CaOx crystallization has received particular attention because in vitro experiments can be done to investigate the contribution of various inhibitors to the total inhibitory activity of urine [8, 11, 25, 28]. Several methods have been used to study inhibitors of CaOx crystal growth, but many are either time consuming or require expensive or specialized equipment [1, 13, 16, 18, 21, 22, 29].

The present study reports a quick, easy and reproducible method for quantitating the inhibition of CaOx crystal growth by various compounds of low molecular weight, i.e. di- and tricarbocylic acids and pyrophosphate. Some of these compounds occur naturally in the urine or are already used in the treatment of nephrolithiasis [3, 6]. The inhibitors' behaviour regarding the medium was studied without any assumptions about their possible mechanisms of action. Their effects will be discussed in relation to the decrease in supersaturation and the consequences for nucleation and crystal growth.

Materials and methods

The effects of additives on CaOx crystallization kinetics were determined in aqueous solution by measurement of the turbidity of the suspension produced by mixing the precipitating reagents. The optical density (OD) of the crystals formed was measured at 3-s intervals at 620 nm using a Uvikon 930 spectrophotometer (Kontron Instruments). In this system, OD_{620} was directly proportional to the mass of crystals formed per unit volume [7, 15, 20].

Formation of calcium oxalate

Stock calcium chloride and sodium oxalate solutions (respectively 20×10^{-3} M and 1.0×10^{-3} M) were buffered at pH 5.5 with 9 mM sodium dimethylarsinate and brought to an ionic strength of 0.15 M

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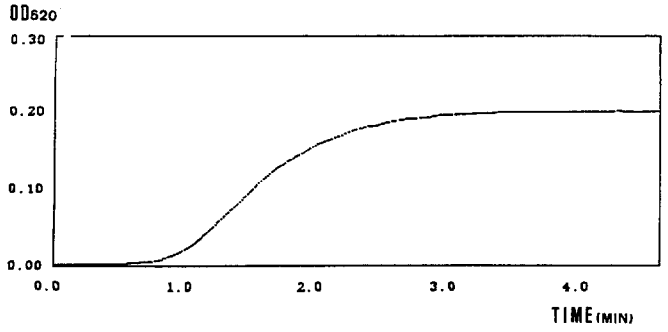

Fig. 1. Typical curve of calcium oxalate crystallization using turbidimetric measurements. Experimental conditions were: calcium concentration, 3×10^{-3} M; oxalate concentration 0.5×10^{-3} M; $T°$, 37° C; pH 5.5; ionic strength, 0.15 M

with sodium chloride. A calcium chloride solution of 6.0×10^{-3} M was prepared from the 20×10^{-3} M solution and a 1.5 ml aliquot of this transferred into a 10-mm light-path cuvette in a cell holder maintained at 30 °C by a constant-temperature circulating bath, with constant stirring at 500 rpm (using a Teflon-covered stirring bar, 7 mm × 2 mm).

After obtaining a stable baseline value of absorbance for 10 s, crystal formation was induced by adding an equal volume of freshly prepared sodium oxalate solution. Thus, the final concentrations in the test were respectively 3.0×10^{-3} M and 0.5×10^{-3} M for calcium and oxalate with a CaOx product of 1.5×10^{-6} mol^{2}l^{-2} and a relative supersaturation [10] of 6.17. These concentrations were chosen because they are close to physiological urinary concentrations; a pH of 5.5 was selected because it is a pH value frequently observed in the first morning urines of calcium stone-formers [2].

OD is measured over 6 min, during which time several kinetic parameters of the reaction are considered, namely the induction time t_i and the "turbidity slope". t_i corresponds to the time between the addition of oxalate and the moment at which the growth is experimentally measurable, i.e. when the change in OD is equal to 0.005 unit (2.5% of the maximal OD value). The slope of the linear portion of the curve is then determined during the time interval when the correlation coefficient of the curve, r^2, exceeds 0.99. The length of this interval varied between the different experiments, being longer in the presence of an effective inhibitor. Both parameters can be analysed in the presence and absence of additives for identical initial calcium and oxalate concentrations.

Effects of various compounds

The effects of citric acid, trisodium citrate and isocitric acid, respectively, on the relative crystal growth rate of CaOx were studied at a final concentration range in the test solution of 0.25–3.0×10^{-3} M for citric acid and trisodium citrate, and 0.25–2.2×10^{-3} M isocitric acid. Because the third ionization (pK = 6.4) of citrate is higher than the experimental pH chosen (5.5), trisodium citrate was tested to see whether it behaves differently to citric acid.

In order to account for a potential inhibitory activity, the following caboxylic acids that occur naturally in urine were tested: tartaric acid, fumaric acid, aspartic acid, maleic acid, glutamic acid, glutaric acid, and α-ketoglutaric acid, at 3.0×10^{-3} M final concentration in the test solution. Moreover, pyrophosphate (PP_i) was studied at a final concentration range in the test solution of $0.05–0.2\times10^{-3}$ M.

Solutions of inhibitors buffered at pH 5.5 with 9 mM sodium dimethylarsinate and brought to an ionic strength of 0.15 m were added to stock calcium chloride solution in order to obtain a calcium chloride solution of 6×10^{-3} M.

