MEASUREMENT OF METASTABILITY AND GROWTH OF CALCIUM OXALATE IN NATIVE URINE BY CALCIUM ELECTRODE: A NEW APPROACH FOR CLINICAL AND EXPERIMENTAL STONE RESEARCH

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Abstract

Crystallization tests are most important for stone research. Since crystallization properties change with urinary dilution, centrifugation and filtration, the test should be performed in freshly voided and non-pretreated urine. Recently, we have developed an automated method to study the growth of calcium oxalate monohydrate in native urine. However, because seed crystals always become coated with urinary macromolecules, the study of crystals directly produced in urine may be more relevant. In a new modification of our test system, calcium oxalate crystallization was induced in freshly voided unseeded urine by the addition of varying exogenous oxalate loads (Oxex) and the decrease of ionic calcium (ΔCa2+) was automatically monitored during crystallization. Calcium chelation was determined by measuring Ca2+ during calcium titration (Caex). With oxalate loads inducing crystallization without any detectable induction time, straight linear regressions of t/ΔCa2+ vs t were observed, allowing the calculation of ΔCa2+ at infinite observation time (∆Ca∞2+) and its half time (h). Further linear regressions were found with respect of Ca2+ vs Caex, ∆Ca∞2+ vs Oxex and 1/h vs ∆Ca∞2+. These regressions allowed calculation of a chelation coefficient (c), a limit of urinary metastability (m) and a growth rate constant (g) as well as the description of oxalate induced crystallization by a simple equation. This new approach has been compared to other methods studying nucleation and growth of calcium oxalate in urine.

Key Words: Urolithiasis, calcium oxalate, crystal nucleation, crystal growth, ion selective electrode.

Introduction

For a long time it has been known that urinary stones are composed of crystals and an organic matrix, but the question of whether stone formation is a process of crystallization with secondary adsorption of urinary macromolecules [20] or the mineralization of a preformed matrix [1] remains open. The finding that even the smallest crystals in urine are coated with urinary macromolecules [1] suggests that crystal and matrix formation are simultaneous, rather than consecutive processes. Urinary macromolecules can inhibit or promote the crystallization of stone minerals. Such a dual effect has been demonstrated with Tamm Horsfall Glycoprotein (THP). With increasing concentration of THP, sodium and calcium, or with decreasing pH, inhibition changes to promotion [9]. Small molecular weight inhibitors like citrate may also have a chelating effect, which can be studied accurately by special software [8]. But the complex crystallization phenomena observed in urine can not yet be simulated by computer calculations. Therefore, the use of crystallization tests is most important in stone research. These tests should be performed in freshly voided urine, because crystallization properties change with urinary dilution [14], centrifugation and filtration [17]. Recently, we have developed an automated method to study the growth of artificial crystals added to native urine [19]. For the mentioned above, stone forming crystals always become coated with urinary macromolecules. Therefore, the study of crystals directly produced in urine may be more relevant for stone research [11, 16]. This paper describes the automated measurement of the formation and growth of calcium oxalate using an ion selective calcium electrode in freshly voided urine without seeding or previous centrifugation and filtration.

