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Simultaneous measurements of calcium oxalate crystal nucleation and aggregation: impact of various modifiers

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Abstract Rates of nucleation and aggregation of calcium oxalate crystals were derived from 20-min time course measurements of OD_{620} after mixing solutions containing $CaCl_2$ and $K_2C_2O_4$ at 37 °C, pH 5.7, ionic strength (IS) 0.21, with constant stirring (500 rpm); final assay concentrations were 4.25 mM calcium and 0.5 mM oxalate, respectively. The maximum increase of OD_{620} with time, termed S_N, mainly reflects maximum rate of formation of new particles and thus crystal nucleation. After equilibrium has been reached, OD_{620} progressively decreases despite ionized calcium staying constant and no new particles being formed, due to crystal aggregation. Rate of aggregation, S_A, is derived from the maximum decrease in OD_{620} with time. S_N and S_A are not independent, as indicated by a positive correlation ($r = 0.844$, $P = 0.0001$). Among the modifiers studied, citrate at 0.5–2.5 mM lowered both S_N and S_A in a concentration-dependent manner ($P < 0.01$ for all comparisons vs control). Chondroitin-6-sulfate at 6.25–25 mg/l moderately lowered S_N, whereas it strongly inhibited aggregation ($P < 0.01$ vs control). At 6.8–20.4 mg/l, albumin did not affect nucleation, whereas it inhibited aggregation in a concentration-dependent manner ($P < 0.005$ vs control for all comparisons).

Key words Calcium oxalate urolithiasis · Crystal nucleation and aggregation · Inhibitors and promoters · Citrate · Chondroitin-6-sulfate · Albumin

When urinary supersaturation with calcium oxalate exceeds the limit of metastability, i.e., the formation product, nucleation of microcrystals occurs as the initial event of stone salt precipitation [4, 12]. In solutions containing stone salt ions, complexes of these ions always form [12]. Whether or not these complexes are being transformed into a stable crystal nucleus depends on changes in free energy [4, 12]; the standard free energy change (ΔG_0) resulting from the formation of a spherical new solid phase can be written as:

$$\Delta G_0 = \frac{\pi l^3}{6}\frac{-mkT}{v}\ln(S) + \pi l^2\sigma \qquad (1)$$

where l is the sphere diameter, m the number of ions, v the molecular volume, k Boltzmann's constant, T absolute temperature, S supersaturation, and σ the surface energy [4]. The higher the supersaturation, the more the volume energy term in Eq. 1, i.e., $(\pi l^3/6) \times (-mkT/v)\ln(S)$, prevails; this favors the liquid-solid phase change, i.e., the formation of a new stable nucleus [12].

Since nucleation lowers urinary supersaturation without producing particles of significant sizes, it has been considered as a phenomenon of uncertain significance for stone formation [2]. However, it is an essential prerequisite for further formation of larger particles within the urinary tract which ultimately might form a stone [2, 4, 12]. Therefore, retarding the rate of nucleation may be important in order to reduce the rate of particle formation.

Nevertheless, it is by means of crystal growth and aggregation that larger crystalline particles do form in renal tubules. Theoretical considerations suggest that growth of single calcium oxalate crystals alone would be too slow to produce clinically significant particles [5], whereas crystal aggregation produces large particles at much faster rates [12, 13]. Therefore, crystal aggregation appears to be the most relevant step in the formation of calcium oxalate renal stones [12, 13]. Indeed, large crystal aggregates can be encountered more frequently in urine samples from recurrent calcium renal stone formers than from healthy controls [18].

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When in vitro measurements of crystallization kinetics are performed in suspensions with constant stirring, crystals always aggregate [9]. In the past, we have presented a spectrophotometric method to measure calcium oxalate crystal aggregation in stirred suspensions at equilibrium solution conditions so that crystals can neither nucleate nor grow [10]. However, since under real conditions urine is mostly supersaturated with respect to calcium and oxalate [4], particles will often nucleate and aggregate simultaneously. The present study considers nucleation and aggregation of calcium oxalate crystals separately but not independently by using a simple spectrophotometric, nonseeded supersaturation decay system.