The percentage inhibition produced by the additives was calculated as $[1-(T_{si}/T_{sc})]\times100$, where T_{sc} was the turbidity slope of control and T_{si} the turbidity slope in the presence of inhibitor.

Six experiments were carried out for each condition, in the presence and absence of inhibitors. Mean values (m), standard deviations (SD) and coefficients of variation (CV%) were calculated within the series obtained for the six turbidity slopes.

Ionized calcium

Ionized calcium was measured using an ionized calcium analyser (Radiometer, Copenhagen, calibrated with calcium chloride aqueous solutions in the initial solution containing calcium only or both calcium and additives. After CaOx crystallization in the presence or absence of inhibitors, ionized calcium was also determined in the cuvette at the stable plateau corresponding to the end of the CaOx crystal growth (ΔOD < 0.002 unit, i.e. 1% OD_{max}).

Let Ca_0 and Cit_0 represent the initial concentration of calcium and citrate; Ca ion., Cit ion. and Ox ion. the concentration of ionized calcium, citrate and oxalate; and CaCit and CaOx the two types of complex formed. The complexation of calcium by citric acid is then represented by:

$$\text{Ca ion.} + \text{Cit ion.} \leftrightarrow \text{CaCit}$$

with $K_f = \text{CaCit}/(\text{Ca ion.}\times\text{Cit ion.}) = 1880$ [21]. Only this equilibrium is considered here. In fact, CaHCit can be neglected because $K_f = 67 \ll 1880$. According to the law of mass action, it can be written.

$$Ca_0 = \text{Ca ion.} + \text{CaCit}$$

$$Cit_0 = \text{Cit ion.} + \text{CaCit}$$

According to Lundager Madsen [19], for an in vitro study using a solution containing, in addition to calcium ions, only chloride and sodium ions, other complexes can be neglected.

Theoretically, ionized calcium is a linear regression as a function of Cit_0 in the concentration range tested (1.8–3 mM), with a slope $a = -0.81$ (see Appendix). Moreover, after the crystallization reaction crystal growth is represented by:

$$\text{Ca ion.} + \text{Ox ion.} \leftrightarrow \text{CaOx}$$

According to the law of mass action, it can be written, $Ca_0 = \text{Ca ion.} + \text{CaCit} + n\text{CaOx}$, where n is the number of moles of CaOx formed from the supersaturated solution.

Theoretically, Ca ion. is also a linear regression as a function of Cit_0 in the concentration range 1.5–2.5 mM, with a slope $a' = -0.78$ (see Appendix).

Scanning electron microscopy

Crystals were collected at a time of when the turbidity slope had its median value, on a Millipore of 0.22 µm pore size under vacuum. This was carried out in the same experimental conditions: first in the absence of inhibitors, then in the presence of 1 mM citric acid. The crystals retained on the filters were observed by scanning electron microscopy (ref: JEOL JSM-840 A) and photographed at a magnification of × 1200.

Results

Formation of calcium oxalate

The amount of crystals formed, as measured by the turbidity of the solution, gradually increased with time until a maximum was reached (Fig. 1). The mean induction time was 0.6 ± 0.05 min (m ± SD). The turbidity slope,

Table 1. Effects of carboxylic acids on calcium oxalate

Inhibitor	t_i (min)	Turbidity slope		$I\%$	Ionized Ca before	Ionized Ca after	pK1	pK2	pK3
		Mean	CV%						
Control	0.6	0.178	5.20		5.98	2.5			
Isocitric acid	2.5	0.027	5.63	85.0	3.75	1.80	3.29	4.71	6.4
Citric acid	2.5	0.041	6.16	77.0	2.26	1.06	3.13	4.76	6.4
Tartaric acid	1	0.109	5.53	38.4	4.45	2.22	2.52	4.16	
Aspartic acid	0.8	0.122	8.80	31.7	5.24	2.31			
Maleic acid	0.8	0.139	6.48	22.1	5.80	2.20	1.92	6.22	
Glutamic acid	0.7	0.148	1.15	17.1	5.98	2.35	2.10	4.07	9.07
Flumaric acid	0.8	0.149	3.06	16.5	5.78	2.71	3.02	4.39	
α-ketoglutaric acid	0.7	0.155	5.20	12.8	5.80	2.65	2.47	4.68	
Glutaric acid	0.7	0.163	6.50	8.6	5.95	2.70	4.34	5.27	

t_i, Induction time; *CV%*, percentage coefficient of variation; *I%*, percentage inhibition; *ionized CA before*, ionized calcium before 1: dilution; *ionized CA after*, ionized calcium after CaOx crystallization; *pK1–3*, values of the carboxylic acids tested
Experimental conditions: calcium concentration 3×10^{-3} M; oxalate concentration, 0.5×10^{-3} M; inhibitor concentration, 3×10^{-3} M, except isocitric acid, 2.2×10^{-3} M; $T°$, 37°C; pH 5.5; ionic strength, 0.15 M. Each experiment was done in sextuplicate

