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ORIGINAL PAPER

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Nucleation and aggregation of calcium oxalate in whole urine; spectrophotometric sedimentation analysis: a new approach to study the aggregation of calcium oxalate dihydrate

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Abstract Spectrophotometric and scanning electron microscopic (SEM) studies of oxalate-induced crystallization have been performed in whole urine with and without continuous magnetic stirring and before and after millipore filtration of urine. With continuous stirring, preferential nucleation was observed and this followed second order kinetics. Important crystal aggregation only occurred after an oxalate load above 1 mmol/l and without stirring. Under these conditions and at an ionic calcium concentration of 2 mmol/l, single crystals and aggregates of calcium oxalate dihydrate and monohydrate of well defined sizes were produced. Single dihydrates, their aggregates and the other particles could be distinguished by their significantly different sedimentation rates. From sedimentation curves an aggregation ratio for calcium oxalate dihydrate (aggregated/total dihydrate particles) was extrapolated. Millipore filtration removing important urinary macromolecules increased this aggregation ratio as well as the size of the aggregates on SEM pictures.

Key words Urolithiasis · Calcium oxalate · Crystallization · Aggregation

Introduction

There is increasing evidence that crystal aggregation is a most important process in stone formation. Microscopic [8] and ultrastructural analyses [12] of kidney stones revealed a highly aggregated structure. Stone formers, contrary to healthy controls were found to excrete large crystal aggregates in their urine [14]. Crystal aggregation occurring within the delay of seconds [4] seems to be the only crystallization process creating, within urinary transit time, particles big enough to be trapped in the renal tubulus [10].

Despite its importance, measurement of crystal aggregation has remained a major problem in stone research [2]. Microscopy is the only technique that enables large single crystals to be distinguished from small aggregates. However, this method is only a semi-quantitative one. Indirect approaches to measure aggregation have been developed. Apparent solubility of crystals at low concentrations differ depending of whether the solubility is measured by crystal growth from a supersaturated solution or by crystal dissolution in an undersaturated medium [11]. This difference seems to increase with aggregation and therefore was used to calculate an agglomeration inhibition parameter [3]. However, the method is limited by the fact that whole urine is mostly supersaturated with respect to calcium oxalate and thus dissolution experiments can not be performed directly.

In further approaches, the reduction of particle number due to aggregation has been measured either by a particle analyzer [17] or turbidometrically [5]. Studying crystal growth with a Coulter counter, this reduction was found in very diluted urine [17]. When nucleating calcium oxalate in whole urine, this phenomenon could not be observed, although microscopy revealed important aggregation [16]. However, at the end of nucleation in artificial solutions, Hess et al have found a marked decrease of turbidity which by scanning electron microscopy (SEM) could be attributed to crystal aggregation [5]. The experiments were performed at slow stirring known to favor crystal aggregation [4].

Using a modified test system of Hess et al., we could find similar crystallization curves in whole urine [1]. Therefore a more extensive spectrophotometric and scanning microscopic study of the formation and aggregation of calcium oxalate in whole urine was performed

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under different initial calcium concentrations, oxalate loads and stirring modalities. A high calcium concentration is known to promote the polymerization of Tamm Horsfall protein and thus crystal aggregation [6]. In artificial solutions by increasing the oxalate loads, we observed a more predominant decrease of turbidity [1]. In our new study we also measured sedimentation rates at the end of crystallization experiments. This procedure has been used by others to study aggregation of preformed crystals in artificial solutions [7]. Some of our experiments were repeated after millipore filtration of urine, in order to remove urinary macromolecules that have an important influence on the crystallization processes [15].

Material and methods

Spectrophotometric analyses of crystallization during permanent slow stirring

Freshly voided urine of ten calcium stone patients was stored at 37 °C and analyzed within 4 h. Then 2 ml urine was pipetted into a cuvette and placed into a thermostatable cell holder (37 °C) of a Perkin Elmer spectrophotometer 550 S (Perkin Elmer, Rotkreuz, Switzerland) connected to a circulating waterbath (JULABO, Exatherm U3, Digitana, Neuchatel, Switzerland). All experiments were monitored at 620 nm wavelength. A mini magnetic stirrer (CUV-O-STIR model 333, Hellma, Müllheim/Baden, Germany) allowed continuous magnetic stirring (500 rpm). Crystallization generally was induced by an oxalate load of 1.5 mmol/l, using a stock solution of 100 mmol/l disodium oxalate and the shift of optical density (OD) was recorded by a Perkin Elmer recorder 561 (Perkin Elmer, Rotkreuz, Switzerland). In six urine samples the test was repeated with oxalate loads of 1 or 2 mmol/l respectively. Five urine samples were tested without adjusting the calcium concentration as well as at an ionic calcium concentration adjusted to 4 mmol/l with a 2 M calcium chloride stock solution.