Materials and Methods

Portions of 14 freshly voided urines from 5 healthy male members of staff were collected at different days, stored at 37°C and directly analysed within 4 hours. 20 ml of the urines were placed in a thermostated (37°C) crystallization
chamber with continuous magnetic stirring (1000 rpm). In this crystallization chamber, the ionic calcium concentration (Ca\(^{2+}\)) was measured every 60 seconds. The main problems in determining Ca\(^{2+}\) in whole urine are the frequent obstruction of the ion selective membrane and the frequent need of calibration [19]. These two problems are avoided by using a commercially available calcium analyser (AVL List GmbH 8020 Graz, Austria) which automatically cleans and calibrates the electrode after each measurement. Since, for each analysis, the sample has to be brought to an aspiration needle which is rotated out in front of the instrument, we have constructed a special sampler. In short, this sampler consists of a sliding carriage which, for each measurement, transports the crystallization chamber from its vertical position to the outwardly rotated aspiration needle of the analyser. Furthermore, we have developed a special software to directly and to coordinate the sampler and the calcium analyser. All results of calcium measurements were transmitted, together with the corresponding time (t) of the Ca\(^{2+}\) analysis, to a PC and stored for further mathematical evaluation. After measuring the initial Ca\(^{2+}\) (Ca\(_i\)), crystallization was induced by the addition of 100-400 µl of a 100 mmol/l sodium oxalate solution. The curves of crystallization were directly followed on a monitor showing a 100 mmol/l sodium oxalate solution. The main problems in determining Ca\(^{2+}\) in whole urine are the frequent obstruction of the ion selective membrane and the frequent need of calibration [19]. These two problems are avoided by using a commercially available calcium analyser (AVL List GmbH 8020 Graz, Austria) which automatically cleans and calibrates the electrode after each measurement. Since, for each analysis, the sample has to be brought to an aspiration needle which is rotated out in front of the instrument, we have constructed a special sampler. In short, this sampler consists of a sliding carriage which, for each measurement, transports the crystallization chamber from its vertical position to the outwardly rotated aspiration needle of the analyser. Furthermore, we have developed a special software to directly and to coordinate the sampler and the calcium analyser. All results of calcium measurements were transmitted, together with the corresponding time (t) of the Ca\(^{2+}\) analysis, to a PC and stored for further mathematical evaluation. After measuring the initial Ca\(^{2+}\) (Ca\(_i\)), crystallization was induced by the addition of 100-400 µl of a 100 mmol/l sodium oxalate solution. The curves of crystallization were directly followed on a monitor showing a plot of Ca\(^{2+}\) vs t (Figs. 1a, 2a and 6). Further details of the method are given in a previous paper describing the growth of calcium oxalate monohydrate in freshly voided urine [19]. In this former study, crystal growth followed eq. (1):

\[
\Delta C a^{2+} = \Delta C a o x^{3+} \times t \times (t + h)^{-1} \tag{1}
\]

where \(\Delta C a^{2+} = C a_{i}^{2+} - C a_{0}^{2+}\) is Ca\(^{2+}\) decrease at time t; \(\Delta C a o x^{3+} = C a^{2+}\) decrease at infinite observation time; \(h = \) half time of \(\Delta C a o x^{3+}\). Data fitting eq. (1) can be linearized by a plot of \(t/\Delta C a^{2+}\) vs t as shown in Figure 1b. From this line, \(\Delta C a o x^{3+}\) and \(h\) can be calculated, the slope of the curve representing \(1/\Delta C a o x^{3+}\) and the intercept \(h/\Delta C a o x^{3+}\). \(\Delta C a o x^{3+}\) and \(h\) are automatically calculated by the software of our test system.

At the end of the crystallization experiments crystals were removed by centrifugation (3000 rpm) and filtration (0.45 µm) and urine (15 ml) was re-titrated to Ca\(^{2+}\) by the addition of calcium chloride (12.5 mmol/l). After each addition of 120 µl of the CaCl\(_2\) solution, Ca\(^{2+}\) was measured and plotted vs the concentration of exogenous calcium in urine (Ca\(_{ex}\)). An example of a titration curve is shown in Figure 3.

All data obtained from the crystallization experiments were treated by the rearranged eq. (1) shown in Figure 1b and analysed by linear regression. Linear regression analyses were also performed for plots of Ca\(^{2+}\) vs Ca\(_{ex}\), of \(\Delta C a o x^{3+}\) vs the concentration of exogenous oxalate in urine (Ox\(_{ex}\)) and of \(h/\Delta C a o x^{3+}\).

