Kidney International, Vol. 34 (1988), pp. 346–350

Modulation of calcium oxalate monohydrate crystallization kinetics in vitro

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Modulation of calcium oxalate monohydrate crystallization kinetics in vitro. The effects of several low and high molecular weight (mol wt) compounds on the kinetics of calcium oxalate crystallization were examined using a seeded crystal growth method in which the solubility, the growth and the agglomeration of calcium oxalate crystals were measured as three separate and system-independent parameters. Citrate, magnesium, phosphate, pyrophosphate, chondroitinsulphate, pentosanpolysulphate and heparin were tested in a wide range of concentrations. The solubility of calcium oxalate crystals was increased only by citrate and magnesium. The crystal growth was inhibited by all compounds tested, but those with the high mol wt had the greatest effect at low concentrations. In contrast, inhibition of crystal agglomeration was achieved only by the low mol wt compounds; citrate was found to be the most potent inhibitor at concentrations likely to be present in normal urine. The high mol wt substances, despite their potent crystal growth inhibitory activity, had no effect on agglomeration. The results show that growth and agglomeration of calcium oxalate crystals are separate processes which are differently modulated by various compounds. They further provide a possible explanation for the pathogenetic role of citrate in hypocitraturic renal stone disease.

Most of the currently employed treatments of renal stone disease are of empirical nature and as a rule aim at reducing the supersaturation of urine with calcium oxalate. The natural history of renal stone disease makes the evaluation of the effective mode of action of any potential therapeutic agent a tedious and long-term commitment. It is, therefore, important to define the processes involved in stone formation before engaging in such studies and to evaluate their relative importance.

Stone formation is a biological process that involves a physicochemical one, crystallization. Crystallization comprises several components, which may all be affected differently by the urine composition. Two main aspects are recognized, a thermodynamic and a kinetic. The first includes the supersaturation which results in nucleation. The second comprises the rates of nucleation, of crystal growth and of crystal agglomeration. At supersaturation, these processes determine whether this results in the development of small crystals, which may be present in normal urine [1], or in the development of renal stones. With the recently described methods for independent measurements of the solubility, the growth and the agglomeration of calcium oxalate monohydrate crystals [2–5], it has become possible to study the biological regulation of these kinetic processes.

In the present study, we have examined how the kinetics of calcium oxalate monohydrate crystallization are affected by low and high mol wt compounds, some of which are naturally occurring in the urine or are used in the treatment of nephrolithiasis.

Methods

The effects of additives on the crystallization kinetics were determined with a seeded crystal growth system which has been described in detail before [2–4]. The solubility product is the lowest concentration product at which growth of added, pregrown, crystals starts to occur, that is, the equilibrium concentration product. It is expressed as the square root of L_c, $\sqrt{L_c}$. The crystal growth inhibition is the actual growth constant in the presence of an additive, $K_{a,inh}$, in relation to the growth constant without additive, $K_{a,c}$, and is expressed as a percentage of the latter, percent G.I. Agglomeration reduces the rate at which mineral components are taken up. It is therefore measured in units of time ([tm]; min). Inhibition of agglomeration by additives increases [tm] relative to that in a solution without additive. The mean [tm] (± SD) in control experiments (that is, the agglomeration proper to the particular seed used) was 58 (± 16) minutes, $N = 357$. Higher values indicate inhibition of agglomeration.

The effect of a compound on agglomeration in our system was determined by the combination of induced changes in zeta potential, viscous binding, van der Waals forces, solid bridge formation and other weaker forces. Shear forces which can affect agglomeration were present equally strong in the control experiment as in the experiment with additive, and thus did not contribute to differences between both experiments.

The essentials of the method, in brief, are as follows. Supersaturated solutions were prepared with initial total concentrations of calcium, $T_{Ca,i}$, and oxalate, $T_{ox,i}$, of 0.372 mM, and were buffered at pH 6.00 with 7.5 mM sodium dimethylarsinate. A tracer amount of ^{45}Ca was added and the solutions were brought to an ionic strength of 0.15 M with sodium chloride. The experiment, performed at 37°C, started by seeding with calcium oxalate monohydrate crystals, in a seed concentration, s, of 0.1395 g/liter. The crystals used were aged, dried and pregrown before each experiment to ensure reproducibility in crystal surface properties between each experiment. The crystals were kept in suspension by shaking. At various times (t) after

Received for publication August 4, 1987
and in revised form April 15, 1988

incubation, the crystals were collected on a millipore filter (0.45 μ) and fractional uptake, U_t, of ^{45}Ca tracer into the crystals was measured. Uptake due to exchange processes was less than 1% in our system [2–4].