Spectrophotometric measurement of sedimentation in crystallization experiments with intermittent stirring

Urine was collected from seven healthy controls and three calcium stone formers. The healthy individuals voided their urine immediately before the test was performed, while stone formers brought their freshly voided morning urine in a Devar-flask to the laboratory. Before performing the crysallization experiments, the ionic calcium concentration of the urine samples was adjusted to 2.0 mmol/l. The urine was placed into the cuvette of the spectrophotometer and stirred at 500 rpm. An oxalate load was applied, immediately after stirring was stopped. For 20 min crystallization was observed during spontaneous sedimentation. Then the sediment was resuspended by stirring for 10 sec. Sedimentation (OD decrease) was remeasured for 20 min. This procedure was repeated two to three times. Experiments were carried out at various oxalate loads ranging from 0.75 to 2 mmol/l, before and after millipore filtration of the urine (Millipore filter, pore diameter 0.2 μm, Millipore, Volketswil, Switzerland).

Millipore filtration always was performed at vacuum traction after centrifugation of urine and as soon as possible after voiding.

Scanning electron microscopy

On completion of the crystallization experiments, 150 μl 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 analyzed by 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 and ×10,000.

Results

Crystallization at continuous stirring

After the oxalate was loaded, OD rapidly increased without any measurable delay and reached a maximum within 20–30 min (Fig. 1a). Even after 1 h, no deflection of the curve could be observed with continuous stirring. Only one urine sample, examined at an ionic calcium concentration of 4 mmol/l and an oxalate load of 2.0 mmol/l, showed a slight decrease of OD, 7 min after reaching the maximum initial peak. In artificial solutions, under identical calcium and oxalate concentrations and continuous stirring, mainly aggregates of up to 20 μm were found (Fig. 1e). Plotting $t/\Delta OD_t$ versus time generally showed a strong linear correlation (Fig. 1b). The ΔOD_t denotes OD decrease at time t. The slope of the curve represents the reciprocal value of $\Delta OD\infty$, which is defined as the optical density being observed at infinite observation time. The intersection of the curve represents $h/\Delta OD\infty$, h being the half-life to reach $\Delta OD\infty$. The crystallization curve obtained when continuous stirring occurs, can be described by the equation given in Fig. 1b. In five out of six experiments performed with varying oxalate loads (Ox), a good linear correlation was found between $\Delta OD\infty$ and Ox (Fig. 1c).

SEM of the sediments revealed small single crystals of calcium oxalate dihydrate with average side lengths of about 3 μm and diameters of about 4 μm (Fig. 1d left). Their density increased with increasing oxalate loads (Fig. 1a). Conversely, only few aggregates of smaller crystals could be observed (Fig. 1d right).

Crystallization with intermittent stirring

Immediately after the oxalate loading, OD sharply increased to a maximum at around 5 min, when sedimentation became visible and was monitored for 20 min. After repeated resuspension of the urinary sediment by a short stirring of 10 sec, characteristic sedimentation curves of high reproducibility could be observed (Fig. 2a). At high oxalate loads (1.5–2 mmol/l) the curves showed three gradients and SEM pictures revealed four main particle types, namely single crystals and aggregates of calcium oxalate dihydrate and of calcium oxalate monohydrate (Fig. 2b right). At low oxalate loads (0.75–1.0 mmol/l) only the second and the third gradient could be observed corresponding mainly to single crystals of calcium oxalate dihydrate and monohydrate on SEM pictures (Fig. 2b left). Prefiltration of urine also had a typical influence on sedimentation curves and on SEM. In millipore filtered urine the second gradient became very short and disappeared with time (Fig. 3a). This was due to a marked increase of crystal aggregation as demonstrated in Fig. 3b. This figure also illustrates why maximal OD attained before sedimentation often was lower in millipore filtered urine. Figure 3b shows that in this prefiltered urine almost all crystals were collected in big aggregates and therefore the number of particles mainly being responsible for OD was minimal. Unlike SEM of native urine, the filter present as the background to the picture was very clear.