**Results**

**The validity of equation (1) to describe oxalate induced crystallization in native urine**

In experiments performed in urines with Ca\(^{2+}\) above 0.8 mmol/l and with Ox\(_{ex}\) of at least 0.75 mmol/l, crystallization started without a detectable induction time (Fig. 1a) and excellent linearity (Fig. 1b). In urines with low Ca\(^{2+}\) and oxalate loads, a time lag of several minutes until crystallization started (Fig. 2a) and poor linearity (Fig. 2b) were observed. However, in these cases, increasing Ox\(_{ex}\) to 2.0 mmol/l produced «ideal» crystallization curves, as shown in Figures 1 and 6 and fitting eq. (1).

**Reproducibility of oxalate induced crystallization**

Repeated experiments with the same urine in the same urine (Figs. 1a and 1b) under the above mentioned “ideal” conditions (without measurable induction time) revealed a coefficient of variation of 2-4% with respect to \(\Delta C a o x^{3+}\) and of 8-10% with respect to \(h\).

**Calcium chelation**

The plot of Ca\(^{2+}\) vs added calcium (Ca\(_{ex}\)) obtained by re-titrating the Ca\(^{2+}\) lost through crystallization showed a strong linear regression. This was also valid for the titration of native urine starting from Ca\(^{2+}\) (Fig. 3). Eq. (2a,b) describing this regression is given below and in Figure 3.

\[
\begin{align*}
\Delta C a^{2+} &= C a^{2+} + (c \times C a_{ex}) \tag{2a} \\
\Delta C a^{2+} &= C a^{2+} + (c \times C a_{ex}) \tag{2b}
\end{align*}
\]

The slope of the regression line represents a chelation coefficient (c), which, dividing \(\Delta C a^{2+}\) by c, allows extrapolation of total calcium decrease (\(\Delta C a\)) from \(\Delta C a^{2+}\).

**The influence of Ox\(_{ex}\) on \(\Delta C a o x^{3+}\)**

Repeated experiments with the same urine but varying oxalate loads showed a straight linear regression of \(\Delta C a o x^{3+}\) and Ox\(_{ex}\). The corresponding equation is given below and in Figure 4.

\[
\Delta C a o x^{3+} = s^{3+} + (r \times Ox_{ex}) \tag{3}
\]

The slope of the regression line (r) represents the ratio of Ca\(^{2+}\) and Ox contribution to the crystallization process. In
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order to get ratios of total calcium and Ox, r has to be divided by c. r/c values of all experiments ranged between 1.0-1.1 but in most cases an r/c of 1.0 was found. These results show that $\Delta Ca^{2+}_{\infty}$ is strongly dependent on the oxalate load and that both values can be calculated from each other by eq. (3).

The correlation of $h^{-1}$ and $\Delta Ca^{2+}_{\infty}$ at different Ox ex

Plotting $h^{-1}$ vs $\Delta Ca^{2+}_{\infty}$ obtained from experiments performed with the same urine but different oxalate loads revealed another linear relationship. An example, together with the corresponding eq. (4) is given below and in Figure 5.

$$h^{-1} = g(\Delta Ca^{2+}_{\infty} - m^{2+}) \quad (4)$$

The correlation of $h^{-1}$ and $\Delta Ca^{2+}_{\infty}$ is characterized by the slope (g) and the intercept with the X-axis ($m^{2+}$). The special meaning of these two parameters will be extensively discussed. The results show that h depends on $\Delta Ca^{2+}_{\infty}$ which has been shown (in eq. (3)) to be a function of Ox ex, the latter being the only variable in our test conditions. g and $m^{2+}$ seem to characterize oxalate induced crystallization independent of $\Delta Ca^{2+}_{\infty}$ or Ox ex.

The validity of g and $m^{2+}$ to describe crystallization experiments independent from Ox ex

In experiments performed with the same urine but with different Ox ex, g and $m^{2+}$ were calculated by eq. (4) given in Figure 5. h in eq. (1) was then substituted by $1/g \times (\Delta Ca^{2+}_{\infty} - m^{2+})$ from eq. (4):

$$\Delta Ca^{2+}_{t} = \Delta Ca^{2+}_{\infty} - \frac{1}{g}(1+g(\Delta Ca^{2+}_{\infty} - m^{2+})) \quad (5)$$

Subtracting $\Delta Ca^{2+}_{t}$ from $Ca^{2+}_{i}$, $Ca^{2+}_{t}$ values were calculated for different times and compared to the experimentally found values. As demonstrated in Figure 6, generally a sufficient fit was found.