Materials and methods

Spectrophotometric crystallization assay

Stock solutions of $CaCl_2$ (8.5 mM) and $K_2C_2O_4$ (1.0 mM), containing 200 mM NaCl and 10 mM sodium acetate, were adjusted to pH 5.70. All chemicals were of the highest purity grade available. Before being used in crystallization experiments, solutions were filtered through Millex-GV membranes with a pore diameter of 0.22 µm (Millipore AG, Volketswil, Switzerland) and warmed up to 37 °C in a circulating water bath (Heto, Denmark). For crystallization experiments, 1.0 ml of the $CaCl_2$ solution was transferred into a 10-mm light path quartz cuvette placed in a Perkin-Elmer Lambda 2 spectrophotometer (Perkin-Elmer, Überlingen, Germany) at 37 °C. In the cuvette, solutions were constantly stirred at 500 rpm (Cuv-O-Stir Model 333, Hellma, Basle, Switzerland), using a Teflon-covered stirring bar, size 7 × 2 × 2 mm (Semadeni, Berne, Switzerland). An additional 1.0 ml of the $K_2C_2O_4$ solution was then added to final assay concentrations of 4.25 mM for calcium and 0.5 mM for oxalate, respectively. Separate stock solutions were prepared accordingly to reach assay concentrations of 2.5 mM calcium and 0.75 mM oxalate, respectively. Control experiments were performed with three different calcium/oxalate concentration ratios, i.e., 4.25/0.75, 4.25/0.5, and 2.5/0.5 mM/mM.

After addition of the oxalate-containing solution, automated time-course measurements of optical density at 620 nm (OD_{620}) were performed, i.e., OD_{620} was recorded every 12 s over 20 min (Fig. 1). After experiments, pH in the crystal suspension was measured at 37 °C by a Metrohm 654 pH meter (Metrohm, Herisau, Switzerland). Before every new assay, the quartz cuvette was rinsed with H_2O and carefully cleaned with BM liquid 3% (Biomed, Munich, Germany) in order to minimize the occurrence of foreign particles; thereafter, the cuvette was rinsed again 10 times with double-distilled water and once with 100% methanol before air-drying.

The time from addition of oxalate until the first stable detectable increment of OD_{620} is named induction time, t_I (Fig. 1); according to Nielsen [17], it can be defined as:

$$t_I = gC^n \quad (2)$$

where C is the square root of the molar ion concentration product of the precipitating salt, and g and n are empirical constants. For calcium oxalate at 25 °C, g equals 1.03×10^{-7} s, and n is -3.33 [4]. In our assay system, t_I reflects the time required to form a stable crystal nucleus and to allow for growth into a size which makes it detectable by turbidimetry. The maximum slope of increase of OD_{620} with time (Fig. 1), determined by linear regression analysis, mainly reflects an increase in particle number in function of time, since OD is an exact measure of particle concentration per unit volume [6]; to a much lesser extent, however, OD also reflects particle size [16]. Therefore, the maximum slope of increase of OD_{620} with time, which we call S_N, mainly represents crystal nucleation.

Finally, after equilibrium, i.e., saturation, has been reached, crystals can neither nucleate nor grow; nevertheless, a progressive decrease of OD_{620} with time can be observed (Fig. 1) despite continuous stirring. The maximum slope of decrease of OD_{620} with time must therefore reflect the rate of decrease in particle number, due to crystal aggregation [10]. This slope has a negative value; for convenience, − slope of turbidity, i.e., a positive number named S_A, will be used for all further comparisons.

Assay suspensions from control experiments with all three different calcium/oxalate concentration ratios that were studied were centrifuged for 10 min at 10 000 rpm in a Heraeus Christ Biofuge A (Heraeus AG, Zurich, Switzerland). Supernatants were removed and crystalline precipitates air-dried before being pooled for analysis by infrared spectroscopy (Perkin-Elmer, Überlingen, Germany).

Measurements of ionized calcium

Free ionized calcium in the assay solution was measured at 0, 90, 210, 330, 540, 840, and 1080 s in three control experiments under standard conditions, at 240 and 360 s in another three control experiments, and at the beginning as well as at the end of experiments with citrate at 1.5 mM (four experiments) and albumin at 13.6 mg/l (three experiments). Two hundred microliters assay solution was aspirated and diluted with 600 µl of a solution containing 200 mM NaCl and 10 mM sodium acetate, pH 5.70, at 37 °C. Ionized calcium was measured by an ion-selective electrode (Ciba-Corning Diagnostics, Medfield, Mass., USA), which consisted of a neutral carrier-based calcium sensor immobilized in PVC. The sensor was in contact with the sample on one side and with the electrode fill solution (4 M KCl) on the other side; electrical connection is via a silver chloride coated wire. The values obtained are corrected for the actual hydrogen concentration, which was simultaneously measured by a pH-selective electrode [15].

