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ORIGINAL PAPER

Aggregation of freshly precipitated calcium oxalate crystals in urine of calcium stone patients and controls

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Abstract Aggregation (AGN) of freshly precipitated calcium oxalate crystals was photometrically studied in urine of 30 calcium stone patients and 30 controls, in solutions containing urinary macromolecules (UMS) and in an inhibitor free control solution (CS). Crystals were produced by oxalate titration and crystallization was monitored measuring optical density (OD). Tests were repeated adding hydroxyapatite (HAP) to urine and UMS and adding citrate and pyrophosphate (PPi) to UMS of the controls. AGN was recognized as a rapid OD decrease being at least three times faster than sedimentation of single crystals ($p < 0.001$) and used to calculate an extent of AGN (EA%). The time between the end of titration and the beginning of AGN was determined as suspension stability (SS). The main effect of urinary inhibitors was retardation of AGN without changing EA, SS being higher in urine than UMS ($p < 0.001$) and in UMS than CS ($p < 0.001$). In urine of 63% of controls but only in 33% of patients, no AGN was recorded ($p < 0.05$). The high inhibitory activity of urine could not be reproduced in UMS even in combination with 3.5 mM citrate or 0.05 mM PPi. 0.05 mg/mL HAP reduced SS in all urine samples to low values and increased the rate of rapid OD decrease, being a measure for the size of aggregates. Retarding AGN of crystals during their passage through the kidney seems to be an important mechanism to prevent stone formation during crystalluria. The promotion of AGN by HAP reveals a new role of Randall's plaques in nephrolithiasis.

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Introduction

Crystal aggregation (AGN) seems to be an important process in calcium nephrolithiasis [1, 2]. During crystalluria, it probably allows the apposition of crystals to Randall's plaques or intratubular crystal deposits and thus leads to crystal retention and to the formation of obstructing stones [3]. Also stone growth seems to be based on crystal AGN [4]. Randall's plaques consist of sub-micron particles of organic material complexed with amorphous hydroxyapatite (HAP), which after erosion to the papillary surface provide a platform to which crystals formed in the urine can attach [5]. Large crystal aggregates being retained in collecting ducts and protruding out to papillary surfaces may also be responsible for stone formation [6]. Free solution crystallization mainly seems to be responsible for nephrolithiasis in cystinuria and some cases of secondary hyperoxaluria [7].

Crystallization processes involved in calcium urolithiasis are influenced by various modulators like citrate, pyrophosphate (PPi) and urinary macromolecules (UM), the latter comprising a large group of proteins and some glycosaminoglycans [8]. Normally, these substances are thought to inhibit crystallization processes and thus to protect from stone formation. Several groups have shown that urine or urinary proteins of some stone formers as compared to healthy controls are less capable of inhibiting crystal AGN [2, 9, 10], and that inhibition of AGN is in correlation to urinary citrate concentration and decreases in direct proportion to the frequency of stone recurrence [2]. In other studies however, no signs for a deficient inhibition of AGN in stone patients was found [11, 12]. Inhibition of AGN

recently was related to UM like albumin [13], Tamm–Horsfall glycoprotein (THG) [14] and prothrombin fragment 1 [15]. In some stone patients or populations with increased stone frequency, these UM were found to be structurally altered [13, 14] and in some cases even promoted AGN [14]. Promotion of AGN by THG seems to occur when THG itself aggregates to polymers with increased viscosity, a process being enhanced by high ionic strength, high THG and Ca concentration and low pH [14, 16]. High citrate concentrations were found to correct the alteration of THG [17].

In most studies, UM were isolated by precipitation at high molar salt concentration [14] or concentrated by dialysis [11], procedures which at least theoretically could alter UM structure [14, 16]. Since crystallization processes are difficult to be reproduced, crystals were thoroughly prepared in standardized solutions without inhibitors and ripened for several hours or even days [9, 10]. However, in stone formation AGN of freshly precipitated crystals seems to be involved. AGN of such crystals could differ from ripened crystals being prepared in inhibitor free solutions. To get further information about the AGN of freshly precipitated crystals, we produced Ca oxalate in urine and in inhibitor containing solutions by slow oxalate titration. Subsequently, we photometrically measured particle sedimentation, being an indicator of AGN [9]. UM solutions (UMS) were prepared by a hemofiltration procedure allowing UM isolation in an almost identical composition and concentration as being present in urine [18]. In a preliminary study, we have found a total inhibition of AGN of freshly precipitated crystals by urine of healthy controls, which could only partially be attributed to UM or citrate [18].

