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Hydroxyapatite induction and secondary aggregation of calcium oxalate, two important processes in calcium stone formation

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Abstract Stone formation has often been ascribed to crystal aggregation and fixed particle growth on kidney calcifications. In this paper, the influence of hydroxyapatite (HAP) and of preformed calcium oxalate (CaOx) aggregates on CaOx crystallization was studied in freshly voided urine. Crystallization was induced by different oxalate loads and precipitates were analyzed by the spectrophotometric measurement of sedimentation time (ST), which decreases with increasing particle size. The fact that the ST of aggregates (ST_A) is significantly lower than the ST of other particles demonstrates that ST_A is a useful indicator for aggregation. At relatively low oxalate loads the addition of HAP to urine increased ST_A by a factor of 4.3 ($P < 0.001$). After a second oxalate load, ST_A decreased by 56% ($P < 0.001$), indicating secondary growth of the preexisting aggregates. HAP induced and primary CaOx aggregation occurred at low pH at which a high ionic calcium concentration (Ca^{+2}) was measured. In urine, crystals are coated by macromolecules creating a negative surface potential with a consecutive accumulation of cations such as Ca^{+2}. This Ca^{+2} accumulation could be responsible for the enhancement of aggregation by preexisting particles, which seems to be important for stone formation and which can otherwise hardly be explained in the presence of coated crystals.

Keywords Urolithiasis · Calcium oxalate · Hydroxyapatite · Crystallization · Aggregation

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Introduction

Since water sparing by urinary concentration is vital for terrestrial life it is not surprising that urine is often supersaturated with respect to poorly soluble substances like calcium oxalate (CaOx). At low fluid and high dietary intake levels of oxalate, CaOx crystalluria is frequent [2]. Under special conditions, crystals can aggregate to big clusters and may give rise to stone disease [16]. At urinary transit, aggregation seems to be the only crystallization process creating particles large enough to be trapped in the urinary tract [12].

Studies on whole urine are mandatory for stone research, because the dilution [17], filtration and even centrifugation of urine markedly change urinary crystallization properties with the latter removing important urinary macromolecules [19]. Therefore, we recently investigated the particular conditions in which crystal aggregation takes place in whole urine [4, 3].

Significantly different sedimentation rates were measured for single crystals of CaOx and their aggregates by spectrophotometry. Since all biological crystals are coated with macromolecules [11], crystals for studying directly were produced in urine by an exogeneous oxalate load. Crystal aggregation was highly inhibited by urinary macromolecules, whereas aggregation markedly increased after these were removed by ultrafiltration. Slow continuous stirring, known to enhance crystal aggregation in artificial solutions [9], inhibited aggregation in urine. Aggregates were only observed at high oxalate concentrations (> 1 mmol/l) and without stirring, because their formation probably took place before the crystals became coated with macromolecules which have a low rate of diffusion. The effect of different diffusion rates between small and macromolecular substances is abolished by stirring.

Stone formation has often been explained by fixed particle growth on papillary calcifications [14] or on crystal aggregates protruding from collecting ducts [12]. Therefore, in the present study, we examined the effect

of hydroxyapatite (HAP) and of preformed CaOx aggregates on CaOx crystallization at different calcium concentrations, oxalate loads and pH-values.

Material and methods

Crystallization tests were performed on several freshly voided urine specimens of five healthy men, at 37°C. For each sample, 2 ml urine was pipetted into a cuvette placed in a thermostatable cell holder of a Perkin Elmer spectrophotometer 550S (Perkin Elmer, Rotkreuz, Switzerland) connected to a circulating waterbath (JULABO, Exatherm U3, Digitana, Neuchatel, Switzerland). All experiments were monitored at 620 nm wave length. Magnetic stirring within the cuvette was performed by a Mini Magnetic Stirrer (CUV-O-STIR 333, Hellma, Müllheim, Germany). Crystallization was induced by an oxalate load using a stock solution of 100 mmol/l disodium oxalate. Stirring was stopped before the application of the oxalate load and changes of optical density (OD) were recorded by a Perkin Elmer 561 recorder. After 20 min, the sediment was resuspended by 10 sec stirring. Sedimentation was measured by the OD decrease over 20 min. The procedure being repeated 1–2 times.