Scanning electron microscopy

At various times in the experiments, 30 µl assay solution was transferred from the spectrophotometric cuvette onto a Millipore GV filter membrane, diameter 47 mm, of pore size 0.22 µm (Millipore AG) under vacuum. On the filter surface, 30 µl crystal suspension produced droplets about 5–6 mm in diameter. For drying, filters were cut to size and mounted on an aluminum holder by means of a double-sided graphitized adhesive tab. A gold layer about 400 Å thick was then sputtered onto the sample. Samples were investigated using a JEOL JSM-840 scanning electron microscope with an acceleration voltage of 25 kV and a working distance of between 15 and 25 mm. Photographs were taken at magnifications of × 500 and × 5000.

Modifiers of calcium oxalate crystallization processes

All experiments with modifiers of calcium oxalate crystallization were performed at assay concentrations of 4.25 mM calcium, 0.5 mM oxalate, 200 mM NaCl, and 10 mM sodium acetate, pH 5.70. Concentrated solutions of modifiers were pipetted into the calcium-containing solution before oxalate was added.

Citrate (CIT) (Tri-potassium-citrate, Sigma, St. Louis, Mo., USA) was studied at final concentrations of 0.5, 1.5, and 2.5 mM by adding double concentrations to the calcium-containing assay solution. In order to reach final concentrations of 6.25, 12.5, and 25.0 mg/l, small volumes of a solution containing chondroitin-6-sulfate (C-6-S)

(chondroitin sulfate C, Sigma, 1000 mg/l standard buffer (200 mM NaCl, 10 mM sodium acetate, pH 5.70), were pipetted into the calcium-containing solution. Bovine albumin (ALB) (albumin RIA grade fraction V powder, 96–99% albumin, Sigma) with a molecular weight of 68 kDa [10] was also dissolved in standard buffer, 1.36 g/l (2×10^{-5} M), and added in small volumes in order to reach final concentrations of 6.8, 13.6, and 20.4 mg/l (corresponding to 1.0, 2.0 and 3.0×10^{-7} M).

Percentage inhibition in the presence of crystallization modifiers was calculated as $[1 - (S_{Nm}/S_{Nc})] \times 100$ for the rate of nucleation and $[1 - (S_{Am}/S_{Ac})] \times 100$ for the rate of aggregation, respectively, where *m* stands for modifier and *c* for control.

Statistics

All values are means ± SE. The nonparametric Mann-Whitney U test for comparisons between groups and Wilcoxon's signed-rank test for paired comparisons within groups were used. For correlation studies, simple and multiple linear regression analysis was used.

Results

Control experiments

Figure 1 represents typical time-course measurements of OD_{620} and ionized calcium under standard conditions (4.25 mM calcium, 0.5 mM oxalate). After induction time, t_I, OD_{620} progressively increased and reached its maximum value at about 500 s. The increment of OD_{620} with time was steepest between 150 and 300 s. During this period, the decrease in ionized calcium with time was also at its maximum. After equilibrium had been reached at about 500 s, OD_{620} progressively fell due to crystal aggregation, whereas ionized calcium remained stable, indicating that saturated conditions had been reached. Infrared spectroscopy of precipitated material collected at the end of control experiments with various Ca/Ox concentration ratios revealed 80% whewellite and 20% whedellite.

In SEM studies, 50 areas of 500-fold magnification were screened on each Millipore filter. In samples taken after 90 s, only 2 out of 50 fields revealed 1 single crystalline particle. Figure 2 demonstrates representative areas of filters with samples collected after 240, 360, 600, and 1080 s. Particle numbers were counted on photographic pictures of SEM fields (Fig. 1, top). At 240 s (Fig. 2a), particle number was 50, whereas it rose to 94 at 360 s (Fig. 2b) and to a maximum of 128 at 600 s (Fig. 2c); at 1080 s (Fig. 2d), the number dropped to 62 particles per area. Particle size also increased with time, but changes were less impressive: at 240 s (Fig. 2a), maximum particle diameter was about 6–7 μm, whereas it rose to 8 μm at 600 s, when maximum particle number was reached (Fig. 2c). At 1080 s, when particle number progressively decreased, maximum particle diameter was about 10–11 μm (Fig. 2d), indicating that crystals were more aggregated. However, aggregated crystals could be detected in all samples, i.e., also in the early phases of the experiments when the slope of increase of OD_{620} with time was the steepest. Thus, since OD_{620} readings are also affected by aggregation, which reduces particle numbers, S_N certainly underestimates to some extent the rate of newly formed crystals. However, as demonstrated on 5000-fold magnifications of SEM pictures (Fig. 3), the largest aggregates were present at 1080 s, i.e., the time point when aggregation rates were actually derived.