To show possible differences between stone patients and controls, we doubled the state of saturation with respect to Ca oxalate in the present work. Contrary to most other studies, urine of unselected patients with a Ca stone in situ was examined. Tests were also performed with addition of HAP crystals, trying to simulate the effect of Randall's plaques. To get an explanation for the high inhibitory activity found in healthy men, we repeated the experiments in UMS of healthy controls after addition of 0.05 mM PPi and of 3.5 mM citrate, a citrate concentration known to influence THG [17].

Methods

Collection and preparation of urine

Urine was obtained from 30 patients (24 men and 6 women aged 16–79, mean 52 years) prior to undergo ESWL for a calcium stone and from 30 controls (22 men and 8 women aged 16–80, mean 52 years) prior to undergo hand surgery.

Table 1 Chemistry of urine after standard dilution and before Ca^{++}, sodium and pH adaptation

	Patients, mmol/L (mean ± SD)	Controls, mmol/L (mean ± SD)	*p*
Na	60.1 ± 24.9	56.2 ± 19.7	n.s.
Ca	1.47 ± 0.77	1.31 ± 0.68	n.s.
Ca^{++}	0.69 ± 0.42	0.63 ± 0.37	n.s.
Ox	0.13 ± 0.08	0.08 ± 0.03	<0.001
Cit	0.80 ± 0.43	0.71 ± 0.43	n.s.

In 60% of patients Ca oxalate monohydrate, in 23% Ca oxalate dihydrate and in 17% Ca phosphate was predominant in stone analysis. 33% of Ca oxalate stones contained 5–20% of apatite. 57% mostly younger patients entered the hospital to treat their first stone episode. The remaining 43% suffered from 1 to 8 recurrences. Hyperuricaemia in two cases and hypocitruria in one case were the only metabolic disorders. Controls had no history of urological or nephrological disease. Patients and controls being on free diet were fasting at least 2 h before starting the urinary collection period, which exactly was recorded. Urine volume was determined and urine with pathology on stick examination (Combur Test UX, Roche, Switzerland) was excluded with the exception of microhaematuria in stone patients. From the collection period, a volume corresponding to a diuresis of 100 mL/h was calculated and urine samples with a lower value were diluted to this volume with distilled water. Only three urine samples with a higher volume were used without this standard dilution. Urine samples were then divided into three portions. With the first portion, urine chemistry as indicated in Table 1 was performed. The second portion was used for crystallization tests and from the third portion UM were isolated.

Preparation of UMS

55 mL of urine were injected into the dialysis fluid compartment of a capillary hemodialyser (Hemoflow F3, Fresenius AG, Bad Homburg, Germany), the exclusion limit of the dialysis membrane being 5 kDa. The compartment was closed and the urine dialyzed against 12 L (200 mL/min) of deionized water [18].

Crystallization tests

UMS and distilled water as control solution (CS) were buffered with 5 mM sodium cacodylate. pH was adjusted in all samples to 6.0. In urine and UMS, Ca and sodium were determined by an ion selective electrode (AVL List GmbH, Graz, Austria). Before performing crystallization experiments in all samples, ionic Ca was adjusted to 2.0 mM and

sodium to 100 mM. Ca oxalate crystals were produced by oxalate titration. Therefore, a quartz macro cuvette containing 2 mL of sample was placed into a thermostable cell holder of a Perkin Elmer spectrophotometer 550S (Perkin Elmer, Rotkreuz, Switzerland) at 37°C. Under continuous stirring, oxalate concentration was raised by 0.1 mM sodium oxalate per minute up to a final addition of 1.5 mM. Crystallization was monitored in the spectrophotometer at 620 nm wavelength by an increase of optical density (OD). At the end of oxalate titration, stirring was stopped and OD was followed during 60 min. Crystallization tests in urine and UMS were repeated adding 0.05 mg/mL HAP which increased OD only minimally [20]. In UMS of healthy controls, test were repeated after adding 0.05 mM PPi or 3.5 mM citrate and in the case of citrate addition after readapting Ca^{2+} to 2.0 mM and pH to 6.0. Neither urine nor UMS were filtered or centrifuged before performing crystallization tests.