Discussion

Our preliminary results confirm that eq. (1) which was originally obtained from seeded crystal growth can also

Figure 1. Reproducibility of oxalate induced crystallization (fourfold repeated experiments in the same urine with a 1 mmol/l oxalate load): (a) Crystallization curve (Ca$^{2+}$ vs t) as continuously monitored by our automated test system; (b) Replotted data from Figure 1a used to extract $\Delta Ca^{2+}_{\infty}$ and h.

Figure 2. Oxalate induced crystallization in urine with low Ca$^{2+}$ (0.58 mmol/l) and Ox ex of 1.0 and 1.5 mmol/l: (a) crystallization curve (Ca$^{2+}$ vs time); (b) replotted data ($t/\Delta Ca^{2+}_{t}$ vs t).
be applied to describe oxalate induced crystallization without seeds, provided that crystallization starts without a detectable induction time. Equation (1) is a modification of the equation of Will et al. [21], in which fractional uptake \( U \) of radioactive calcium into crystals was replaced by \( \Delta Ca^{2+} \) [19]. After division of eq. (1) by \( c \), eq. (6) describes the depletion of urine with respect to total Ca or Ca uptake to crystals respectively, at the observation time \( t \):

\[
\Delta Ca_t = \Delta Ca^{\infty} \times t \times (t + h)^{-1}
\]

The chelation coefficient \( c \) which was found to be valid in all ranges of \( \Delta Ca^{2+} \) of our experiments is a simple estimate of calcium chelation. Otherwise, calcium chelation has to be determined by chemical analyses of several urinary compounds and by special computer calculations [8]. The strong linear regression of \( \Delta Ca^{\infty} \) and \( Ox_{\text{ex}} \) found in all experiments showed that oxalate induced crystallization almost exclusively produced calcium oxalate with an equimolar contribution of calcium and oxalate \( (r/c = 1.0) \). Values above 1.0, which were rarely observed might be attributed to a coprecipitation of calcium phosphate. The correlation of \( \Delta Ca^{\infty} \) and \( Ox_{\text{ex}} \) allows, together with \( c \), to calculate the oxalate load being necessary to produce any desired \( \Delta Ca^{\infty} \) or crystal mass respectively. This might be important for inhibitor research, because the crystal mass being present in a test system essentially can influence results of inhibitor measurements [5]. \( s^{2+} \) in eq. (3) of Figure 4 probably reflects the state of initial supersaturation of urine before performing the oxalate load. However, this interpretation has to be confirmed studying the correlation of \( s^{2+} \) and the relative urinary supersaturation calculated by Equil II [8].

By the differentiation of eq. (6) with respect to time, eq. (7) is obtained, where \( d\Delta Ca/dt \) represents the growth of calcium oxalate in urine.
rate of crystals at time $t$, a term often used in stone research [12, 18, 21]:

$$\frac{d\Delta Ca}{dt} = (h \times \Delta Ca^{\infty})^{-1} \times (\Delta Ca^{\infty} - \Delta Ca)^3 \quad (7)$$

As already shown, $h^{-1}$ which depends from $\Delta Ca^{\infty}$ or OX$_{es}$ respectively, can be replaced by $g \times (\Delta Ca^{\infty} - m^2)$ of eq. (4) or $g \times c \times (\Delta Ca^{\infty} - m)$:

$$\frac{d\Delta Ca}{dt} = g \times c \times (\Delta Ca^{\infty} - m) / \Delta Ca^{\infty} \times (\Delta Ca^{\infty} - \Delta Ca)^3 \quad (8)$$