Two types of experiments were performed. In the first, solubility of calcium oxalate monohydrate in the additive containing system was measured. Growth time was fixed and initial concentrations of calcium and oxalate were varied. In this way the concentration product at which net growth of added crystals started to occur with increasing concentrations of calcium and oxalate (equilibrium product) was measured. From this value the uptake required to reach equilibrium, U_{eq}, was calculated. It should be noted that although the thermodynamic solubility product based on equilibrium activities is contant at constant temperature, the apparent solubility product, which is based on concentrations, as in our system, can change considerably [3]. For example ionic strength, complexation of calcium or oxalate by other compounds or the presence of very small crystals can all influence the concentrations which can be retained at equilibrium.

In a second experiment, initial concentrations of calcium and oxalate were constant but growth time was varied. Variation of the incubation time yielded a hyperbolic relationship between uptake and growth time described by the following equation:

$$\frac{U_t}{U_\infty} = \frac{t}{(t + tm)} \qquad (1)$$

Where U_∞ denoted the uptake at infinite growth time as extrapolated from the measured growth curve and tm was the time where uptake equals $U_\infty/2$.

The thermodynamically expected U_∞ depended only on the difference between initial and equilibrium concentration products and equaled U_{eq}. In practice, however, we found that the measured U_∞ was always lower than the U_{eq} [2]. This difference was correlated with the seed crystal concentration [2–4]. At infinite seed concentration the U_∞ could be established by extrapolation. This was called $[U_\infty]$ and equals U_{eq} at all supersaturations tested. This was expected, because such systems should reach equilibrium in an infinitely short time. The difference between U_∞ and U_{eq} could be described by the parameter [tm], the time where uptake equals $[U_\infty]/2$ according to the following equation:

$$[tm] = tm \cdot \left(\frac{U_{eq}}{U_{eq} - U_\infty}\right) = tm \cdot \frac{[U_\infty]}{[U_\infty] - U_\infty} \qquad (2)$$

As the U_∞ thermodynamically could only depend on the starting supersaturation, the correlation with the seed concentration must be due to an influence of the seed material on the supersaturation. This may be caused by a process which was not regulated in our system, and which was a property of the seed crystals used, their natural tendency to agglomerate. It was conceivable that inside agglomerates, inclusions could occur which were not freely accessible to the bulk solution. As a consequence of the hindered diffusion, the crystal surface surrounding these inclusions would 'see' a lower ambient supersaturation than that existing in the bulk solution. Overall for the total crystal mass, the ambient supersaturation would be somewhat lower than could be expected on the basis of the total calcium and oxalate concentration present. Thus, agglomeration of calcium oxalate crystals could cause the decrease in uptake as described by [tm] [4]. To validate this conclusion, we have used an independent system, a laserflowcytometer, to examine the agglomeration process. With the laserflowcytometer actual (changes in) crystal-size distribution were measured. The agglomeration data obtained from the laserflowcytometer experiments agreed completely with those obtained from the tracer experiments, thus justifying the use of [tm] as a parameter for agglomeration [6]. The tm was a kinetic parameter measured directly in a particular growth experiment as the time at which uptake equaled $U_\infty/2$. It was specific for the particular supersaturation and the seed concentration used, as well as for the agglomeration state of the particular seed. The parameter [tm] described the specific agglomeration state of a particular seed throughout experiments, independent from the actual supersaturations and seed concentrations that were applied.

By differentiating equation one to the time and knowing the effects of changes in solubility and agglomeration on the growth rate, a final description of the kinetics of calcium oxalate monohydrate was obtained [4]. In this equation the growth constant K_a is characteristic for the type of seed used and was constant under changing solution conditions. Rearrangement of the obtained formula yielded the following equation, from which K_a was calculated.

$$\frac{1}{K_a} = \frac{[U_\infty] \cdot s \cdot tm}{U_\infty} \cdot \left(\frac{T_{Ca,i}}{T_{Ca,eq}} + \frac{T_{ox,i}^2}{T_{ox,eq}}\right) \qquad (3)$$

The use of ^{45}Ca tracer for following calcium uptake into the crystals has been compared to a system where uptake of calcium and oxalate was measured through changes in the conductivity of the suspension [6]. Both systems were found to produce the same growth kinetics.

The type of adsorption of inhibitors on the crystal surface could be derived from the dose dependent changes in crystal growth inhibition with a Langmuir type isotherm. As shown before [7] a linear relationship between the logarithms $\ln((K_{a,c}-K_{a,inh})/K_{a,inh})$ and $\ln(T_{inh}/M)$ indicated a monolayer type of adsorption, while non-linearity pointed to other adsorption types. $K_{a,c}$ and $K_{a,inh}$ were the growth constants in the control experiment and in the experiment with inhibitor added, respectively. T_{inh} denoted the total molar inhibitor concentration.