Table 1 summarizes values of t_I, S_N and S_A in control experiments with various Ca/Ox concentration ratios. With decreasing Ca × Ox product (from left to right in Table 1), t_I progressively increased, whereas S_N and S_A decreased. When considering all 27 control experiments together, simple linear regression analysis revealed inverse correlations between t_I and S_A ($r = -0.599$, $P = 0.001$) and between t_I and S_A ($r = -0.556$, $P = 0.003$). In addition, S_A was positively correlated with S_N ($r = 0.844$, $P = 0.0001$). Using multiple regression analysis, a significant correlation ($r = 0.846$, $P = 0.0001$) of S_A with both t_I (partial $F = 0.34$) and S_N (partial $F = 34.3$) was noted, indicating that the rate of aggregation primarily depends on the number of particles, but much less on induction time. The mean pH value in control assay suspensions at the end of experiments was 5.70 ± 0.01.

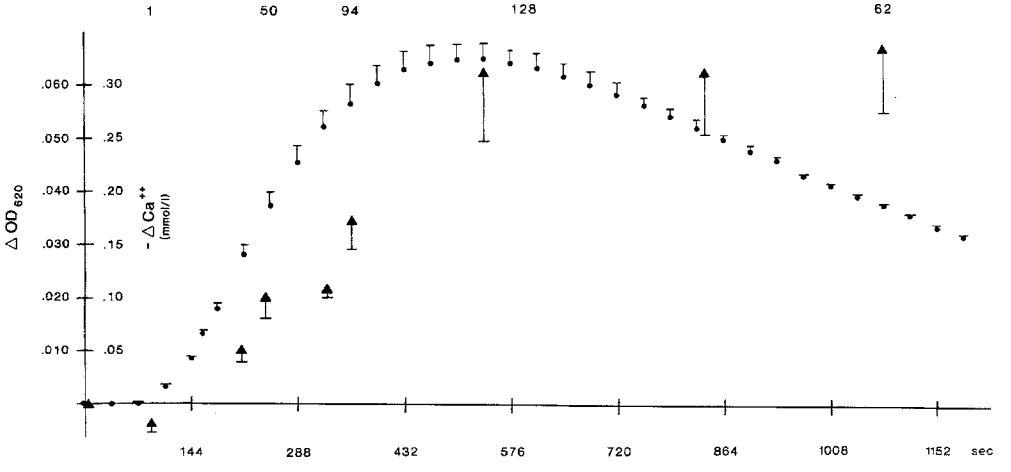

Fig. 1 Time-course measurements of OD_{620} (*circles*) and deviations of Ca^{2+} from zero values (*triangles*) at various times in three control experiments at standard conditions (Ca/Ox = 4.25/0.5 mM/mM). For technical reasons, values at 240 and 360 s had to be obtained from three other experiments performed under identical conditions. Values are means ± SE. *Numbers on top* indicate crystalline particles counted on photographic pictures of 500-fold SEM magnifications (see Fig. 2)