Evaluation of spectrophotometric curves

From the starting time of OD increase and the titration rate the so called metastable limit (ML) was calculated. If the initial Ca and oxalate concentrations are known, ML is a measure for the critical supersaturation necessary to induce crystallization. At the end of oxalate titration, the maximal OD (mOD) being a measure for particle concentration [9] was determined, stirring was stopped and the further course of OD was monitored. OD decreases were measured as dOD/dt (min^{-1}) as soon as they became straight lines. OD increase caused by the addition of HAP was subtracted from mOD. Where gradient of OD decrease significantly changed, OD was marked and the corresponding time was determined. Scanning electron microscopy of sediments demonstrated that slow OD decrease after oxalate titration mainly could be attributed to the sedimentation of single crystals, whereas a rapid OD decrease was due to AGN [3, 18]. The time between the end of the titration period and the start of rapid OD decrease or AGN, respectively, was denominated suspension stability (SS), because AGN is known to destabilize suspensions and emulsions. Since OD mainly reflects particle number per volume, the difference of OD after a period with rapid OD decrease and a virtual OD without AGN corresponds to the particle concentration involved in AGN. This difference was an estimation of the extent of AGN (EA) and expressed by the equation present in Fig. 1 in percentage of the virtual OD which would be present without AGN.

Statistics

Results are given as mean ± SD. Probabilities were calculated by Mann–Whitney *U* test and by Fisher's test.

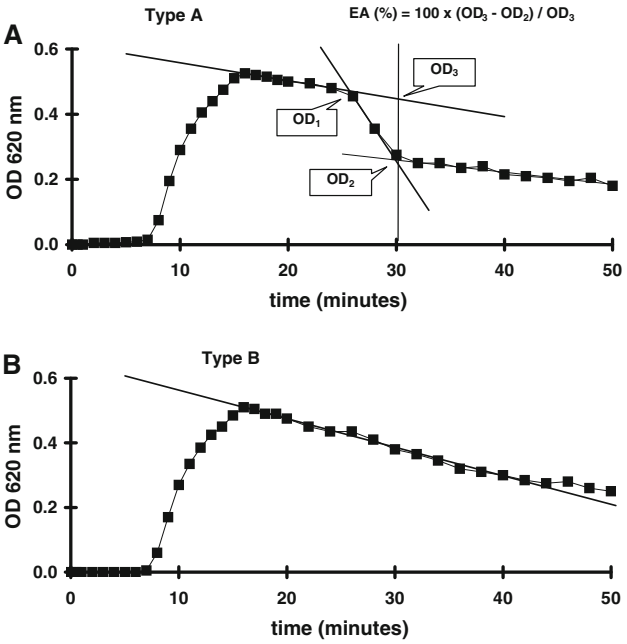

Fig. 1 Crystallization curves in urine with a phase of rapid decrease of optical density (OD) indicating AGN (**a**) and with exclusively slow sedimentation of single crystals (**b**). OD_1 start of AGN, OD_2 end of AGN, OD_3 virtual OD without AGN, *EA* extent of AGN as indicated in the equation

Results

Chemistry of urine after standard dilution and before ionic Ca, sodium and pH adaptation is presented in Table 1. Before the adaptation, Ca^{2+} was always below 2 mM and sodium only in three urine samples above the desired concentration of 100 mM. Stone patients as compared to controls showed a significantly increased oxalate concentration. But mean urinary oxalate concentration was also in patients below 10% of the later oxalate addition to the test system by titration. HAP addition to urine decreased Ca^{2+} on average by 0.07 mM and increased pH by 0.05. The other chemical parameters showed no measurable change. In UMS after HAP addition an increase of Ca^{2+} by 0.1 mM and of pH by 0.1 was observed. Although these measurements showed in urine some Ca phosphate growth and in UMS some HAP dissolution, the OD increase of 0.12 produced by the addition of HAP remained constant in both solutions during an observation period of more than 30 min. After citrate addition to UMS and Ca^{2+} adaptation to 2.0 mM, pH increased on average to 6.2 which was corrected before performing crystallization tests.

Crystallization curves obtained by spectrophotometry revealed two different types (Fig. 1). Type A showed after a period of slow sedimentation a sharp OD decrease being characteristic for AGN, which started very abruptly and

ended after a 20–50% OD decrease again by a phase of slow sedimentation. Type B was characterized by a continuous slow OD decrease starting shortly after the end of oxalate titration and showing no signs of AGN during an observation period of 60 min. Type B without signs of AGN was exclusively found in urine without HAP addition and was more frequently in urine of controls than of patients (Table 2, $p < 0.05$). After HAP addition to urine as well as in all other experiments, type A was observed.