Beginning from the right, the individual terms of this new equation (8) may be interpreted as follows: $(\Delta Ca^{\infty} - \Delta Ca)$ represents the amount of calcium which at time $t$ is in solution and which by further crystallization can be deposited until urine reaches the state of saturation. Therefore, this term is a measure for urinary supersaturation at time $t$. $(\Delta Ca^{\infty} - \Delta Ca)^3$ confirms that calcium oxalate crystallization under the above mentioned conditions is still a second order reaction as found for the growth of calcium oxalate in artificial solutions [12, 18, 21]. $(\Delta Ca^{\infty} - m)/\Delta Ca^{\infty}$ seems to represent an index for metastability. Figure 5 shows, that below a critical $\Delta Ca^{\infty}$ denoted by $m^2$ no crystallization will be measurable ($h = 0, h = \infty$). It is well known that for a detectable nucleation, supersaturation has always to exceed a critical value, the metastable limit. Supersaturations above this limit are called labile. Thus, the index of labile supersaturation seems to indicate the potential of an urine after the oxalate load to produce nidus for crystal growth. $c$, as already mentioned, is a coefficient for calcium chelation. Finally $g (1 \times \text{mmol}^3 \times \text{min}^{-1})$, is a parameter characterizing oxalate induced crystallization independent of supersaturation, metastability and calcium chelation. Equation (8) represents a second order reaction of crystal growth with the important prerequisite that all crystal nidus have been formed at $t = 0$.

Apart from the limit of metastability, nucleation can be characterized by induction time (the time between the start of an experiment and the first observation of crystallization) and by nucleation rate. Many years ago, we tried to measure induction time as well as the limit of metastability by the infusion of sodium oxalate into whole urine and by nephelometry monitoring the start of crystallization [2]. However, experiments performed with variable infusion rates showed that metastability increased with increasing infusion rate or decreasing incubation time respectively [5]. Only at very high infusion rates or short induction times respectively, the influence of incubation time on the limit of metastability was minimal. Further approaches are to incubate series of urines with increasing supersaturations and to look at which supersaturation crystallization has occurred after a given incubation time [13] or to determine the induction time at constant supersaturation [10]. In our experience these methods were not ideal for clinical use and gave only limited information on crystal growth. Nucleation rate can exactly be determined by a continuous crystallizer method recently adapted for whole urine [11]. In this test system, concentrated solutions of calcium chloride and sodium oxalate are infused together with centrifuged or filtered whole urine in a crystallization chamber with an overflow. In the solution obtained from the overflow, the number and size of crystals are determined with a Coulter Counter. After a delay of about 30 minutes a steady state with respect to number/size distribution of the crystals is reached allowing to calculate nucleation rate (number per ml and minute) as well as growth rate (micrometer per minute). However, both parameters are dependent on the supersaturation present in the test system which, on the other hand, is a function of transit time or perfusion rate, respectively [15].

In our new approach, we tried to characterize nucleation and growth by the two parameters $g$ and $m$ independently from the supersaturation given by the oxalate load. At the high initial supersaturation generally used in our experiments nucleation was so rapid, that no induction time could be observed and crystallization after the first calcium decrease followed the second order reaction known from crystal growth in artificial solutions [12, 18, 21]. Therefore, $g$ seems mainly to represent a growth rate constant, whereas nucleation properties of urine almost exclusively seem to be characterized by $m$. $m$ is obtained by dividing $m^2$ by $c$. The supersaturations applied in our test system were certainly beyond normal physiological limits. However, other methods studying the formation of calcium oxalate have also to work with initial oxalate concentrations of 1.5 to 2.4 mmol/l to get measurable crystallization [7, 11, 16]. Also, the crystal concentrations $(\Delta Ca^{\infty} / c)$ produced by oxalate induced crystallization were with 0.5 to 1.5 mmol/l in the range of seed concentrations used in other test systems [12, 18, 21]. $g$ and $m^2$ together with Ca$^{2+}$ (given by the individual urine) and $\Delta Ca^{\infty}$ (given by the oxalate load) exactly described oxalate induced crystallization, provided that crystallization started without a detectable induction time. Continuous monitoring of crystallization by a plot of Ca$^{2+}$ vs $t$ as shown in Figures 1a, 2a, and 6 allows the correction of OX$_{es}$ after only a few calcium measurements in order to achieve the “ideal” test conditions. In our experience with various test systems [3, 4, 6, 19], the ion selective calcium electrode has turned out to be the most sensitive instrument to detect and to monitor crystallization. The electrode works independently of natural urinary impurities (e.g., mucus, cells, crystals), which in other test
systems have to be removed by centrifugation and filtration before performing the experiments. Therefore, our tests can directly be performed in freshly voided urine. The disadvantage of the method is that no information can be gained on crystal agglomeration, a process being thought to be most important for stone formation.