The compounds tested in this paper were four low mol wt substances: phosphate (Na_2HPO_4, E. Merck, Darmstad, FRG), citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$, E. Merck), magnesium ($MgCl_2 \cdot 6H_2O$, E. Merck), pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$, E. Merck), and three high mol wt substances: pentosanpolysulphate (Sigma Chemical Co., St. Louis, Missouri, USA, mol wt 5000 daltons), chondroitinsulphate (Sigma, mol wt 50000 daltons) and heparin (Diosynth, Oss, The Netherlands, mol wt 16000 daltons). A wide range of concentrations was tested, including those likely to be present in normal urine (for the urinary constituents).

Results

Effects on solubility

Amongst the seven compounds tested, only citrate and magnesium affected the solubility. This is shown in Figure 1. The solubility of the calcium oxalate monohydrate crystals in the control experiments was 0.167 mM. Increasing citrate

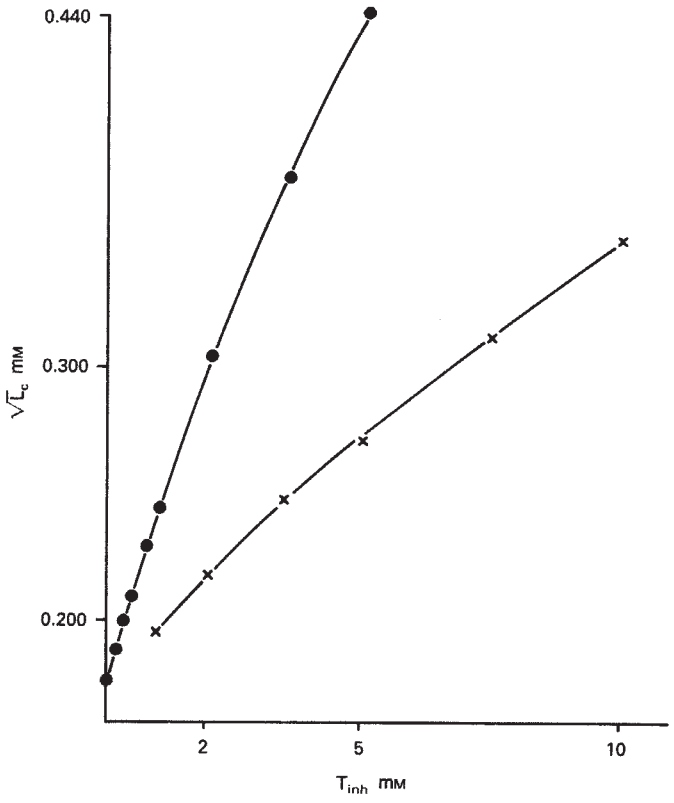

Fig. 1. *The effects of citrate (●) and magnesium (×) on the apparent solubility of calcium oxalate monohydrate.*

concentration from 0.05 mM to 5.0 mM increased the solubility from 0.174 to 0.441 mM. Similarly, raising magnesium concentration from 0.5 mM to 10.0 mM increased solubility from 0.167 to 0.350 mM. Phosphate in concentrations of 0.3 to 30 mM increased solubility from 0.167 to 0.184 mM only. Pyrophosphate (1 to 100 μM), pentosanpolysulphate (0.1 to 100 μM), Chondroitinsulfate (0.05 to 10.0 μM) and heparin (0.02 to 10.0 μM) were without effect.

Effects on crystal growth

As shown in Figure 2, all of the tested substances inhibited crystal growth. A 50% inhibition of crystal growth was exerted approximately by: 20 mM phosphate, 7 mM Mg, 0.4 mM citrate, 20 μM pyrophosphate, 2 μM pentosanpolysulphate, 1.0 μM chondroitinsulphate and 0.03 μM heparin, respectively. Thus, the high mol wt substances inhibited crystal growth at low concentrations.

The Langmuir isotherm, relating $\ln((K_{a,c}-K_{a,inh})/K_{a,inh})$ with $\ln(T_{inh}/M)$ was linear in the cases of the high mol wt substances, pentosanpolysulphate (slope 0.24), chondroitinsulphate (slope 0.6) and heparin (slope 0.24), and in the case of pyrophosphate (slope 1). This indicated a monolayer type of adsorption. Their mode of action could thus involve a simple surface-kink poisoning, the macromolecules in view of their low effective concentrations having a preference for the growth sites. The Langmuir isotherms for phosphate, citrate and magnesium were not linear, indicating perhaps a different adsorption behavior.