Sedimentation curves showed different gradients correlating to particle size. The slopes of the gradients, which increased with particle size, were measured as sedimentation time (ST), which is the time in minutes for a 0.05 OD decrease. When sedimentation curves and scanning electron microscope (SEM) micrographs of sediments were compared, only experiments with CaOx aggregates showed gradients with an ST of 2.1 ± 0.7 (mean ± SD). Therefore, an ST of ≤ 2.8 (ST_A) was taken as indicator for CaOx aggregation. Since this study focussed on maximal aggregation, the start of maximal deflection combined with the absorbance 2 min later was used to calculate the maximal measurable ST. In half of the experiments, the validity of ST_A as an aggregation indicator was controlled by SEM pictures of the corresponding sediments. For SEM analysis, at the end of crystallization experiments, 150 µl of the resuspended assay solution was millipore filtered (pore size 0.45 µm, under vacuum) and dried at 37°C. For SEM investigation a gold layer of about 5–10 nm was then sputtered onto the sample. Samples were analysed using a LEO 435 VP scanning electron microscope (LEO Electron Microscopy, Cambridge, England) with an acceleration voltage of 5–20 kV and photographed at magnifications of ×2000.

Ionic calcium concentration (Ca^{+2}) was measured using a commercially available calcium analyzer (AVL List, Graz, Austria) which automatically cleans and calibrates the electrode after each measurement. The pH was measured with a pH-Meter (Metrohm E 603, Metrohm, Herisau, Switzerland) and adjusted by the addition of solutions of 1N NaOH or 1N HCl.

Differences of ST_A and pH were analyzed by a Students *t*-test. The incidence of ST_A found in the different groups of experiments were compared by the χ^2-test.

Study of HAP induced CaOx aggregation

Being aware of the high interdependence of phosphates and pH, we examined the influence of HAP on urinary composition and OD at different pH values. The pH was adjusted to 5.0–6.5, then 10 mg/ml of HAP (tri-calciumphosphate, Merck, Darmstadt, Germany) was added to the urine and stirred for 30 min. Total concentrations of calcium, Ca^{+2} and phosphate were determined before and after incubation with HAP.

To study the effect of different concentrations of HAP on the OD, 10 mg/ml HAP suspended in NaCl 0.15 M by short and rapid stirring was added to 2 ml of urine in final concentrations of 0.05–0.25 mg/ml. Sedimentation was examined as described above. Based on these results, 0.05 mg/ml HAP was used to study CaOx crystallization. After the addition of HAP to urine stirring was stopped and an oxalate load of 1 mmol/l, a concentration that rarely induced measurable aggregation [4], was applied.

Study of secondary CaOx aggregation

Initial CaOx aggregates were produced in urine by initial oxalate loads of 2 mmol/l. This was always found to induce significant aggregation [4]. Sedimentation of the initial precipitate was observed by monitoring the OD decrease. After 20 min, the supernatant (1.5 ml) of the urine was removed and replaced by new urine from the same sample. Additional oxalate loads were performed in concentrations of 1–2 mmol/l, secondary crystallization was monitored and ST_A calculated.

Results

HAP induced CaOx aggregation

Figure 1 shows the influence of 30 min HAP incubation at pH 5.0, 5.5, 6.0 and 6.5 respectively, in seven urine