Details of crystallization curves obtained in urine, UMS and CS are presented in Fig. 2. The ML, being the critical oxalate addition where crystallization starts, was almost identical in urine and UMS, but significantly higher than in CS ($p < 0.01$). HAP addition to urine or UMS had no influence on ML. Maximal OD (mOD) reached at the end of titration and reflecting particle concentration was significantly higher in urine of patients and controls than in the corresponding UMS ($p < 0.001$) and was also higher in UMS than in CS ($p < 0.001$). HAP addition had no significant influence on mOD. Figure 2 clearly demonstrates, that in all experiments showing a rapid OD decrease the corresponding dOD/dt_R was 3–10 times faster than the dOD/dt of the slow sedimentation phase ($p < 0.001$). dOD/dt_R was lower in urine of patients and controls ($p < 0.001$) than in the corresponding UMS. The addition of HAP increased dOD/dt_R in urine as well as in UMS ($p < 0.01$). SS, the time between the end of oxalate titration and the start of rapid OD decrease, was much longer in urine of patients and controls than in UMS (patients $p < 0.001$, controls $p < 0.01$). SS in UMS was also higher than in CS ($p < 0.001$).The highest SS was found in urine of stone patients exceeding about three times the values observed in UMS and being also significantly higher than in urine of the controls ($p < 0.01$). However, as Table 2 shows, in urine of 63% of controls but only in urine of 33% of patients no AGN was recorded. Addition of HAP produced in all urine samples crystallization curves of type A, being characteristic for AGN and reduced SS in all urine samples to values found in UMS. In UMS, no significant influence of HAP on SS was observed. The EA was similar in urine, UMS and CS.

Table 2 Frequency of crystallization curves with AGN (A) and without AGN (B) in urine of patients and controls without and with addition of 0.05 mg/mL hydroxyapatite (+HAP)

	A	B
Patients	20/30	10/30
+HAP	30/30	–
Controls	11/30	19/30
+HAP	30/30	–

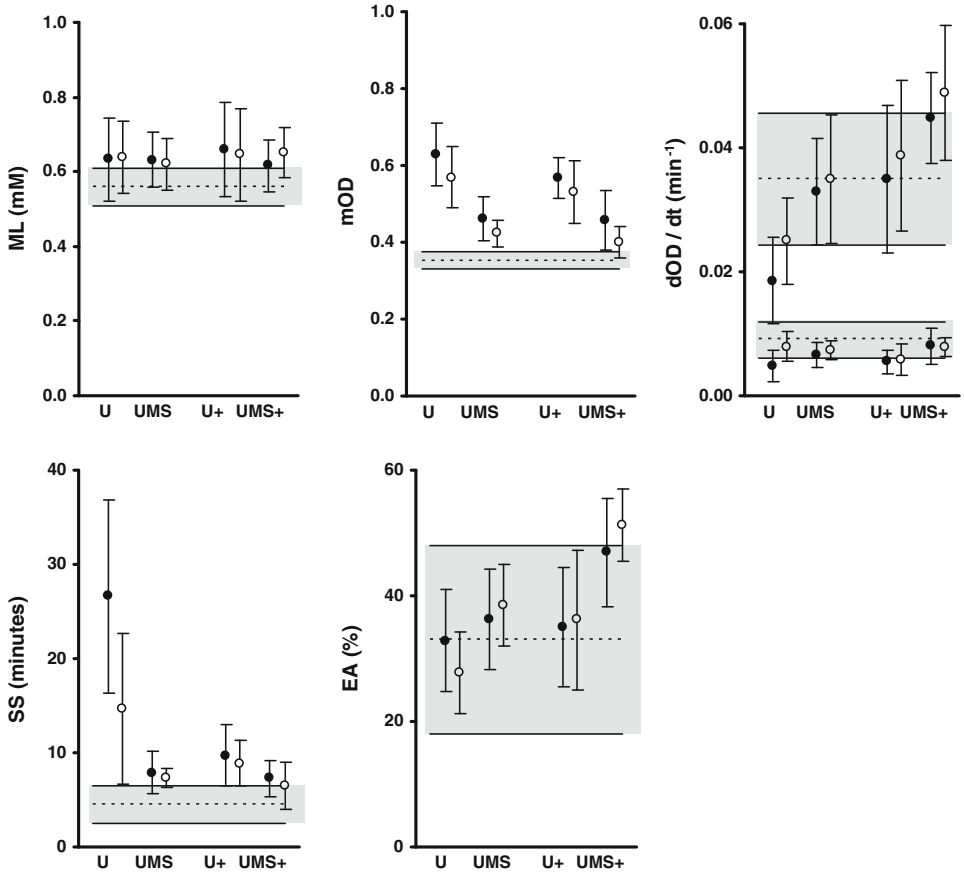

Fig. 2 Crystallization parameters (mean ± SD) in urine (U), in solutions of urinary macromolecules (UMS) of stone patients (*closed circles*) and controls (*open circles*) without and with HAP addition (+) and in a control solution (CS, *shaded area*). *ML* metastable limit, *mOD* maximal OD after oxalate titration, *dOD/dt* rate of rapid (*above*) and slow OD decrease (*below*), *SS* starting time of AGN after end of titration, *EA* extent of AGN (see text and Fig. 1)