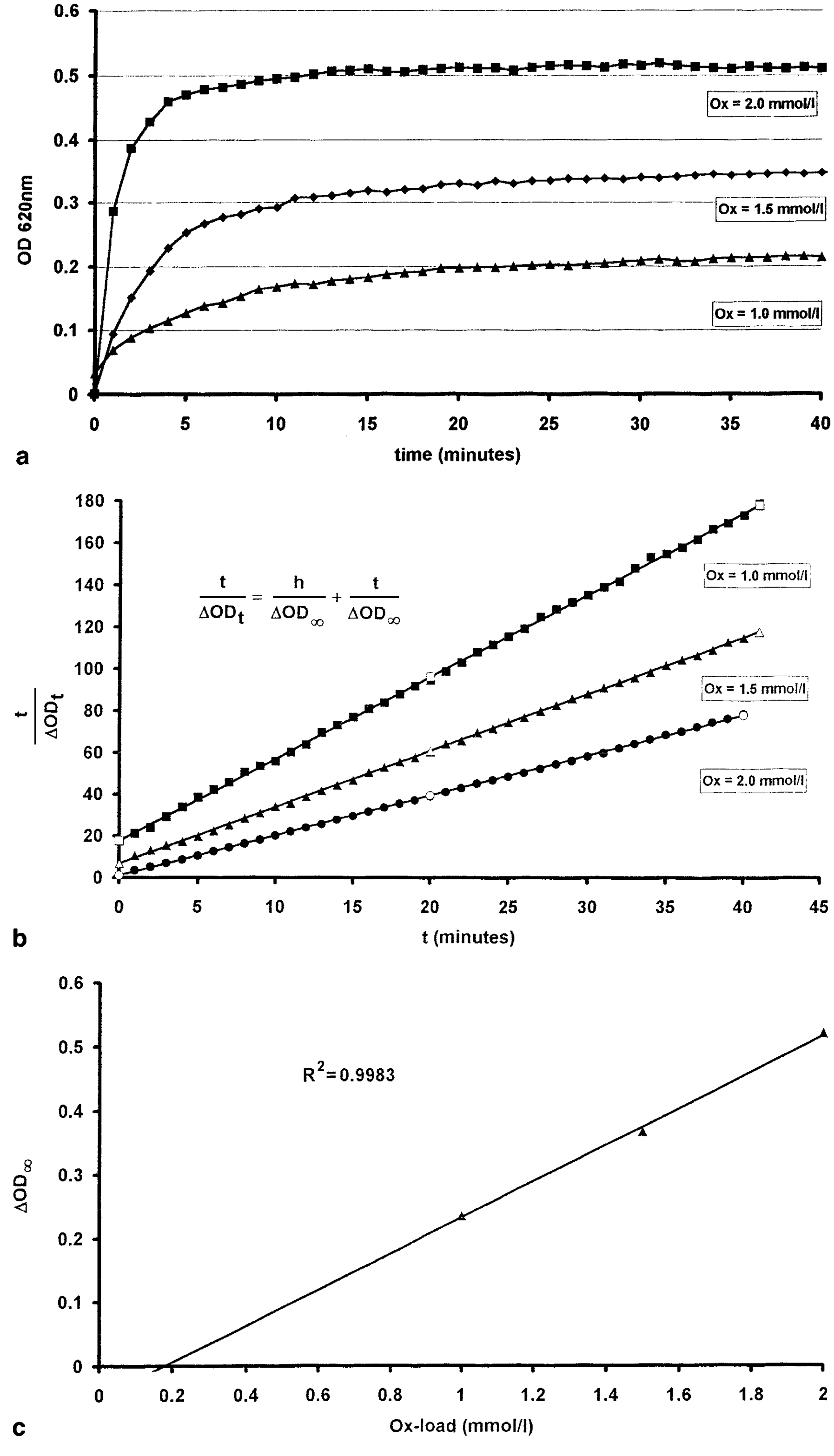