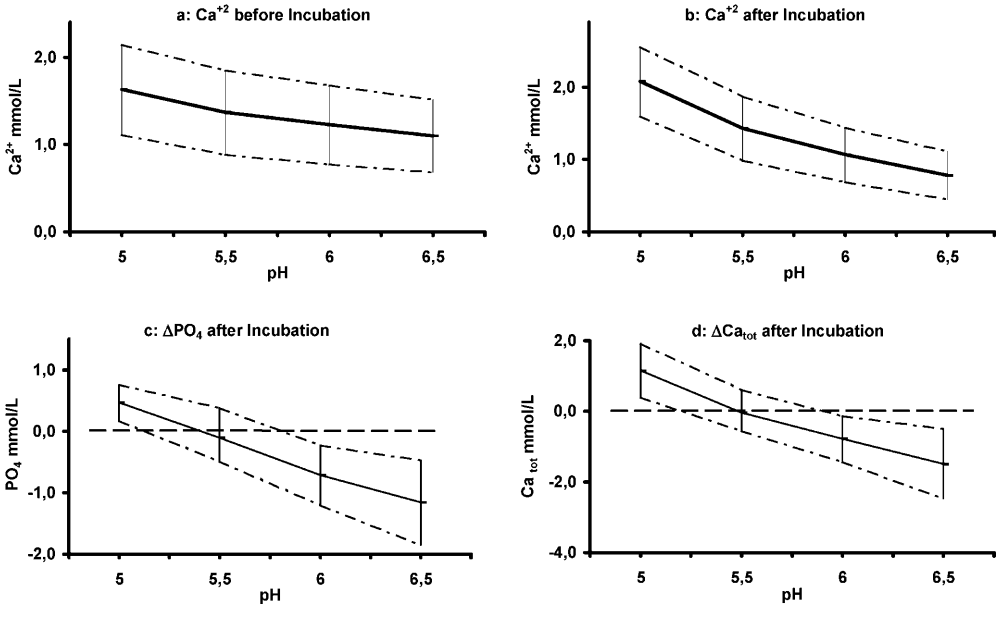

Fig. 1a–d Influence of pH alone (**a**) and pH and HAP incubation (**b–d**) on the composition of seven urine samples (mean ± SD) (Δ, change of concentration)

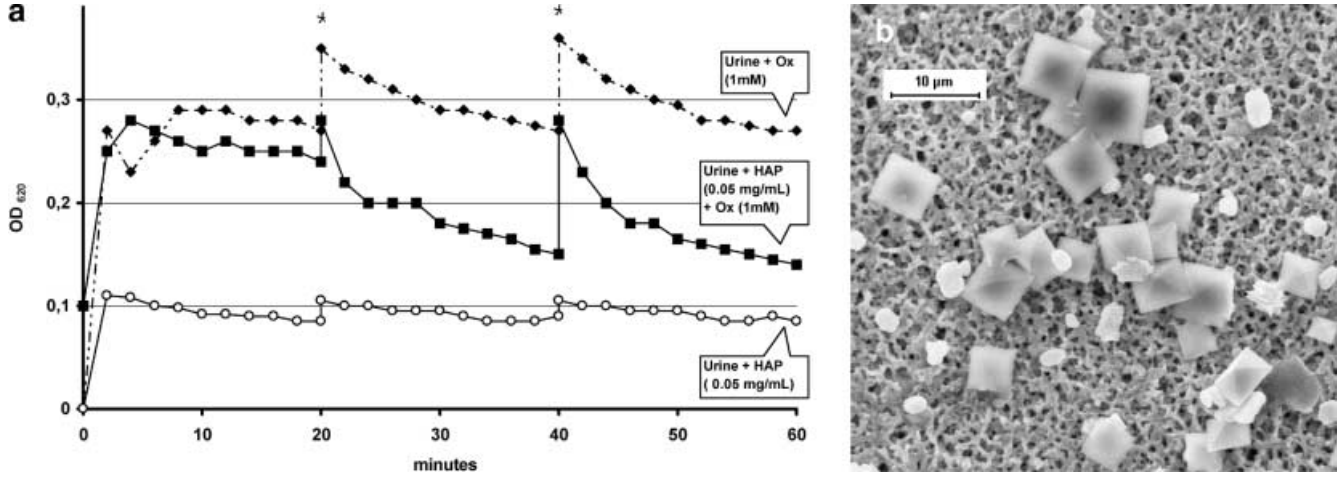

Fig. 2a, b **a** The influence of HAP (*open circles*), HAP and oxalate (*closed squares*) and oxalate (*closed diamonds*) addition at time 0 on the sedimentation curves (*stirring period of 10 s). **b** SEM of urinary sediment with 0.05 mg/ml HAP (*ovals*) 60 min after oxalate load of 1 mmol/l

samples with concentrations of calcium ranging from 2.8–7.6 mmol/l and of phosphate from 6.3–24.8 mmol/l. Increasing urinary pH from 5.0 to 6.5 before the addition of HAP produced a 33% decrease of Ca^{+2} (Fig. 1a). HAP dissolved during incubation at low pH (< 5.5), as shown by an increase in the concentrations of calcium and phosphate. At higher pH values (> 5.5) urinary calcium and phosphate concentrations decreased, due to calcium phosphate precipitation (Fig. 1c, d). An increase in pH from 5.0 to 6.5 provoked a 2.7-fold decrease of Ca^{+2} in urine incubated with HAP (Fig. 1b).