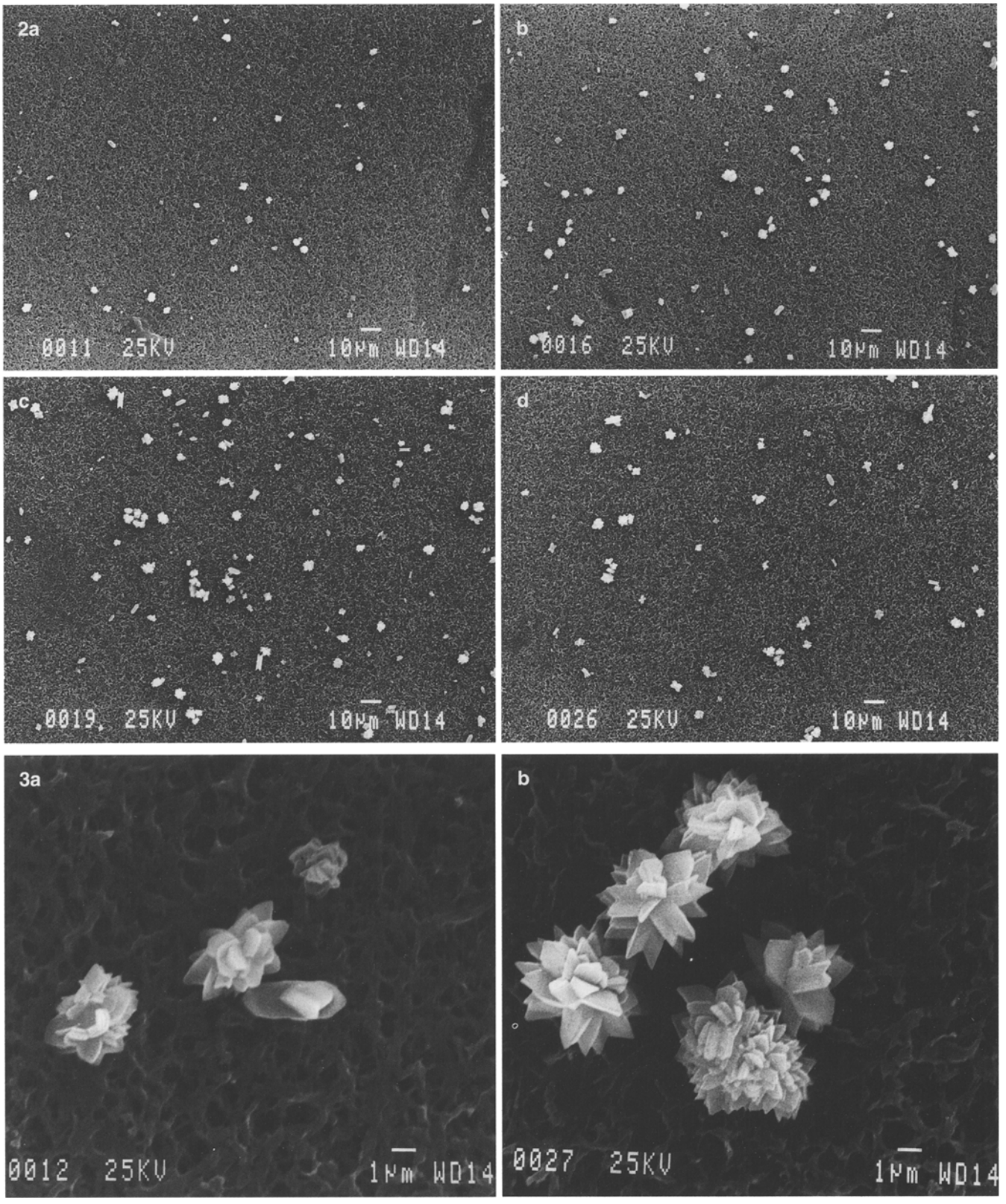

Fig. 2a–d Representative photographs (×500) of SEM studies on samples obtained at various times of standard crystallization experiments: **a** 240 s, **b** 360 s, **c** 600 s, **d** 1080 s. For details, see text

Fig. 3 Photographs (×5000) of SEM studies from samples obtained at 240 s (**a** magnification of Fig. 2a) and 1080 s (**b** magnification of Fig. 2d). For details, see text

Table 1 Values of t_I, S_N, and S_A at three different calcium/oxalate ratios in control experiments. For abbreviations, see text. *In italics*, standard conditions applied for all studies with modifiers of crystallization. Values are means ± SE. For convenience, the positive value of S_A, which is basically a negative number, is indicated (see "Materials and methods")

Measurement	Calcium/Oxalate (mM/mM)		
	4.25/0.75 ($n = 4$)	*4.25/0.5* ($n = 20$)	2.5/0.5 ($n = 3$)
t_I (s)	40 ± 3**	*109 ± 5*	293 ± 9*
S_N ($\times 10^{-3}$/s)	11.92 ± 0.49**	*3.65 ± 0.14*	2.32 ± 0.20*
S_A ($\times 10^{-3}$/s)	0.90 ± 0.03**	*0.61 ± 0.02*	0.54 ± 0.03