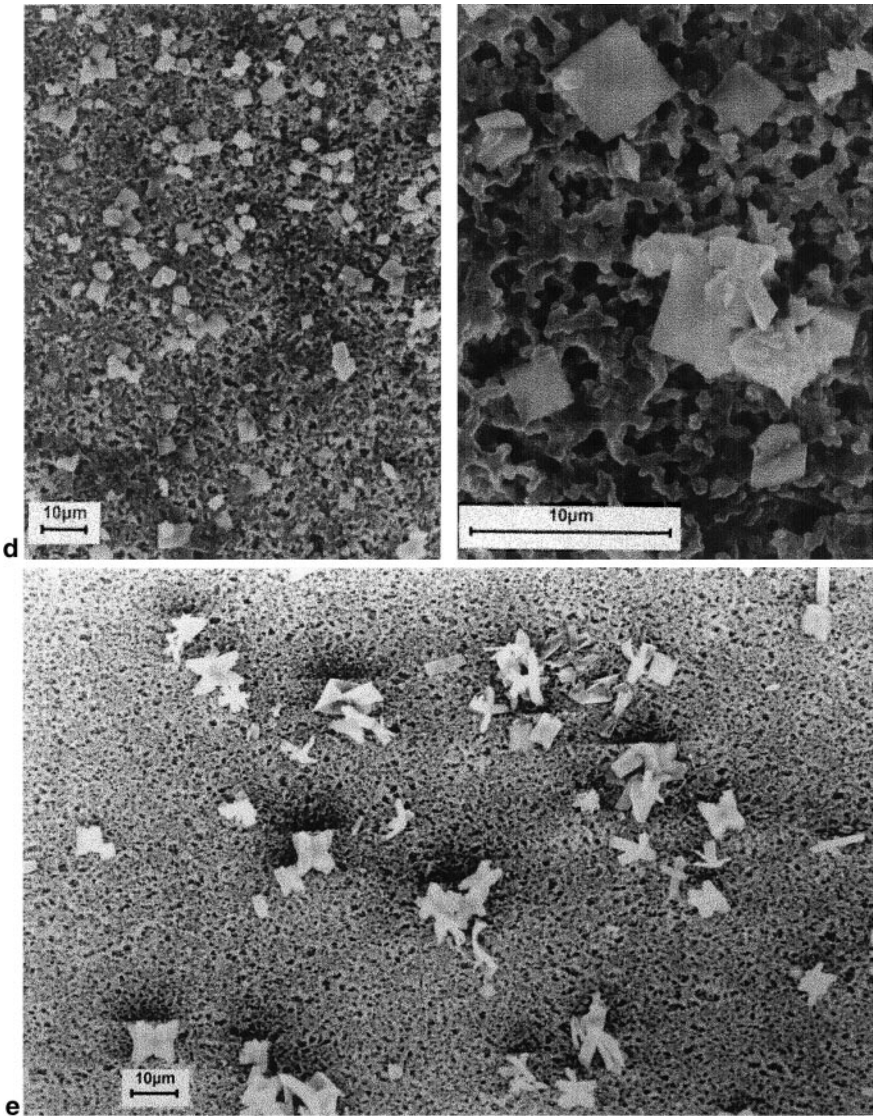