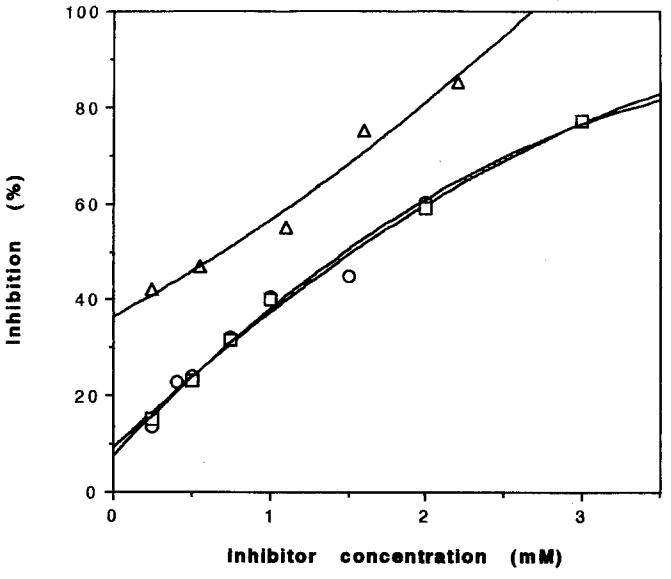

Fig. 2. Percentage of calcium oxalate crystal growth inhibition as a function of inhibitor concentration (○, citric acid; □, trisodium citrate; △, isocitric acid). Experimental conditions were: calcium concentration 3×10^{-3} M; oxalate concentration, 0.5×10^{-3} M; $T°$, 37°C; pH 5.5; ionic strength, 0.15 M

0.178 ± 0.009 Abs/min, was determined with good reproducibility ($n=6$; CV = 5.2%).

Effect of various compounds on calcium oxalate

In the presence of all the compounds studies, t_i increased and the slope of CaOx crystal growth decreased.

Citric acid. The inhibitory effect of citric acid on the kinetics of CaOx crystal growth was proportional to the concentration of citric acid, as shown in Fig. 2. Within a final concentration range in the test solution of 0.25–3.0×10^{-3} M, t_i varied from 0.7 to 2.5 min and the percentage inhibition from 15% to 77%.

Trinatrium citrate. The inhibitory effect of trinatrium citrate was completely superimposable with the effect of citric acid at pH 5.5 (Fig. 2).

Isocitric acid. Under the conditions of the study, the effect of isocitric acid was more marked than that of citric acid. A 0.25×10^{-3} M concentration of isocitric acid led to a 42% inhibition, and a concentration of 2.2×10^{-3} M gave a 85% inhibition (Fig. 2). Moreover, in this latter solution t_i was the same as the t_i of citric acid 3×10^{-3} M.

Other carboxylic acids (Table 1). Among the other organic acids tested at the final concentration of 3×10^{-3} M, the most effective inhibitors were tartaric acid and aspartic acid, leading to an inhibition of 38.4% and 31.7%, respectively. It must be noted that these acids exhibit weak inhibitory properties at pH 5.5, since their effect on growth rate appears at much higher concentrations (3×10^{-3} M) than the values observed in normal urine (traces). Moreover, the inhibition varied from 8.6% to 40%, depending on the compound considered. t_i was slightly higher than the t_i determined in the absence of inhibitor.

Pyrophosphate (Table 2). Over the range of concentrations studied ($0.05–0.2\times10^{-3}$ M in the test), the percentage inhibition rose from 21.5% to 31.7%, which means that PP_i is a rather good inhibitor. However, despite this variation in turbidity slope, t_i does not increase significantly (0.7 min to 0.8 min, compared with 0.6 min without inhibitor).

Table 2. Effects of pyrophosphate on calcium oxalate crystallization

PP_i (mM)	t_i (min)	Turbidity slope		$I\%$
		Mean	CV%	
0	0.6	0.178	5.20	
0.05	0.7	0.1383	2.24	21.58
0.1	0.7	0.1321	3.39	25.11
0.15	0.7	0.1218	4.02	30.93
0.2	0.8	0.1204	2.29	31.76

PP_i, Pyrophosphate; t_i, induction time; *CV%*, percentage coefficient of varriation; *I%*, percentage inhibition. Experimental conditions: calcium concentration, 3×10^{-3} M; oxalate concentration, 0.5×10^{-3} M; PP_i concentration, 0.05 to 0.2×10^{-3} M; *T*°, 37°C; pH 5.5; ionic strength, 0.15 M. Each experiment was done in sextuplicate

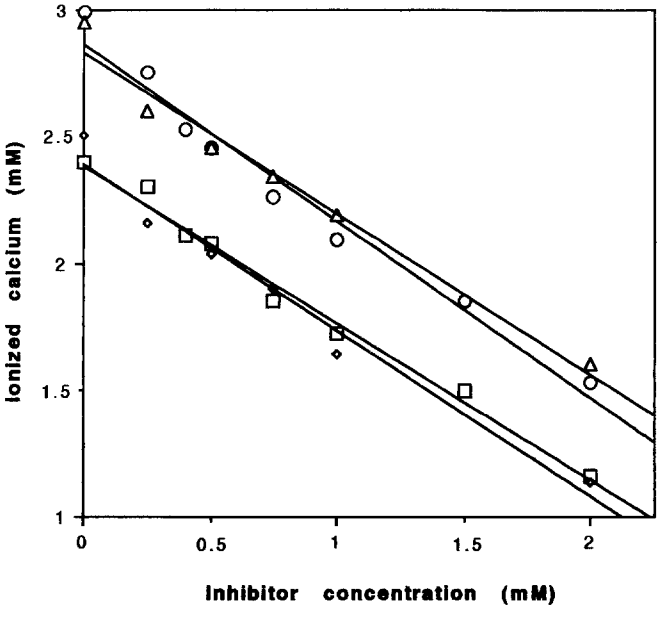

Fig. 3. Ionized calcium before and after calcium oxalate crystallization in the presence of citric acid (○ and □ respectively) and of trisodium citrate (△ and ◊, respectively) as a function of inhibitor concentration (mmol/l)