Table 3 Crystallization parameters (mean ± SD) as indicated in Fig. 2 in solutions of urinary macromolecules of controls (UMS) without and with 0.05 mM pyrophosphate (+PPi) or 3.5 mM citrate (+Cit)

	UMS	+PPi	+Cit
ML (mM)	0.62 ± 0.07	0.62 ± 0.08	0.59 ± 0.04
mOD	0.42 ± 0.04	0.39 ± 0.04	0.44 ± 0.02
dOD/dt_R	0.035 ± 0.01	0.036 ± 0.05	0.040 ± 0.05
SS (min)	6.7 ± 2.2	7.3 ± 0.8	6.7 ± 0.8
EA (%)	39 ± 7	38 ± 9	41 ± 2

HAP produced in UMS of patients and controls an increase of EA ($p < 0.001$). In CS being free of chelators and inhibitors reproducibility especially of EA was poor. Table 3 shows that the addition of PPi and citrate to UMS of controls had no significant influence on crystallization parameters. Results of crystallization tests revealed neither a correlation to stone composition nor to stone episodes. Summarizing the results of crystallization experiments, we found that the main difference between stone patients and controls was a reduced blockage of AGN in urine samples of patients. On the other hand, the remaining urine samples of controls showing AGN revealed, compared to the urine of the patients, a significantly reduced SS. HAP addition to urine produced always AGN and SS was reduced to the low values found in UMS. In urine and UMS, dOD/dt_R significantly increased after HAP addition. The addition of citrate and PPi to UMS of controls had no effect on crystallization parameters and especially could not restore the blockage of AGN or the high SS found in urine.

Discussion

To study crystal AGN as it may occur in stone formation during crystalluria, we produced by oxalate titration crystals directly in urine and in UMS. We used diluted urine, which due to low concentrations of its compounds allowed by additions to adapt Ca^{2+}, sodium and pH to identical values. By the following oxalate titration to a final concentration, exceeding largely urinary oxalate, we tried to perform all tests at a similar initial supersaturation. Under these conditions, the metastable limit (ML) defined as critical oxalate addition to induce crystal formation was similar in urine and UMS. Both showed a higher ML than CS and therefore inhibition of Ca oxalate nucleation. But contrary to other studies where urine was centrifuged to remove debris being potential nucleators and where an upper limit of metastability was calculated from critical oxalate addition and urine chemistry [11, 12], we could not find a different ML between stone patients and controls. Crystal concentration produced in the test system was assessed measuring maximal optical density (mOD) after oxalate titration. A mOD being in urine 30% higher than in UMS could hardly be explained by urinary oxalate which on average was less than 10% of the oxalate added by titration. In UMS, mOD was also higher than in CS despite of an almost identical initial supersaturation in both solutions. An elevated mOD at identical initial supersaturation means that more but smaller crystals are formed. This effect can be explained by an inhibition of crystal growth where during the state of labile supersaturation more Ca oxalate is available for nucleation than without inhibition. HAP addition to urine and UMS interestingly neither decreased ML nor increased mOD indicating heterogeneous nucleation of Ca oxalate by HAP as being demonstrated under crystallization conditions similar to those in renal collecting ducts [19].