Fig. 1 **a** Spectrophotometric curves of calcium oxalate crystallization induced in whole urine at continuous magnetic stirring by different oxalate loads (*Ox*) OD 620 nm optical density measured at 620 nm. **b** Linearization of crystallization curves of **a** by plotting $t/\Delta DOD_t$ versus t. ΔDOD_t increase of OD at time t, $\Delta DOD\infty$ increase of OD at infinite observation time, *h* half-life to reach $\Delta DOD\infty$. **c** $\Delta DOD\infty$ calculated using the equation given in **b** and plotted against the corresponding oxalate loads. **d** Scanning electron microscopy (SEM) of urinary sediment obtained at continuous stirring 20 min after a 2.0 mmol/l oxalate load. For magnification see the scale of 10 μm on the micrographs. **e** SEM of sediment obtained in an artificial solution (Ca^{2+}: 2 mmol/l) at continuous stirring after 1.5 mmol/l oxalate load

Fig. 1 (Continued)



In general micrographs obtained from native urine generally showed dark shadows on the filter, probably due to the retained urinary macromolecules (Fig 1d and Fig. 2b).

After oxalate loads of 1.5–2.0 mmol/l, in nine out of ten urines characteristic and reproducible sedimentation curves were observed. A total of 37 sedimentation curves was evaluated and all discernable gradients treated by regression analysis as shown in Fig. 4. Results given in Table 1 demonstrate the existence of three significantly different gradients. For practical reasons, results were given as the reciprocal of sedimentation rates, namely the sedimentation time (minutes) for 0.05 OD decrease. In 12 sedimentation curves, probably due to an almost complete aggregation of dihydrates, the second gradient was not visible or too short for evaluation. In three sedimentation curves that showed poor aggregation and long second gradients, the third gradient could not be observed when resuspending the sediment within 20 min. Placing at the end of resuspension a vertical axis into the sedimentation curves and prolonging the individual gradients back to this axis, the three segments a, b, c shown in Fig. 4 could be seen. They seem to represent the contribution of different particle types to the total OD observed in the suspended sediment. A measure for dihydrate aggregation was extrapolated from these segments forming a ratio of OD attributed to aggregated dihydrates (a) and to all dihydrate particles being in the suspension (a + b). A ratio of 1.0 denotes total aggregation; a ratio of 0 indicates the absence of any aggregation. In the native urines an average aggregation ratio of 0.49 ± 0.06 was found. This ratio increased after filtration in four out of five urine samples to 0.82 ± 0.11 (mean ± SE).

SEM pictures were taken at the end of the 14 experiments and evaluated for the average sizes of the single crystals and the number of crystals in the individual aggregates. Results are given in Table 2