Conclusions

Inducing calcium oxalate crystallization by an adequate oxalate load and monitoring crystallization by an ion selective calcium electrode has turned out to be a simple, rapid and reproducible method to study the formation of calcium oxalate in freshly voided urine. Determining a limit of metastability (m) and a growth rate constant (g) allowed characterization of crystallization properties of urine independently of supersaturation, which was the only variable in our test conditions. For inhibitor research, even this variable can be standardized by repeating the experiment with a precalculated oxalate load producing a given crystal mass. Urinary centrifugation and filtration, which removes some macromolecules that may be important for crystallization processes and stone formation, are not needed in this method, which is a further advantage for research on inhibitors and promoters of crystallization.

References


Discussion with Reviewers

H.-G. Tiselius: The measurements of Ca\(^{2+}\) following the low oxalate load (1 mmol/l) in Figure 2a reflects a situation where the supersaturation apparently is insufficient to cause a CaOx nucleation. How would you explain the great variability in $\Delta$Ca\(^{2+}\) obvious from Figure 2b?

Authors: Figure 2b is a plot of $t/\Delta$Ca\(^{2+}\) versus time, where, for mathematical reasons, a minimal $\Delta$Ca\(^{2+}\) (during first 7 minutes in the order of 0.01 mmol/l) has a maximal influence on the curve.

H.-G. Tiselius: The nucleation is reflected in the limit of metastability. Regarding the high supersaturation necessary to induce the crystal formation, what importance do you think that this value can be given from a clinical point of view?

Authors: The limit of metastability is known to increase by the action of inhibitors and to decrease in the presence of promoters of crystallization. In our previous work, we have shown a high metastability too with respect to the growth of crystal seeds in whole urine. The high supersaturation necessary to induce crystal formation may explain the generally low recurrence rate of stone disease and the slow growth of residual fragments after extra shock-wave lithotripsy (ESWL), although urine being almost always supersaturated with respect to calcium oxalate.

W. Achilles: Twenty-four hours (24-h) urine collections are regarded to be most useful as standard samples for the determination of urinary properties also with respect to stone formation. The authors, however, recommend the use of freshly voided, i.e., spontaneously collected urine, which has the disadvantage of large variation of composition compared to a 24-h collection.

Authors: We agree that in metabolic studies, the collection of 24-h urine might compensate for individual variations of urinary composition during the day. However, with respect to crystallization tests, the question remains open whether results obtained of single urine portions or of 24-h urine are more adequate to physiopathology. Problems arising from storing of urine are enzymatic disintegration (e.g., pyrophosphate), polymerization of macromolecules (e.g., THP), spontaneous crystal precipitation (e.g., calcium phosphate) with the possibility of heterogeneous nucleation of calcium oxalate, unknown effects on macromolecules and crystallization conditions by preservatives used against bacterial overgrowth.

W. Achilles: Do you have any independent experimental evidence that your parameters m and g are really related to nucleation and growth of crystals, i.e., have you determined the course of crystal number and crystal size as a function of time, e.g., by Coulter Counter, in parallel to electrometric Ca\(^{2+}\) measurements using the same solutions?