Crystal AGN, being the topic of this study, was monitored by an abrupt change of OD decrease which was observed in some urine samples even more than 30 min after the end of oxalate titration. OD decrease occurs when particles by sedimentation disappear out of the observation field of the spectrophotometer and during AGN, when the concentration of individual particles is reduced by the formation of large aggregates. Since sedimentation rate increases by particle diameters in square, aggregates show a very rapid sedimentation [3]. The rate of rapid OD decrease (dOD/dt_R) which showed a reverse relation to mOD or crystal concentration respectively (Fig. 2) mainly seems to reflect the size of the aggregates. The lowest dOD/dt_R was found in urine without HAP addition. At the high mOD of urine probably relative small single crystals may have formed also relative small aggregates with a low sedimentation rate or dOD/dt_R. HAP addition to urine and UMS, known to induce Ca oxalate AGN [20, 21] on the other hand increased dOD/dt_R without a significant change of mOD. HAP induction of AGN seems therefore mainly to increase the number of crystals in the aggregates. To characterize AGN, not only the size of aggregates which can be estimated by dOD/dt_R, but also the share of crystals involved in AGN should be known. Although possible errors are inherent in every extrapolation, as a measure for the EA we calculated the relative OD disappearance during rapid OD decrease. A third characteristic was the starting time of AGN after the end of oxalate titration which was denominated SS. AGN is the process which destabilizes suspensions and emulsions [22].

Experiments performed in CS gave despite of a poor reproducibility with respect to dOD/dt_R and EA an interesting insight in the ability of freshly nucleated crystals to aggregate. These crystals showed a delayed and limited AGN with an average SS of 4.5 min and EA of 33%. Urine and UMS prolonged the delay of AGN but had no influence on EA. The effect of urinary inhibitors could therefore be explained by retarding a spontaneous transformation of

crystals to a state being suitable for AGN which can base on particle attraction by Van der Waal's forces, on viscous binding by UM coats of crystals or on Ca-bridging between particles [23]. In most urine samples, an extremely high retardation or even a blockage of AGN was found. This high inhibitory activity could not be reproduced in UMS. Also the addition of high concentrations of PPi and citrate to UMS did not increase the inhibition by UM. Urine of controls showed with respect to the delay of AGN some discrepancies. In 66% of urine samples, no signs of AGN were found whereas in the remaining urine samples SS was even lower than in the urine of the stone patients. A similar observation was made studying the AGN of preformed Ca oxalate crystals in solutions of dialyzed urinary proteins where the proteins of male patients showed a stronger inhibition of AGN than proteins of the controls [11]. The extremely high retardation and especially the blockage of the AGN of freshly precipitated crystals in urine cannot exclusively be explained by the action of current inhibitors and therefore deserves further exploration. Theoretically, other low molecular weight substances than citrate or PPi could enhance or stabilize UM as it was described for citrate with respect to THG [17].

A severely delayed start of AGN, and thus the stabilization of the crystal suspension in urine, seems to be an important mechanism to prevent stone formation during crystalluria where some crystals may show a very slow passage through the kidney. Near tubular walls this passage may be delayed up to more than 1 h by fluid drag or sticking of crystals to the wall [24]. The concept of urine as a colloidal system protecting from stone formation was already put forward in 1952 [25]. Our finding that in urine AGN was often totally blocked confirms this theory. The fact that blockage of AGN was not exclusively found in urine of controls but also in 33% of stone patients and was lacking in 37% of controls illustrates, that urolithiasis generally is a multifactoral disease with a high prevalence. Our finding of HAP-induced AGN in urine reveals Randall's plaques in a new light. HAP deposits in Randall's plaques generally are thought to present a platform for overgrowth by calcium oxalate [7]. However, during crystalluria the plaques could also induce AGN and thus accumulate preformed crystals, a process probably being more efficient than simple overgrowth. When interpreting our study it has to be pointed out that results obtained from stone patients might have been influenced by stones being in situ which could have altered urinary composition by microhaematuria, by obstruction and by adsorption of some compounds to the stone. Red blood cell membrane fragments, as example influence growth and AGN of calcium oxalate crystals [26]. Our results may thus not be representative for the beginning of stone disease but for the situation during stone growth where AGN seems to be an important mechanism [4]. We believe that the production of calcium oxalate crystals directly in urine with the evaluation of spectrophotometrical crystallization curves as described in our paper is an interesting tool to study AGN. However, further experiences will show whether this procedure is suitable as a routine method. The main drawback of our method was the necessity of urine dilution to get comparable states of supersaturation or crystal concentration, respectively.

Conclusions

Aggregation of freshly precipitated crystals is a process starting with some delay and ending with the integration of only about 30% of crystals in the aggregates. Urinary inhibitors retard AGN without changing crystal integration. The extremely high inhibitor activity of urine which could not be reproduced by UM even in combination with high concentrations of citrate and PPi, seems to be an important mechanism to prevent stone formation during crystalluria and deserves further research. HAP which reduced SS in all urine samples to the relative low values observed in UMS and increased particle number in aggregates is a potent promoter of AGN. This observation reveals a new role of Randall's plaques in nephrolithiasis.

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