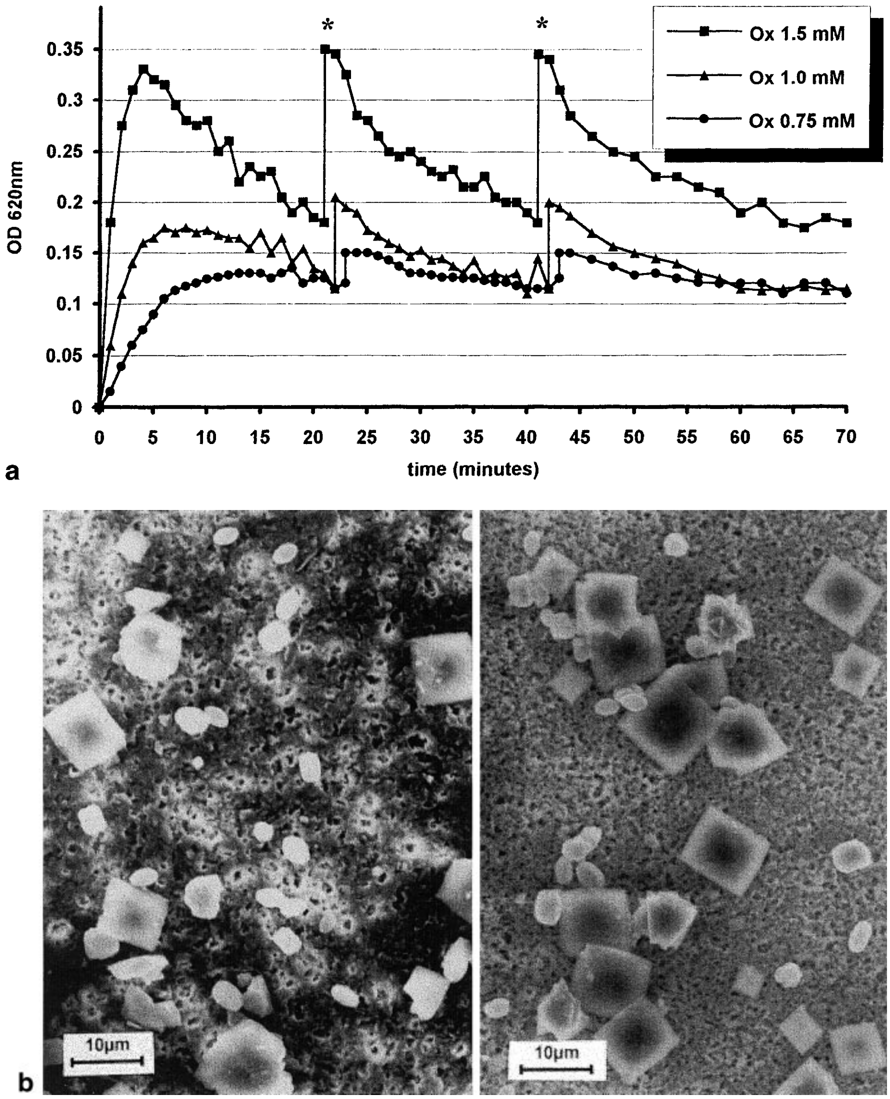


Fig. 2 **a** Spectrophotometric curves of calcium oxalate crystallization in whole urine induced by different oxalate loads (Ox) and at intermittent stirring over 10 s(*). **b** SEM of urinary sediments obtained 20 min after an oxalate load of 1 mmol/l (*left*) and of 1.5 mmol/l (*right*). On the right, all crystal types obtained in whole urine can be seen, namely single crystals of calcium oxalate monohydrate (ovals of about 4 µm long), calcium oxalate dihydrate (envelopes of about 10 µm diameter), aggregates of both crystal types and rare rosettes of small crystals

that shows, that under intermittent stirring, crystals and aggregates of a rather constant size were formed.

Discussion

This study confirmed that particular test conditions, especially the pretreatment of urine [15] but also stirring, influenced the results of crystallization experiments in whole urine. Experiments using continuous stirring were performed exclusively in urine of stone formers, because we tried to confirm a rare preliminary finding that there is a decrease of the turbidity curve in urine of such patients [1]. However, in whole urine of nine patients at continuous stirring, an oxalate load mainly produced small single crystals and only a few small aggregates (Fig. 1d). Spectrophotometry of crystallization with continuous stirring of whole urine measured crystal nucleation preferentially. The maximum OD increase obtained in most experiments showed a strong linear correlation with the oxalate load or the initial supersaturation respectively (Fig. 1c). The increase of OD is known to reflect mainly the number but not the size of particles [5]. Linearization of turbidity curves (plotting $t/\Delta OD_t$ versus t) showed that the curve represents a second order reaction. This best can be demonstrated differentiating the equation given in Fig. 1b: $dOD/dt = \Delta OD\infty/h(\Delta OD\infty - \Delta OD_t/\Delta OD\infty)^2$. Second order kinetics have been shown already for ionic calcium decrease under similar experimental conditions in whole urine [1].

The inhibitory effect of urinary macromolecules seemed to be responsible for the lack of aggregation in continuously stirred whole urine. In experiments performed on a shaker, aggregation was enhanced after removing urinary macromolecules and especially Tamm Horsfall protein by millipore filtration of urine [15]. These macromolecules may inhibit aggregation by coating the single crystals [9]. At high oxalate concentrations and at slow diffusion rates of macromolecules,