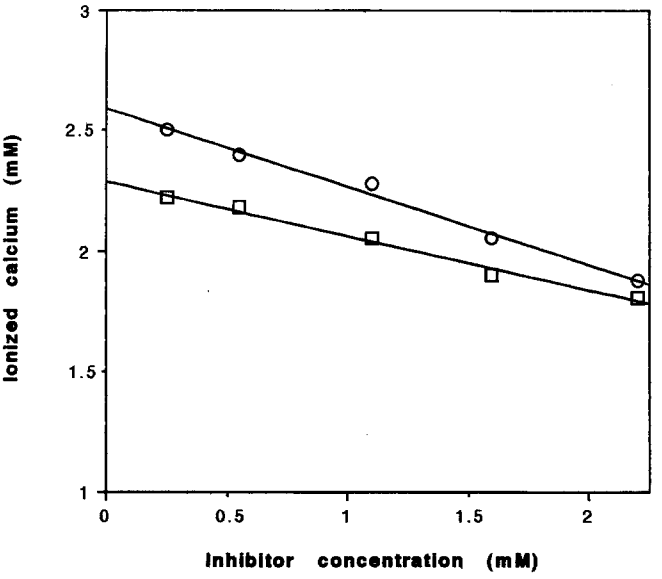

Fig. 4. Ionized calcium before (○) and after (□) calcium oxalate crystallization in the presence of isocitric acid as a function of inhibitor concentration (mmol/l)

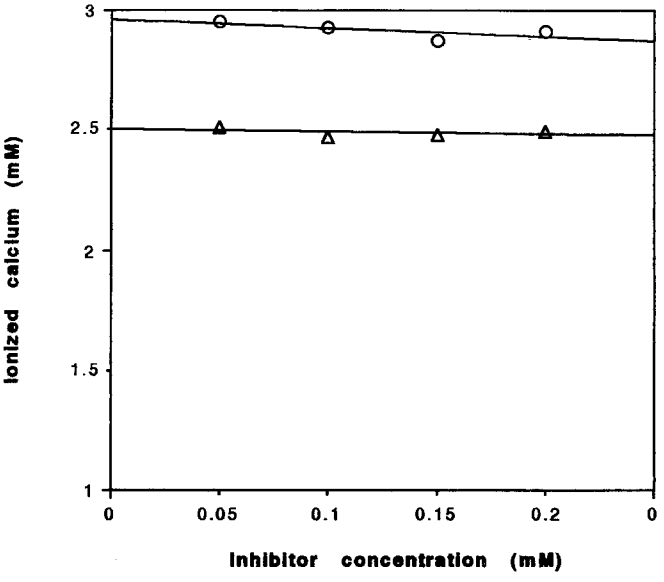

Fig. 5. Ionized calcium before (○) and after (△) calcium oxalate crystallization in the presence of Pyrophosphate as a function of inhibitor concentration (mmol/l)

Ionized calcium

Measurement of ionized calcium was carried out to assess (1) whether calcium complexation and surface phenomena contribute to the overall inhibition process, and (2) whether the inhibitory effect reduces the mass of crystal precipitated or produces only a delay in CaOx crystal growth.

Detailed data obtained with citric acid, trisodium citrate, isocitric acid and PP_i at various concentrations are presented in Figs. 3–5. It can be seen that ionized calcium both before and after the crystallization reaction decreases when inhibitor concentration increases.

Experimental data before crystallization in the presence of citric acid or trisodium citrate (Fig. 3) showed a linear relationship with a slope of −0.8. This value is in complete agreement with the theoretical value calculated in the Appendix (−0.8). The *y* intercept of this relation corresponds roughly to the Ca_0 value, i.e. 3.0×10^{-3} M.

After crystallization, the linear regression of the experimental data paralleled the first regression. The slope α was always at −0.8, and the intercept at 2.45. Therefore, the amount of calcium complexed in presence of citric acid or trisodium citrate is a constant which is equal to the