After adding freshly suspended HAP crystals in concentrations of 0.05–0.1 mg/ml to urine, reproducible turbidity curves were found at pH 5.0 and at 6.5 (not shown). A HAP concentration of 0.05 mg/ml demonstrated only a small effect on turbidity and a slight sedimentation (Fig. 2a) and was therefore used in the following crystallization experiments.

A typical experiment is shown in Figure 2a. An oxalate load of 1 mmol/l produced a sharp increase of OD with relatively slight sedimentation at a pH of 6.0, being characteristic for the formation of a large amount of single crystals. With the same oxalate load, in the presence of HAP, a reduction in the OD increase and a faster sedimentation was observed. Both effects, as demonstrated by a SEM micrograph of the corresponding sediment (Fig. 2b), are typical for aggregation.

Results of 22 crystallization experiments performed with HAP and an oxalate load of 1 mmol/l are summarized in Table 1. A total of 21 experiments were repeated as controls without the addition of HAP. In the presence of HAP, an ST_A 4.3× more frequent than in the controls was observed (82% vs 19%; $P < 0.001$). No aggregation with HAP was found in four samples, all with pH 6.5. In the controls, aggregation occurred in four samples with a pH which was significantly lower than in the controls without aggregation (5.1 vs 5.9; $P < 0.02$). Thus, low pH seems to enhance and high pH to inhibit aggregation.

Secondary CaOx aggregation

Typical curves of primary and secondary CaOx crystallization are shown in Figure 3a. During the first 20 min after the oxalate load, in which urine and oxalate stock solution were mixed and the main crystallization occurred, curves were often irregular and therefore difficult to interpret. After 20 min, when the precipitate was resuspended, fairly reproducible sedimentation could be observed. After the second oxalate load, OD as well as sedimentation rate increased markedly, demonstrating that apart from the formation of new particles (OD increase) also larger aggregates (Fig. 3b) had assembled from preexisting material.

In 12 experiments performed on five urine samples with Ca^{+2} concentrations ranging from 0.85 to 2.1 mmol/l and secondary oxalate loads of 1.0, 1.5 and 2.0 mmol/l, the secondary ST_A with 0.93 ± 0.36 (mean ± SD) represented only 44% of primary ST_A

Table 1 HAP induced CaOx aggregation. ST_A sedimentation time of aggregates ≤ 2.8 min for 0.05 OD-decrease, mean ± SD. Percentages are given in brackets

	ST_A present			ST_A absent	
	n	pH	ST_A	*n*	pH
HAP	18 (82)	5.6 ± 0.6	1.7 ± 0.7	4 (18)	6.5
Control	4 (19)	5.1 ± 0.2	2.1 ± 0.7	17 (81)	5.9 ± 0.6