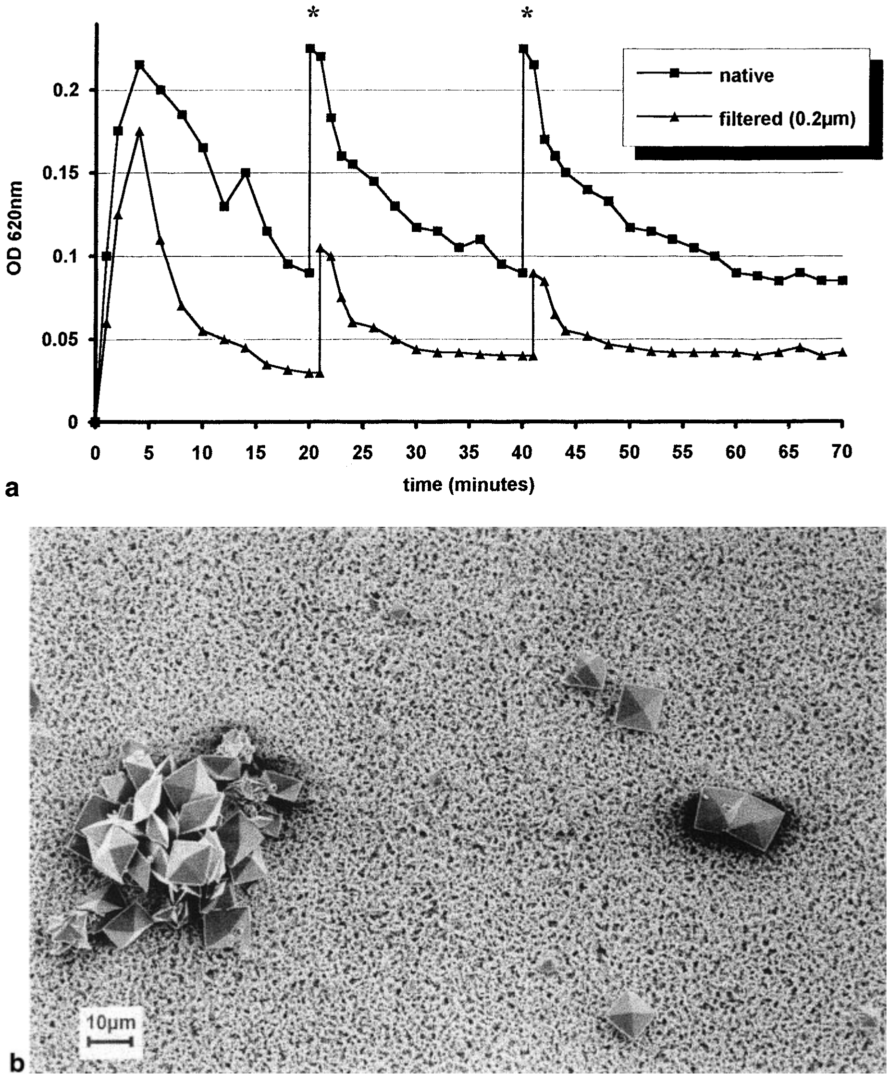


Fig. 3 **a** Spectrophotometric crystallization curves induced by an oxalate load of 2.0 mmol/l in native and millipore filtered urine at intermittent stirring (*). **b** SEM of urinary sediment at the end of the experiment shown in **a** performed with millipore filtered urine

crystal aggregates could be formed before the coating phenomenon becomes active. Stirring obviously abolishes the effect of the different diffusion rates between low- and macro-molecular substances.

In a second approach we tried to produce significant crystal aggregation in whole urine of stone formers as well as in that of healthy controls without continuous stirring. These experiments showed that the degree of aggregation was dependent on the crystal density being present; by increasing the oxalate load, both crystal density and aggregation markedly increased. This phenomenon was observed in whole urine (Fig. 2a, b) as well as in artificial solutions [1]. At our particular test conditions (Ca^{2+} 2 mmol/l, oxalate load 1.5–2.0 mmol/l, no stirring) we were able to produce in urine of stone patients and controls aggregates of calcium oxalate dihydrate with an average diameter of 22 µm. Coulter counter studies of crystalluria in renal stone formers revealed a peak in the particle number of the same range of particle size, namely 19.3–24.3 µm [13]. Spectrophotometric measurement of sedimentation rates was used before to study the aggregation of preformed crystals in artificial solutions [7]. In our experiments, particles of well defined sizes were directly produced in urine. Single crystals of calcium oxalate dihydrate and their aggregates could be attributed to two significantly different sedimentation rates. At low oxalate loads the first gradient of the sedimentation curve was absent (Fig. 2a) and on SEM pictures no aggregates of calcium oxalate dihydrate were found (Fig. 2b left). Conversely the second gradient was abolished in millipore filtered urine (Fig. 3a) when almost all of the dihydrate crystals were aggregated (Fig. 3b). Cosedimentation of some aggregates of calcium oxalate monohydrate in the second gradient together with single dihydrate crystals can not be excluded. However this seems to be of minor importance because SEM revealed on average only 1.7 of such aggregates but at least 16 single dihydrate crystals per picture (Table 2). The introduction of an OD-ratio derived from aggregated dihydrates and from all dihydrate particles being present in the suspended precipitate seems to be a promising tool to study crystal aggregation