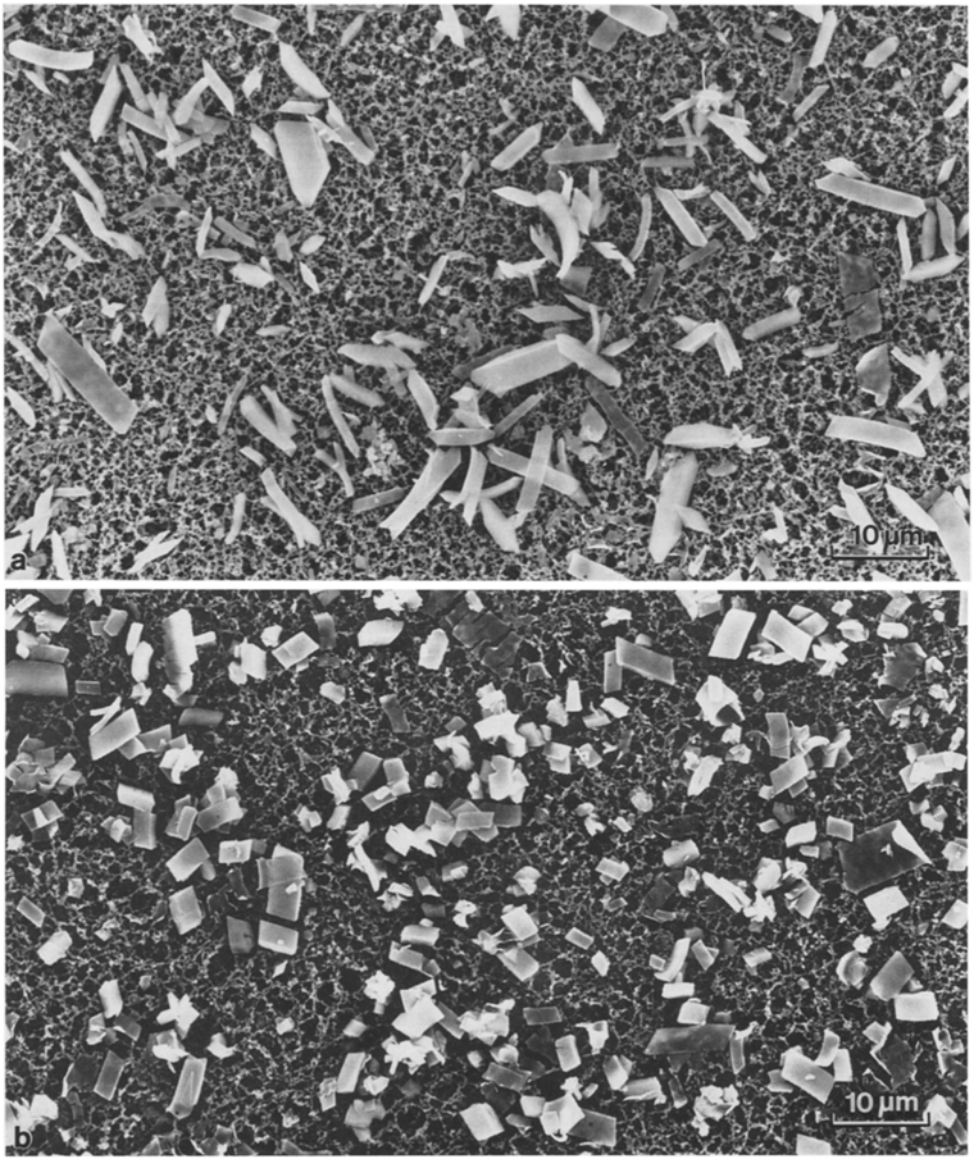

Fig. 6a, b. Scanning electron micrographs in the absence (**A**) and in the presence (**B**) of citric acid. Experimental conditions were: calcium concentration, 3×10^{-3} M; oxalate concentration 0.5×10^{-3} M; citric acid concentration in **b**, 1×10^{-3} M; $T°$, 37° C; pH 5.5; ionic strength, 0.15 M. Medium time in the turbidity slope. ×1200

difference between the two linear regressions (Fig. 3). By contrast, such a parallelism before and after crystallization has not been observed with isocitric acid (Fig. 4).

The results for the other carboxylic acids tested at 3.0×10^{-3} M are given in Table 1. Ionized calcium was reduced with tartaric acid and aspartic acid before the crystallization reaction (4.45 and 5.24×10^{-3} M), in contrast to the other caboxylic acids tested (ionized calcium varied from 5.78 to 5.98×10^{-3} M).

Finally, as regards PP_i two horizontal parallel regressions are obtained (Fig. 5): there is no change in the ionized calcium concentration before the reaction, even after PP_i was added (roughly equal to 2.9×10^{-3} M). The ionized calcium concentration after the reaction is constant too (roughly equal to 2.5×10^{-3} M).

Scanning electron microscopy

On the two electron micrographs taken (Fig. 6) the number of crystals appears to be comparable, but the mean size is smaller in the presence of citric acid. The difference in morphology of the crystals is more striking: the length/width ratio is lower in the presence of inhibitor than in its absence.

Discussion

Formation of calcium oxalate crystals

The formation of CaOx crystals has been directly followed in a spectrophotometer cuvette by the light scattered after mixing solutions of calcium cloride and sodium oxalate. The dispersion of light by turbid solutions depends on the number, mean size and distribution of particle sizes present, according to the theory of turbidimetry in aqueous solution [7, 20] and to the scanning electron micrographs.

The present method is easy and rapid to perform. Furthermore, the reproducibility of the measurements is

good (CV% < 9%, whatever the conditions in the presence or absence of inhibitors), suggesting that under the same experimental conditions an identical distibution of particle number and size was produced.

Previous investigations used seeded crystal growth systems [18, 21, 22] or gel matrices [1] to study inhibitors of CaOx crystallization kinetics. It is difficult to compare crystal formation of CaOx in the present model with results reported in the literature because previous workers took into account only the growth of CaOx. The present report of spontaneous precipitation involves both nucleation and crystal growth, which in our opinion is of interest and a better reflection of phenomena in the urine, because generally the two processes occur simultaneously.