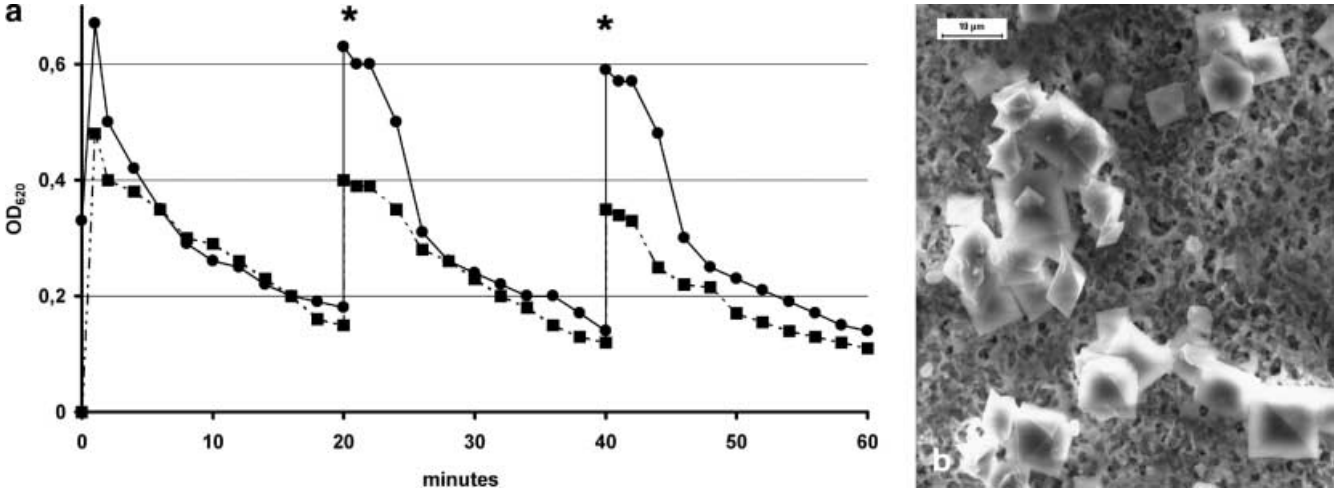

Fig. 3a, b **a** Sedimentation curves of primary (*closed squares*) and secondary (*closed circles*) oxalate load of 2 mM for the same urine sample at time 0. (*stirring period of 10 s). **b**. SEM of urinary sediment 60 min after secondary oxalate load of 2 mmol/l

$(2.1 \pm 0.72;\ P < 0.001)$ and therefore demonstrated secondary growth of aggregates. Secondary ST_A showed a negative correlation with the oxalate load but not with urinary Ca^{+2} concentrations (Fig. 4). However, on SEM micrographs, at low Ca^{+2} concentrations (≤ 1 mmol/l), CaOx monohydrate crystals (ovals in Fig. 5) were observed in addition to CaOx dihydrates.

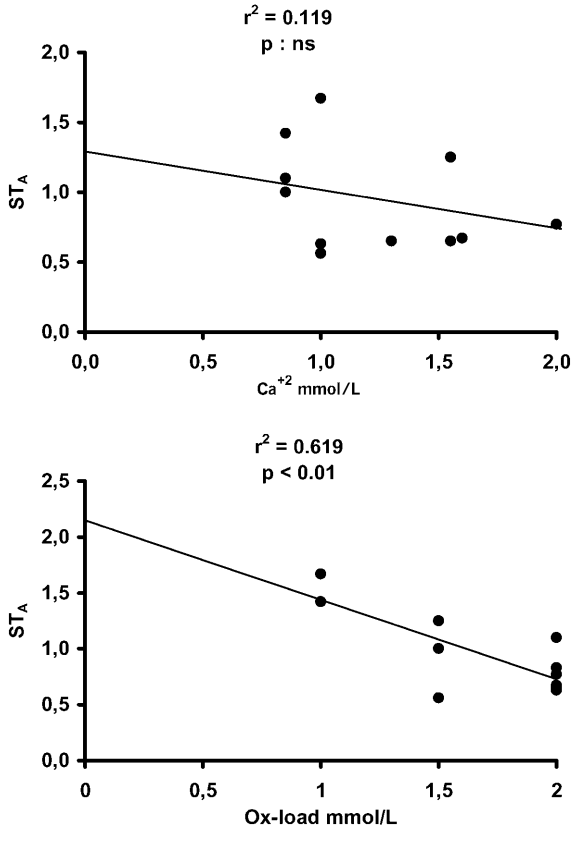

Fig. 4 Influence of urinary Ca^{+2} and oxalate load on ST_A

Discussion

For the first time in this study, HAP induced and secondary CaOx aggregation were examined in whole urine, which provides a high inhibitory potential against aggregation mainly due to urinary macromolecules [18, 20]. These macromolecules might inhibit aggregation by coating the individual crystals. In the absence of stirring, which abolishes the effect of different diffusion rates between small and macromolecular substances, inhibition is overwhelmed by high concentrations of calcium and oxalate [4] or, as shown in this study, by preexisting particles such as HAP or CaOx aggregates. HAP increased approximately fourfold the finding of ST_A, which proved to be an important indicator of aggregation. After secondary CaOx crystallization, ST_A decreased to 40%, demonstrating secondary growth of aggregates. The crystallization tests used in our study