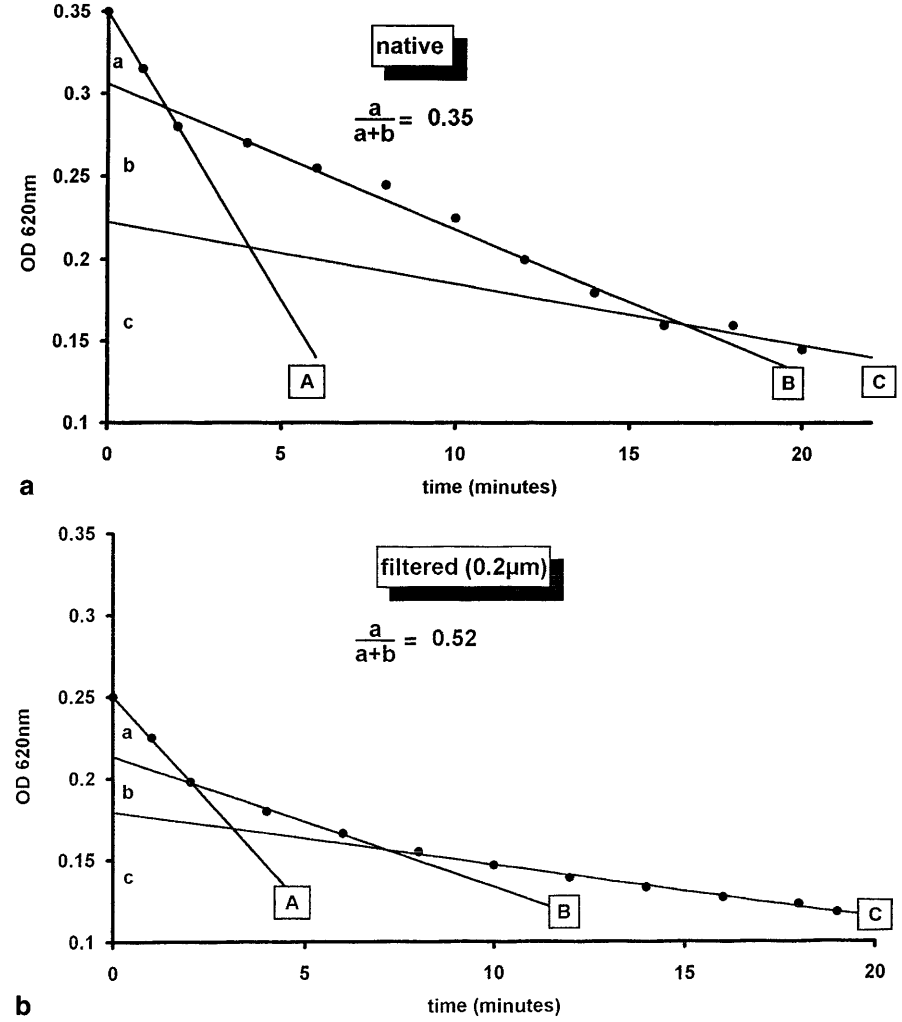


Fig. 4 **a** Regression analyses of a sedimentation curve in native urine showing the three gradients *A*, *B* and *C* and corresponding segments (*a*, *b*, *c*) on a vertical axis placed at the end of intermittent stirring. **b** Regression analyses of an experiment performed with the same urine as in **a** but after millipore filtration

Table 1 Evaluation of 37 sedimentation curves. (For details, see Fig. 4a, b)

Gradient	*n*	min/0.05ΔOD ± SE	*P*
A	37	2.10 ± 0.12	A vs B, $P < 0.001$
B	25	7.96 ± 0.47	B vs C, $P < 0.001$
C	34	18.05 ± 1.14	

Table 2 Evaluation of 14 SEM pictures. *n* sum of all identical particles, mean ± SE

Single crystals	*n*	Length (μ)	Diameter (μ)
Dihydrate	229	8.6 ± 0.5	11.4 ± 0.5
Monohydrate	357	4.4 ± 0.4	2.6 ± 0.2
Aggregates	*n*	Crystals/Aggregate	
Dihydrate	61	4.7 ± 0.7	
Monohydrate	24	6.1 ± 0.7	

in whole urine. However, only further studies will reveal whether this ratio will be helpful in stone research and in the management of stone disease.

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