Authors: The Coulter Counter exclusively measures global changes of the particle number (increase by nucleation and decrease by aggregation) and of the crystal size (increase by growth and by aggregation). Therefore, results obtained by a Coulter Counter and by electrometric Ca\(^{2+}\) measurement hardly can be compared. As mentioned in the Discussion, our eqs. (7 and 8) represent a second order reaction known to describe crystal growth of calcium oxalate. Comparing the decrease of Ca\(^{2+}\) in the solution with the increase of optical density (spectrophotometric method of Hess et al. [10]), we found reciprocal curves with comparable h values. Nucleation parameters always have to be extrapolated, because nucleation can only be detected after crystal nidus have grown to a measurable size. Our “limit of metastability” (m\(^2\)) is such an extrapolation.

W. Achilles: Do you have any experimental data on the different effects of untreated, centrifuged or filtered urine samples on the parameters determined by your method?

Authors: The clinical implication of our new method including the study of the influence of urinary pretreatment on test results is under investigation.

W. Achilles: The authors have not mentioned which crystal phase of calcium oxalate (monohydrate (COM), dihydrate (COD) or trihydrate (COT)) was mainly formed in their experiments and whether there was a difference in crystal phase distribution between different urine samples. Please give more detailed information on this.

Authors: Microscopically, COD and COM were found in the precipitates after the oxalate load. Scanning electron microscopy (SEM) and infrared spectrometry have not yet been used.

W. Achilles: Crystal aggregation seems to be a process which is of great importance in urinary stone formation. Could one get any information about this phenomenon using your method?

Authors: As mentioned in the Discussion, unfortunately, the method does not provide any information about crystal aggregation.

A.L. Rodgers: Do you have any evidence (e.g., scanning electron microscopy, X-ray powder diffraction, etc.), to support your contention that calcium phosphate co-precipitates with calcium oxalate in some cases?

Authors: We have not checked the precipitates for traces of calciumphosphate. Our supposition is based on the frequent finding of calcium phosphate crystalluria in freshly voided urine by Hermann et al. (Urol Res (1991) 19: 151-158).
A.L. Rodgers: Suppose that oxalate depletion instead of calcium depletion is measured. Could your mathematical approach be applied? If so, what analogous parameters would emerge?

Authors: Since the formation of calciumoxalate seems to be an equimolar process, we suppose that, substituting $\Delta Ca$ by $\Delta Ox$, eqs. (6, 7 and 8) can also be applied to describe oxalate depletion.

J.P. Kavanagh: I would like to discuss further the physical interpretation of the nucleation parameter appearing in your rate eq. (8). We are well used to the concept of a metastable limit which is often expressed as the oxalate concentration required to initiate heterogeneous nucleation; the lower the metastable limit the more labile is the solution. You have arrived at an analogous parameter ($m^2+$) by extrapolating back from crystallization initiated by oxalate concentrations greater than the metastable limit (Figure 5) and you have gone on to demonstrate that this parameter appears in your rate eq. (8), such that lower metastable limits lead to higher growth rates at any given supersaturation. This second order equation is comparable to those found for seeded calcium oxalate crystallization, in which nucleation is not a factor, but often includes the concentration or surface area of seed crystals as a proportionality factor. Do you think it reasonable to infer that $m^2+$ is inversely related to the concentration/surface area of heterogeneous nucleators, which give rise to the incipient seed crystals?

Authors: The term $(\Delta Ca^{\infty}-m)$ in eq. (8) represents the solid phase in mmol/l which after the oxalate load maximally (until the metastable limit is reached) can be deposited by nucleation. In other words, $(\Delta Ca^{\infty}-m)$ is related to the crystal surface being available for growth at time zero. This growth specific surface is inversely related to $m$. $m$ increases in the presence of inhibitors poising growth sites and it decreases in the presence of crystal seeds furnishing preformed surfaces for further growth.