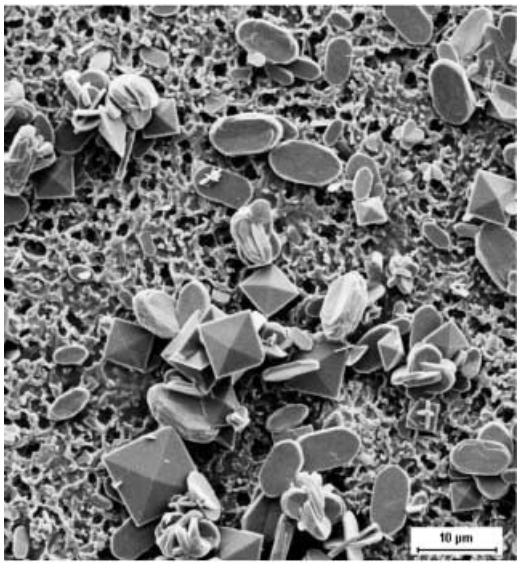

Fig. 5 SEM of sediment in urine with low Ca^{+2} (1 mmol/l) 60 min after secondary oxalate load (2 mmol/l)

were influenced by various physicochemical parameters such as increased supersaturation, induced nucleation, growth and aggregation and crystal coating by urinary macromolecules. Since this study focussed on aggregation, ST_A measurements and SEM were performed at least 20 min after the oxalate load, when almost all crystallization had occurred and sedimentation curves did not change under repeated suspension.

Aggregation is based on crystal attraction by Van der Waals forces and on the viscous binding of crystals by macromolecules [5, 6]. In urine, a normally electropositive charge on crystal surfaces becomes negative by the chemisorption of highly anionic macromolecules such as glycosaminoglycanes, Tamm Horsfall proteins or nephrocalcin [5]. Negative potentials inhibit aggregation by the electrostatic repulsion of other negatively charged crystals. In artificial solutions, the complex interaction of Van der Waals forces and electrostatic repulsion can be described by the DLVO (Derjaguin, Landau, Verwey, Overbeek) theory [5]. However, this theory is difficult to apply to whole urine with its complex and only partially known macromolecular composition. On the other hand, the pH dependency of aggregation observed in this study may provide some explanation as to how particles that are coated by macromolecules can enhance aggregation. Primary and HAP induced aggregations were observed at low pH, where, due to the high protonization of chelators, a high Ca^{+2} concentration was present. The negative surface potential of preexisting particles certainly leads to the accumulation of Ca^{+2} in their surroundings. This may create, together with a high urinary oxalate concentration, ideal conditions for the formation and aggregation of new CaOx crystals before they become coated with low diffusing macromolecules. HAP incubation amplified the pH dependent change of urinary Ca^{+2} (Fig. 1b), which may explain the high enhancement of CaOx aggregation by HAP found at low pH.

A significant correlation between oxalate load and ST_A confirmed urinary oxalate to be a very important factor for CaOx crystallization [1, 15]. Beside the extreme oxalate loads necessary to obtain a particle density sufficient for spectrophotometric measurement, almost physiological conditions were applied in our test system. Experiments extrapolating the limits of metastability for the nucleation and growth of CaOx in whole urine showed that, in the presence of CaOx seeds, crystallization started at high physiological oxalate concentrations [3]. Some aggregation may also occur without stirring under these conditions.

HAP induced aggregation in whole urine gives further evidence that calcium stone formation could start from kidney calcifications [7, 14]. Even so called pure oxalate stones often contain traces of apatite [14]. However, stone formation might also be initiated by intracellular or intratubular crystallization with important crystal-cell interactions [20] which were neglected in our study. On the other hand secondary aggregation, which was examined apart from the oxalate load under physiological conditions, is likely to be a suitable model for stone growth. It explains how crystals are retained and integrated into a preexisting stone during crystalluria. At urinary oxalate concentrations above the limit of metastability, CaOx crystallization might occur preferentially in the Ca^{+2} rich surrounding of preexisting particles that are negatively charged by their macromolecular coat. Initially, newly formed crystals known to be charged positively [6] probably stick to these particles not only by Van der Waals forces but also by electrostatic attraction. In a second phase, when new crystals become coated by low diffusing macromolecules, viscous binding and matrix formation might predominate. Reduced Ca^{+2} concentration and aggregation observed at high pH may give a further explanation for the benefit of alkali therapy in calcium stone metaphylaxis. Until now, the effect of this treatment was mainly ascribed to an increase of the calcium chelator and crystallization inhibitor citrate [10] and not to the direct effect of an increased pH. Our findings suggest that aggregation can be prevented by dietary oxalate restriction and increased fluid intake, both of which reduce urinary oxalate concentration, and also by alkali treatment.

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