* $P = 0.006$, ** $P = 0.002$ vs standard conditions

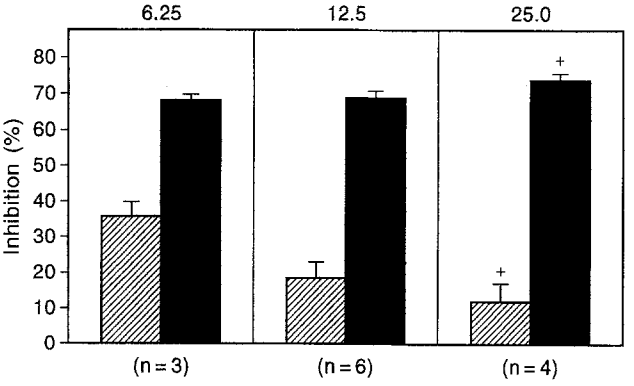

Fig. 5 Percentage inhibition of calcium oxalate crystal nucleation and aggregation by chondroitin-6-sulfate. Values are means ± SE; $P < 0.010$ for all comparisons with controls except nucleation at 25.0 mg C-6-S (NS). $^+$ $P < 0.05$ vs 6.25 mg/l C-6-S. ▨ Nucleation; ■ aggregation

Modifiers of crystallization

In the presence of 1.5 mm CIT, $[Ca^{2+}]$ at the beginning of the experiments was 3.33 ± 0.04 mM, compared with 4.22 ± 0.06 mM in controls ($P = 0.003$), and was 3.30 ± 0.02 mM, almost identical, at the end of the experiments, still clearly lower than in controls (3.91 ± 0.06 mM, $P = 0.018$). This indicates a reduced driving force for crystallization due to chelation of calcium. Indeed, CIT increased t_I at all concentrations studied, from 109 ± 5 s in controls to 130 ± 7 s at 0.5 mM ($P = 0.042$ vs control), 129 ± 4 s at 1.5 mM ($P = 0.022$ vs control), and 154 ± 2 s at 2.5 mM ($P = 0.014$ vs control). Additionally, CIT decreased both S_N and S_A, i.e., it inhibited crystal nucleation and aggregation, as demonstrated in Fig. 4 ($P < 0.01$ for all comparisons of nucleation and aggregation with controls). When pooling all experiments with the three conditions together ($n = 12$), no significant correlations of either S_N and S_A with t_I were found; S_N and S_A, however, were positively correlated with each other ($r = 0.667$, $P = 0.018$).

C-6-S increased t_I to a greater extent than CIT, i.e., from 109 ± 5 s in controls to 204 ± 9 s with 6.25 mg/l ($P = 0.006$ vs control), 165 ± 5 s with 12.5 mg/l ($P = 0.0005$ vs control), and 182 ± 14 s with 25.0 mg/l of C-6-S ($P = 0.0003$ vs control). The effect on rates of nucleation, however, was different from that of CIT (Fig. 5): at the lowest concentration of C-6-S, 6.25 mg/l, nucleation inhibition was 36% ($P = 0.006$ vs control), but it fell with increasing concentrations to a level no different from that of the control at 25.0 mg/l. On the other hand, crystal aggregation was inhibited by about 70% at all concentrations of C-6-S that we studied ($P < 0.01$ for all comparisons with controls). When pooling all data ($n = 13$), there was neither a correlation between t_I and S_N or S_A nor were S_N and S_A related to each other.

ALB did not affect free ionized calcium concentration: at the beginning of experiments, $[Ca^{2+}]$ in the presence of ALB at 13.6 mg/l was 4.17 ± 0.04 mM, no different from the 4.22 ± 0.06 mM in controls; at the end, it dropped to 3.83 ± 0.03 mM, similar to the 3.91 ± 0.06 mM in controls. ALB raised t_I from

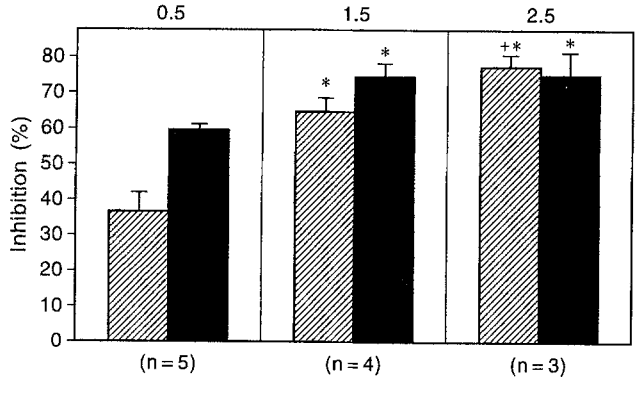

Fig. 4 Percentage inhibition of calcium oxalate crystal nucleation and aggregation by citrate. Values are means ± SE; $P < 0.01$ for all comparisons with controls; $^+$ $P < 0.05$ vs 1.5 mM; * $P < 0.025$ vs 0.5 mM. ▨ Nucleation; ■ aggregation