The nucleation period is characterized by the time of appearance of optically measurable crystals that have reached a critical size in the solution [5]. This time can be considered as equal to t_i, previously defined as the time when the variation in OD is experimentally measurable [11]. In our investigation t_i increased with the addition of inhibitors, reflecting their effect on crystal nucleation.

The rising portion of the curve (Fig. 1) is essentially related to crystal growth although nucleation continues even after t_i. The slope of the turbidity curve was directly related to the kinetics of the reaction and was reduced in the presence of known inhibitors of CaOx formation [1, 18, 21, 22].

Moreover, most authors worked at different pH values; however, CaOx crystallization and in particular the effect of inhibitors must be evaluated in relation to pH [1, 23, 26, 27].

Effects of various compounds

Because their chemical structure is analogous to that of citric acid, various di- and tricarboxylic acids were tested in this system. PP_i was also studied since it is a well-known inhibitor in human urine. In presence of these compounds the increased t_i and the decreased slope of the curve corresponded to inhibition. For citric acid, trisodium citrate, isocitric acid and PP_i the inhibition was proportional to the concentration of inhibitor present in the reaction medium (from 0.25 to 3 mM); this is in agreement with previous studies demonstrating at pH 6, in seeded crystal growth systems with lower supersaturation, 50% inhibition at a citrate concentration of 0.1 or 0.4 mM [16, 18, 22]. For isocitric acid, Achilles [1] demonstrated in gel matrices an inhibition varying from 4% to 17% for 0.1–1 mM concentrations.

Moreover, it has been demonstrated that inhibitory activity with regard to CaOx crystal growth must be evaluated in relation to the pH of urine [23, 27]. Thus, the inhibition index of citric acid was found to increase in the pH interval from 5.5 to 7.5 [26]. At pH 5.5 the inhibitory effect is significant for other carboxylic acids at high concentration (3×10^{-3} M) (varying from 8% to 40%). At this concentration, tartaric acid and aspartic acid exhibit respectively a 40% and 32% inhibition. These data are in accord with previous reports [1, 22]. Moreover, as previously reported by Garti et al. [12] and recently by Kohri et al. [17], we found an inhibitory effect of glutamic acid and aspartic acid on CaOx crystallization in vitro.

Concerning PP_i, the induction time is not affected at all by the presence of this inhibitor in the test. However, the slope is significantly decreased. As t_i represents the efficiency of nucleation, it seems to us that PP_i does not inhibit this phase but more likely crystal growth. Thus, this rejects the hypothesis of inhibition by complexation.

Ionized calcium

The effect of citric acid and trinatrium citrate can be attributed mainly to their complexation ability towards the calcium ion. In fact, in the presence of these inhibitors the decrease in ionized calcium in solution indicated that the observed decrease in crystal growth rate was mainly due to complexation, as shown by the parallelism of the curves observed before and after the crystallization reaction (Fig. 3). However, it does no exclude another mechanism having a minor role (Fig. 6b).

Theoretical results on the complexation of calcium by citrate have been verified experimentally, as shown in the Appendix. Therefore, our approximations appear to be justified, and it can be assumed that the observed inhibition affects only the kinetic aspect and produces a delay only in crystallization, without modifying the precipitation equilibrium.

The results with isocitric acid suggest a different mechanism of action. Although most of its effect is attributable to ion pairing, part appears to depend on another mechanism such as adsorption phenomena, according to the experimental results obtained. The convergence of the curves shows that calcium complexation probably increases with the concentration of isocitric acid, and that in urine the main effect is thus an adsorption effect with regard to urinary concentration.

With the other dicarboxylic acids tested, the ionized calcium in the initial solution was reduced by a lesser amount, although the concentrations of these compounds tested were higher than that of citric or isocitric acid. These results suggest either a different mechanism of action or unfavourable experimental conditions, particularly the pH. Indeed, it has been demonstrated that glutamic acid and aspartic acid appeared in relatively high proportions in the organic matrices of CaOx stones. It may therefore be inferred that these amino acids were preferentially absorbed on CaOx crystals [12]. Moreover, the pH must be considered as a factor because all these acids have different pK values and could possibly have a more marked inhibitory effect at another pH. Finally, comparing the structure of the dicarboxylic acids tested, the present data suggest that the isomeric position of –COOH did not appear to contribute to the effect of CaOx crystallization growth. Indeed, results obtained with fumaric acid and maleic acid were not significantly different ($\alpha = 0.05$).

The results obtained for PP_i are no less interesting: two horizontal lines are obtained, meaning that calcium is not complexed by PP_i (at least not significantly). This confirms the hypothesis of another inhibitory mechanism.

Scanning electron microscopy

According to the theory of turbidimetry [7, 20] we were expecting a more striking difference in the number, mean size and distribution of sizes of crystals between the experiments in the absence and presence of citric acid. The real contrast, however, lies in the morphology of the crystals, which has already been noted in the literature [14]; we can therefore assume that inhibition by surface adsorption phenomena is added to complexation.