Effects on agglomeration

The results in Figure 3 indicate that citrate is a potent inhibitor of agglomeration even at low concentrations. Magnesium inhibits agglomeration only slightly, and only at high concentrations, while phosphate had no effects. Pyrophosphate had no effect at low concentrations, but at very high concentrations it inhibited agglomeration strongly. Among the macromolecules only chondroitinsulphate had a slight inhibitory effect, and pentosanpolysulphate and heparin had none.

Discussion

It is generally thought that supersaturation of urine is the prime element responsible for calcium oxalate stone formation. Consequently, management and prevention of renal stone disease have been focused on lowering the urinary calcium and oxalate concentrations. These concentrations are, however, normal in the urines of a number of stone formers and the calcium oxalate product is not different from that in normal urine. It may be that in those patients the effective concentration product is increased by diminished excretion of substances which complex calcium or oxalate. From the present solubility measurements and as generally assumed, it would appear that reduced citrate and magnesium could have this effect. On the other hand, it is unlikely that nucleation alone can be responsible for the renal stone formation, as crystalluria is as frequent in normal urines as in stone former urines [1]. In addition, particles must reach a certain size in order to remain in the urinary tract and form a stone, while small particles can be excreted with no consequences. Therefore, other processes affecting the size of particles must be involved. Two separate physicochemical processes govern particle size, namely growth and agglomeration of calcium oxalate crystals, and these were here independently assessed.

Low concentrations of high mol wt substances were found to inhibit the growth of crystals, but it appeared that they had hardly any inhibitory effect on their agglomeration. On the other hand, the low mol wt compounds, which only inhibited growth when concentrations were high, exerted a strong inhibitory action on the agglomeration. Thus, effects of different compounds on growth and agglomeration of calcium oxalate monohydrate crystals were not unidirectional (they could even be opposing [6, 7]). Crystal growth and crystal agglomeration, therefore, are distinct processes which can be dissociated.

This is in agreement with our previous studies, where it was found that urines from stone formers with frequent recurrence and from healthy persons, diluted 1:5, inhibited crystal growth to the same extent, but the difference found in their ability to inhibit crystal agglomeration was striking [8, 9]. Crystal agglomeration is, therefore, a separate central element in calcium oxalate stone formation. There is additional evidence in support of that premise. For example, though total crystalluria is the same in healthy subjects and in stone formers, the percentage of large particles, especially agglomerates, is higher in the stone former group [1]. In recurrent stone formers, there is close correlation between the number of particles (mainly agglomerates) larger than 12 μ excreted and the number of stone episodes per year [1]. It has also been calculated [10] that the influence of crystal growth on the size of a single crystal during the short transit time of urine in the urinary tract is not large

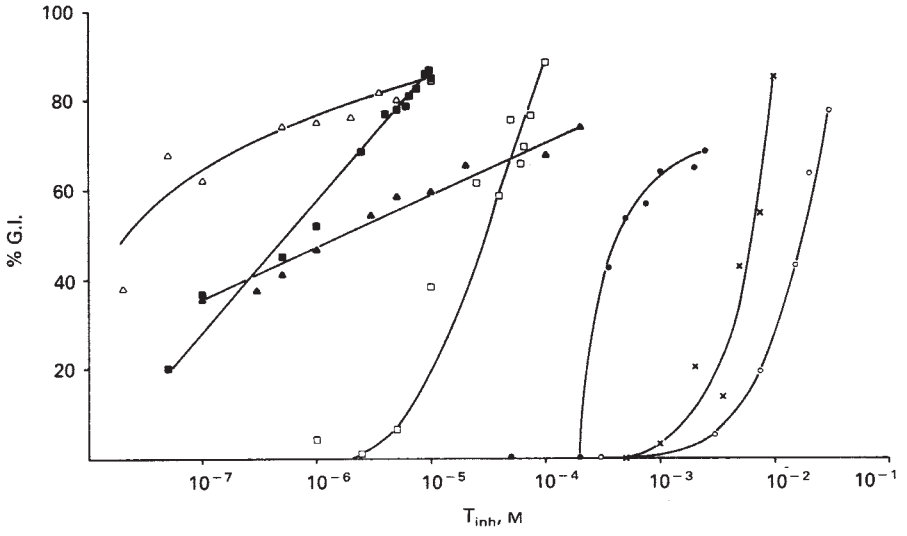

Fig. 2. *Crystal growth inhibition, % G.I., was achieved by all substances tested.* Symbols are: (○) phosphate 0.3–30 mM; (X) magnesium 0.5–10 mM; (●) citrate 0.05–2.5 mM; (□) pyrophosphate 1–100 μM; (▲) pentosanpolysulfate 1–100 μM; (■) chondroitinsulfate 0.1–10 μM; (△) heparin 0.05–10 μM.