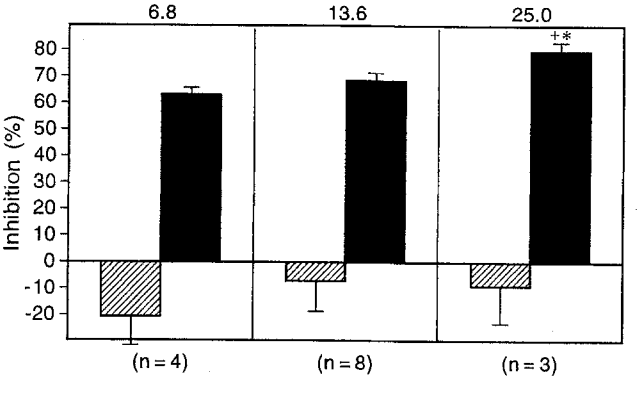

Fig. 6. Percentage inhibition of calcium oxalate crystal nucleation and aggregation by albumin. Negative values indicate promotion. Values are means ± SE; P = NS for all comparisons of nucleation with controls; $P < 0.020$ for all comparisons of aggregation with controls; $^+$ $P < 0.05$ vs 13.6 mg/l; $^*P < 0.025$ vs 6.8 mg/l

109 ± 5 s to 141 ± 6 s at 6.8 mg/l ($P = 0.006$ vs control), 125 ± 5 s at 13.6 mg/l ($P = 0.027$ vs control) and 169 ± 8 s at 20.4 mg/l ($P = 0.003$ vs control). As depicted in Fig. 6, ALB did not alter the rate of nucleation significantly, whereas it inhibited crystal aggregation in a dose-dependent manner, reaching 79% inhibition at 20.4 mg/l ($P < 0.005$ for all comparisons with controls). Induction time was correlated neither with S_N nor with S_A, nor were S_N and S_A related to each other.

Discussion

Whereas turbidimetry has previously been used for kinetic measurements of crystal nucleation and growth in supersaturated non-seeded crystallization systems [1, 7, 8], this study represents a novel approach for measuring rates of nucleation and aggregation of calcium oxalate crystals in a single experiment. Using a similar system, Hennequin et al. [8] described two kinetic parameters: first, induction time, t_I, was taken as an index for crystal nucleation, where any prolongation was related to inhibition of nucleation [8]. However, since t_I indicates only the beginning of nucleation of particles that are detectable by turbidimetry [8, 12], prolongation of t_I may not only represent retardation of nucleation of detectable particles, but also signify increased rate of nucleation of very small particles which are undetectable by the analytical precision of the assay system. Indeed, calcium oxalate nuclei are of the order of 100 Å or less [12], i.e., much smaller than what can be detected by turbidimetry.

In addition, Hennequin et al. [8] described the maximum increase in OD_{620} with time as being related essentially to crystal growth, although nucleation was not totally excluded. It should be noted, however, that in polydisperse suspensions such as in our system, turbidity is directly proportional to particle concentration N (particle number per unit volume) and to a scattering coefficient Q; the latter is a function of two parameters, i.e., of particle size, α, and of the ratio of refractive indices of particles and medium, m [16]. Therefore, N affects turbidity measurements directly and to a greater extent than α, which has only an indirect impact on turbidity through its contribution to Q. Indeed, OD is an exact measure of particle concentration per unit volume [6], and we have demonstrated that OD_{620} directly relates to concentrations of dry calcium oxalate monohydrate crystals per unit volume in aqueous suspensions [10]. Accordingly, the present SEM studies (Fig. 2) suggest that the increase in particle number during the steepest rise of OD_{620} with time (Fig. 2a–c) is relatively more pronounced than increases in particle sizes. Altogether, this indicates that S_N primarily reflects the rate of newly formed, detectable particles, i.e., crystal nucleation, and much less the rate of crystal growth.

We took advantage of the fact that aggregation is inevitable in stirred crystal suspensions, especially at lower stirring rates [9]. Therefore, the aggregation parameter was derived from the same time-course measurements by calculating the slope of decrease of OD_{620} with time after saturation had been reached, as previously described [10]. As indicated by our SEM studies, however, crystal aggregation was also present early during experiments when OD_{620} was increasing at maximal rates. Nevertheless, crystals were most aggregated late during experiments when OD_{620} progressively decreased.