In conclusion, this fast and simple method can easily be used to study the inhibitory effect of various substances on CaOx crystal growth rate. The inhibitory effect of various low molecular weight di- and tricarboxylic acids of the tricarboxylic acid cycle allows an estimation of the efficiency of these compounds as inhibitors. In addition it is an approach to the study of the mechanisms of inhibition of CaOx crystal growth.

In the present study citric acid, trisodium citrate, isocitric acid and PP_i were found to be more potent inhibitors of CaOx crystal growth than were the other carboxylic acids which were tested at a concentration similar to that of citric acid in normal urine. If citric acid exerts its overall effect mainly through ion pairing, it appears that PP_i and the other di- and tricarboxylic acids act via another mechanism. For isocitric acid and tartaric acid, that mechanism is in part attributable to ion pairing, in part to crystal adsorption. For PP_i and the other dicarboxylic acids the latter mechanism is probably mainly responsible for the inhibitory effect. Of course, such in vitro data cannot be directly extrapolated to the more complex conditions in the urine in vivo, where many other physiologically occurring substances may modulate the effect on CaOx crystallization. However, they allow us to extend our knowledge of the behaviour of each individual substance.

Appendix

Let Ca_0 and Cit_0 represent the initial concentrations of calcium and citrate; Ca ion. and Cit ion. represent the concentrations of ionized calcium and citrate; and CaCit and CaOx represent the calcium citrate and calium oxalate complexes.

The calcium citrate equilibrium

Ca ion. + Cit ion. $\leftrightarrow$ CaCit, with $K_f = \text{CaCit}/(\text{Ca ion.} \times \text{Cit ion.}) = 1880$

We need consider only this eqilibrium: CaHCit can be neglected because $K_f = 67 \ll 1880$.

According to the law of mass action, it can be written

$$Ca_0 = \text{Ca ion.} + \text{CaCit} \quad (1)$$

$$Cit_0 = \text{Cit ion.} + \text{CaCit} \quad (2)$$

Therefore, according to Eq. 2:

$$\text{Cit ion.} = \frac{Cit_0 - \text{Cit ion.}}{k_f \times \text{Ca ion.}}$$

$$\text{Cit ion.} = \frac{Cit_0}{1 + K_f \times \text{Ca ion.}} \quad (3)$$

The expression for Ca ion., according to Eqs. 1 and 2, is:

$$\text{Ca ion.} = Ca_0 - \text{CaCit} = Ca_0 - Cit_0 + \text{Cit ion.}$$

And according to Eq. 3:

$$\text{Ca ion.} = -\left(1 - \frac{1}{1 + K_f \times \text{Ca ion.}}\right) Cit_0 + Ca_0 \quad (4)$$

Therefore, theoretically, Ca ion. as a functon of Cit_0 gives a half parabola with horizontal axis. We can, however, approximate this parabola with a linear regression for the concentration range tested (1.8 mM < Ca ion. < 3 mM). Therefore

$$-0.85 < -\left(1 - \frac{1}{1 + K_f \times \text{Ca ion.}}\right) < -0.77$$

i.e.

$$-0.85 \times Cit_0 + Ca_0 < \text{Ca ion} < -0.77 \times Cit_0 + Ca_0$$

We can approximate

$$\text{Ca ion.} = -0.81 \times Cit_0 + Ca_0 \quad (5)$$

Ionized calcium is thus a linear regression as a function of Cit_0, with a slope $a = -0.81$.

The comparison with the experimental results shows acceptable results. In fact, a linear regression is observed: Ca ion. $= \alpha \times Cit_0 + K$ with $\alpha = -0.8$, therefore $a = \alpha$ and $K = Ca_0$.

Addition of oxalate

$$\text{Ca ion.} + \text{Ox ion.} \leftrightarrow \text{CaOx} \quad (6)$$

According to the law of mass action, this can be written

$$Ca_0 = \text{Ca ion.} + \text{CaCit} + n\text{CaOx}$$

or

$$\text{Ca ion.} = Ca_0 - \text{CaCit} - n\text{CaOx}$$

where n is the number of moles of calcium oxalate formed from the supersaturated solution.

According to Eqs. 2 and 3

$$\text{Ca ion.} = -Cit_0 + \left(\frac{1}{1 + K_f \times \text{Ca ion.}}\right) Cit_0 + Ca_0 - n\text{CaOx}$$

Thus

$$\text{Ca ion.} = -\left(1 - \frac{1}{1 + K_f \times \text{Ca ion.}}\right) Cit_0 + Ca_0 - n\text{CaOx} \quad (7)$$

The precipitation equilibrium (Eq. 6) influences only nCaOx according to Eq. 7, i.e. the y intercept of the relationship of Ca ion. versus Cit_0. In contrast, there is no particular effect on the slope of this linear regression. In the same way as before, we can consider that Ca ion. is a linear regression as a function of Cit_0, in the concentration range tested.

Here, 1.5 mM < Ca ion. < 2.5 mM. Therefore

$$-0.82 < -\left(1 - \frac{1}{1 + K_f \times \text{Ca ion.}}\right) < -0.74$$

This can be approximated as

$$\text{Ca ion.} = -0.78 \times Cit_0 + Ca_0 - n\text{CaOx}$$

The comparison with the experimental data shows a linear regression parallel with the first regression:

$$\text{Ca ion.} = -0.8\ Cit_0 + K', \quad \text{where } K' = Ca_0 - n\text{CaOx} = 2.45$$

Therefore nCaOx is a constant equal to the difference between the two linear regressions.