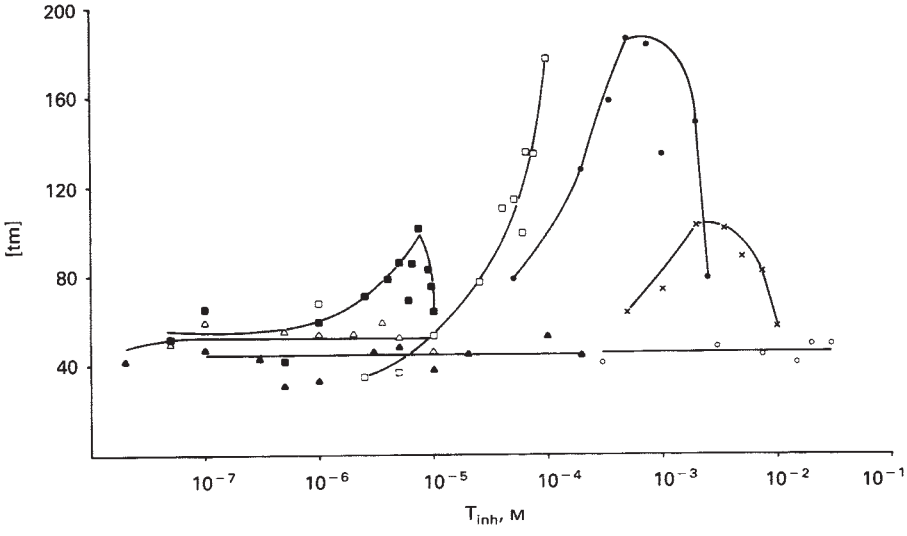

Fig. 3. *Effects on crystal agglomeration, high values of [tm] indicate inhibition relative to the control situation.* Symbols are: (○) phosphate 0.3–30 mM; (X) magnesium 0.5–25 mM; (●) 0.05–25 mM; (□) pyrophosphate 1–100 μM; (▲) pentosanpolysulfate 1–100 μM; (■) chondroitinsulfate 0.1–10 μM; (△) heparin 0.05–10 μM.

enough to cause the crystal to remain in the system. It has, therefore, been proposed that crystal retention, increasing transit times, is necessary (fixed particle mechanism) [10, 11]. The rapidity, however, with which crystal agglomeration is accomplished may suffice to explain why the particles may become large enough for retention. Large agglomerates have a greater chance to stick to walls or to be retained in dead volumes sites, than single crystals. Finally, the average renal stone has an agglomerate structure indeed, rather than consisting of a single large crystal [12–15].

Among the several candidates we tested, magnesium, citrate and pyrophosphate were the only ones found with an inhibitory effect on agglomeration. These three have all previously been implicated as acting on "growth and agglomeration" [16, 17]. It is now evident that here mainly the agglomeration is involved. Direct comparison of our data with other previously published material is not possible. For the measurement of the crystallization processes, well designed systems must be used. Both experimental parameters (ionic strength, pH, seed type, seed concentration) and physicochemical processes (solubility, growth, agglomeration) determine measured growth rates and crystal size distributions. It is therefore important that all these must be precisely defined and accounted for before any attempt at comparing data obtained by various methods is made.

In the present study citrate was found to be the most potent inhibitor of calcium oxalate crystal agglomeration at concentrations similar to those found in normal urine. At higher concentrations, representing citrate/calcium ratios which can be found under no physiological or pathological condition, this effect was not sustained. Of course, these in vitro data cannot directly be extrapolated to the more complex urinary conditions, where several substances which may modulate the effect on calcium oxalate crystallization are also present. However, these results conform to our previous findings in studies with urines from stone formers. The urines from these hypocitraturic patients, which had lost their ability to inhibit crystal agglomeration,

regained this ability after the citrate content was increased [8, 18].

It is, therefore, clear that growth and agglomeration of calcium oxalate crystals are differently modulated and must not be considered collectively. In this way the physicochemical basis of treatments aiming at inhibiting crystallization of calcium oxalate can be better defined and more rational ways of preventing renal stone formation can be developed.

Acknowledgments

The support of The Dutch Kidney Foundation is gratefully acknowledged. We thank Mr. I. Que for technical assistance and Mrs. F. Boegborn for her secretarial assistance.

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