On a theoretical basis the rate of aggregation depends on particle number per unit volume and thus on the rate of nucleation [4]. Indeed, multiple regression analysis revealed that S_A was significantly correlated with t_I and S_N in control experiments; S_N, however, was much more strongly predictive for S_A, indicating that larger particles more easily form aggregates than microcrystals, which remain undetected by turbidimetry.

Citrate increases induction times and reduces rates of nucleation in a concentration-dependent manner. This was expected, since citrate chelates calcium ions, which efficiently lowers supersaturation, the driving force for crystallization [4]. We also demonstrate that citrate inhibits aggregation of newly forming crystals; this is in keeping with others who used a metastably supersaturated seeded crystal growth assay [14]. On the other hand, these results are in apparent contradiction to those of a previous study of ours in which citrate did not change the rate of crystal aggregation under saturated conditions [10]. The reason is that, under saturated conditions, we solely measured the tendency of preformed crystals to aggregate, and the lack of effect of citrate simply indicated that citrate did not alter the surface characteristics of these crystals to an extent that allowed for aggregation inhibition in this system [10].

In the present study, we tested the effects of citrate on small, freshly nucleating, growing, and aggregating crystals in supersaturated solutions. Inasmuch as citrate lowers supersaturation, whereby the number of nucleating crystals is reduced, the apparent inhibition of crystal aggregation is expected. Compared with control conditions, however, 1.5 mM citrate reduced the concentration of ionic calcium by 21%, i.e., from 4.22 to 3.33 mmol/l, whereas the rate of crystal aggregation dropped by 74% (Fig. 4). In control experiments without citrate, where the calcium concentration was only 2.5 mM, mean rate of aggregation was reduced by only 11.5% in comparison with controls at 4.25 mM calcium (Table 1), despite calcium concentration being even lower than in the presence of 1.5 mM citrate. This indicates that – under the conditions of this assay system – citrate affects crystal aggregation not only by lowering supersaturation, but to quite a large extent also by another mechanism,

most probably by binding to specific sites on crystal surfaces [12].

Polymeric urinary glycosaminoglycans such as C-6-S affect crystallization by adsorbing on the crystal surfaces [22]. In our system, C-6-S only moderately inhibits crystal nucleation, with progressive loss of inhibitory activity with increasing concentrations. This is in agreement with two previous studies showing that chondroitin sulfate at concentrations equal to those used in our study even increased total mass of precipitated crystals, when compared with other macromolecules [11, 19]. Furthermore, by using scanning electron microscopy, Kohri et al. [11] have demonstrated that – in comparison with heparin or no additives – chondroitin sulfate allows for the generation of significantly larger calcium oxalate crystals in a mixed suspension, mixed product removal crystallization system. In addition, we found marked aggregation inhibition by C-6-S, which has also been demonstrated by Ryall et al. [20, 21]. Altogether, C-6-S may promote nucleation of calcium oxalate crystals at high concentrations, whereas it strongly inhibits crystal aggregation.

We found that albumin does not significantly affect crystal nucleation, whereas it efficiently inhibits crystal aggregation in a concentration-dependent manner. This is in agreement with Ryall et al. [21], who found even slight promotion of calcium oxalate crystal nucleation by albumin added to ultrafiltered human urine, as well as with Edyvane et al. [3], in whose studies albumin inhibited crystal aggregation in an aqueous inorganic medium. Our previous studies using saturated conditions [10] also revealed inhibition of aggregation of preformed calcium oxalate monohydrate crystals by albumin. Since albumin binds to calcium oxalate crystal surfaces [23], it is expected to affect surface properties and thus rates of crystal aggregation.

In order to understand the apparently contradictory effects of modifiers on the different steps of calcium oxalate crystallization that we observe, it has to be emphasized that the process of crystallization always comprises several steps that are not independent and occur simultaneously, such as nucleation, growth, aggregation, and phase transformation of crystals [12]. At a given level of supersaturation, the same crystal mass can theoretically be precipitated either in the form of a large number of micronuclei, or as a small number of large aggregates. Therefore, it is not surprising that the inhibitory effects of chondroitin sulfate and albumin on crystal aggregation lead to the precipitation of larger numbers of small crystal nuclei, measurable as an apparently lower inhibition of crystal nucleation [21].

In conclusion, our findings indicate that nucleation and aggregation of calcium oxalate crystals can be measured separately but not independently in single experiments by time-course readings of OD_{620} in highly supersaturated calcium oxalate solutions, and that such a system holds promise for the study of distinct effects of various modifiers on crystal nucleation and aggregation.

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