References

1. Achilles W (1987) Crystallization in gel matrices: a new experimental model of calcium stone formation. Contrib Nephrol 58:59
2. Berg C, Tiselius HG (1986) The effect of pH on the risk of calcium oxalate crystallization in urine. Eur Urol 12:59
3. Berg C, Larsson L, Tiselius HG (1990) Effects of different doses of alkaline citrate on urine composition and crystallization of calcium oxalate. Urol Res 18:13
4. Berland Y, Olmer M, Boistelle R, Barlatier A (1984) Influence respective du calcium et de l'oxalate sur la cristallisation des oxalates de calcium dans l'urine. Nephrologie 5:175
5. Boistelle R (1985) Concepts de la cristallisation en solution. Act Nephrol. Flammarion Medecine Sciences Ed:159
6. Cowley DM, McWhinney BC, Brown JM, Chalmers AH (1989) Effect on the urinary excretion of calcium and oxalate: relevance to calcium oxalate nephrolithiasis. Clin Chem 35:23
7. Dezelic G, Dezelic N, Tezac BA (1963) A simple method for particle size determination by turbidity measurement. J Coll Sci 18:888
8. Fellström B, Backman U, Danielson BG, Holmgren K, Lunghall S, Wikström B (1982) Inhibitory activity of human urine on calcium oxalate crystal growth: effects of sodium urate and uric acid. Clin Sci 62:509
9. Finlayson B (1978) Physicochemical aspects of urolithiasis. Kidney Int 13:344
10. Gardner GL (1975) Nucleation and crystal growth of calcium oxalate trihydrate. Crystal Growth 30:158
11. Gardner GL, Doremus RH (1978) Crystal growth inhibitors in human urine: effect on calcium oxalate kinetics. Invest Urol 15:478
12. Garti N, Tibika F, Sarig S, Perlberg S (1980) The inhibitory effect of polymeric carboxylic amino-acids and urine on calcium oxalate crystallization. Biochem Biophys Res Commun 97:1154
13. Gill WB, Karesh IW, Garsin L, Roma MJ (1977) Inhibitory effects of urinary macromolecules on the crystallization of calcium oxalate. Invest Urol 15:95
14. Grases F, Costa-Bauza A (1990) Study of factors affecting calcium oxalate crystalline aggregation. Br J Urol 66:240
15. Hess, B, Nakagawa Y, Coe FL (1989) Inhibition of calcium oxalate monohydrate crystal aggregation by urine proteins. Am J Physiol 257:F99
16. Kok DJ, Papapoulos SE, Blomen LJ, Bijvoet OLM (1988) Modulation of calcium oxalate monohydrate crystallization kinetics in vitro. Kidney Int 34:346
17. Kohri K, Umekawa T, Kodama M, Katayama Y, Ishikawa Y, Takada M, Katoh Y, Kataoka K, Iguchi M, Kurita T (1990) Inhibitory effect of glutamic acid and aspartic acid on calcium oxalate crystal formation. Eur Urol 17:173
18. Ligabue A, Fini M, Robertson WG (1979) Influence of urine on in vitro crystallization rate of calcium oxalate: Determination of inhibitory activity by a ^{14}C oxalate technique. Clin Chim Acta 98:39
19. Lundager Madsen HE (1984) Aspects physicochimiques de la lithiase urinaire. Nephrologie 5:151
20. Melik DH, Fogler HS (1983) Turbidimetric determination of particle size distributions of colloidal systems. J Coll Interf Sci 92:161
21. Meyer JL, Smith LH (1975) Growth of calcium oxalate crystals. II. Inhibition by natural urinary crystal growth inhibitors. Invest Urol 13:36
22. Minero C, Maggiorotti P, Barbeni M, Pelizetti E, Tizzani A, Casseta G, Piana P, Giona C (1987) Effect of inhibitors on the crystal growth of calcium oxalate. Contrib Nephrol 58:65
23. Pak CYC (1987) Citrate and renal calculi. Miner Electrolyte Metab 13:257–266
24. Robertson WG, Peacock M, Marshall DH, Nordin BE (1976) Saturation-inhibition index as a measure of the risk of calcium oxalate stone formation in the urinary tract. N Engl J Med 294:249
25. Scurr DS, Robertson WG (1986) Modifiers of calcium oxalate crystallization found in urine. III. Studies on the role of Tamm–Horsfall mucoprotein and of ionic strength. J Urol 136:505
26. Sutor DJ (1969) Growth studies of calcium oxalate in the presence of various ions and compounds. Br J Urol 41:171
27. Tiselius HG (1981) The effect of pH on the urinary inhibition of calcium oxalate crystal growth. Br J Urol 53:470
28. Werness PG, Bergert JH, Lee EK (1981) Urinary crystal growth: effect of inhibitor mixtures. Clin Sci 61:487
29. Will EJ, Bijvoet OLM, Van Der Linden HB (1976) Inhibition of calcium oxalate crystal growth: a simple method of measurement and preliminary results. In: Fleisch H, Robertson WG, Smith LH (eds) Plenum Press, New York, p 367