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MICROPHOTOMETRIC QUANTIFICATION OF CRYSTAL GROWTH IN GELS FOR THE STUDY OF CALCIUM OXALATE UROLITHIASIS

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Abstract

This paper gives a review on an efficient microtechnique (Gel Crystallization Method; GCM) which quantifies crystal growth in gel matrices, and its application to research into urinary stone formation. Relative crystal growth rates (Vcr) of calcium oxalate hydrates (CaOx) were determined by simultaneous multiple measurements in 96-well microplates using computer controlled microscopic photometry. Efficiency: 120 kinetic measurements/h; imprecision: <2% at standard conditions. The method was applied to study the effects of diverse small-ionic and macromolecular constituents of human urine on the crystal growth rate of CaOx. The 'inhibitory activity' of urine could be evaluated by comparison of the Vcr measured in native and corresponding artificial samples. Macromolecules, at physiological concentration, played only a minor role as inhibitors in the model under regard. The GCM was employed to study the in-vivo effects of different therapeutic measures on CaOx growth in urine of normal volunteers. While the application of alkali citrate resulted in a >70% decline of Vcr, no beneficial effects could be found with three magnesium compounds, and a dietary fiber preparation. In a study including 20 male recurrent CaOx stone formers and 29 well-matched controls, among all parameters under investigation, the Vcr showed the most significant difference ($p<0.001$) between the both groups. In conclusion, the method described here has been proven to be of high value with respect to a series of applications in the study of urolithiasis. However, though the crystal growth rate of CaOx is of undoubted importance in stone genesis, other phenomena, like crystal agglomeration and adhesion on surfaces, must yet be taken into account as causative factors.

Key words:
Calcium oxalate urolithiasis, Crystal growth, Crystals, Gel matrices, Human urine, Hydrated calcium oxalate, Inhibition, Microscopic photometry, Microtechnique

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Introduction

Conventional determinations of growth rates of crystal phases relevant in urinary stone formation usually are carried out in aqueous solutions or crystal suspensions (Fleisch, 1985; Baumann, 1988). However, urinary stones form via the growth of crystals fixed in the urinary tract (Finlayson, 1982). Corresponding to the results of Iwata et al. (1985, 1986, 1989), this growth occurs in a gel-like state. The gel is formed by a mucinous macromolecular layer, which encrusts on the surface of the calculi forming the well-known organic stone matrix. From this point of view, it seems to be reasonable to study the growth of crystals relevant in urolithiasis in gel matrices rather than in solutions or suspensions, respectively. Moreover, conventional methods for the determination of crystal growth rates are time-consuming and, as a rule, require large test volumes. Often, urine has to be highly diluted in order to test the so-called inhibitory activity because a direct application of the methods to undiluted urine is not possible (Fleisch, 1985; Baumann, 1988).

Taking into account these aspects of crystal growth experiments in research into urinary stone formation, we have developed a photometric micromethod (Gel Crystallization Method; GCM) for the efficient determination of relative crystal growth rates in gel (Achilles, 1984,1985,1989). It has been aimed to overcome the disadvantages referred to above or to complement the methods hitherto practiced .

This paper reviews the experimental design of the technique and its principal applications to the research of urinary stone formation with respect to calcium oxalate (CaOx) hydrates.

Methods and Materials

Description of the Method

From the two components of a precipitating solid phase (e.g. calcium oxalate), one is dissolved as a soluble compound (e.g. sodium oxalate) in a sol. By addition of a small amount of the counter-ion (e.g. as calcium chloride), sufficient supersaturation is gained in order to generate seed crystals. Subsequently, the sol is pipetted into the cavities of a 96-well microplate, thus forming a horizontal gel matrix with seed crystals. Crystal growth, which occurs

by one-dimensional double diffusion in the gel matrix, is started by pipetting a suitable solution containing the counter ion (i.e. calcium in artificial or natural urine) onto the gel phase. Subsequently, growing crystals are followed by vertical light path photometry as a function of time. The dark field of the microscope producing scattered light has been proven to be the most useful mode of measurement. High efficiency and precision in measurements is achieved by: (1) a strictly standardized procedure of gel preparation; (2) multiple sample handling of small volumes; (3) fast sequential photometry in 48 (-96) positions of the microplate, repeating the measuring cycle in prescribed intervals up to 15-30 times, thus collecting time-dependent measuring data; (4) the application of a computer-controlled microphotometer, and (5) current reference to a kinetic standard taking into account blank values. The principle of the method is demonstrated in Figure 1.

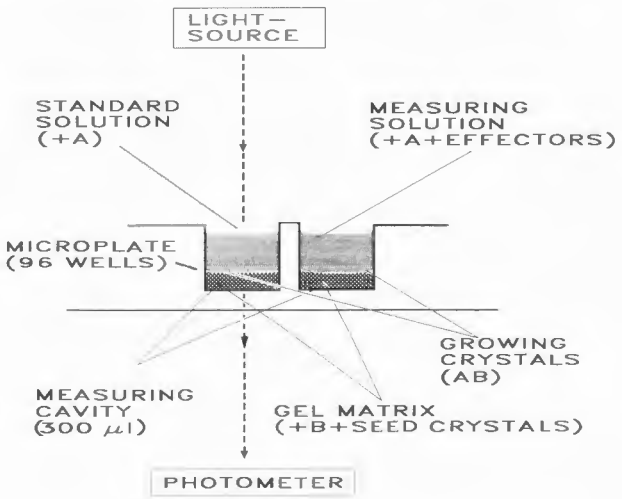

Figure 1: Principle of the optical micromethod for the determination of relative crystal growth rates in gel matrices (Gel Crystallization Method; GCM).

Measuring Device

An automated microphotometric system for transmitted light was used comprising the following parts: a) inverted microscope IM35 equipped with power-stabilized halogen light source HB100, rapid (50 µm step) scanning stage adapted to 96-well microplates, and photometer SFD; b) electronic control unit MPC 64; c) camera Contax RTS and video camera LDH 2107 (Philips) for the observation and evaluation of crystal images within the gel; and d) on-line microcomputer HP 9816S for photometer control, aquisition, processing and display of data. Computer programs were written in HP-BASIC 5.0. The equipment is shown in Figure 2.

Vcr, the parameter of relative crystal growth, is defined as the ratio of the maximum slope of any measuring curve to the maximum slope of the current standard curve. In case of quasi-linearly shaped curves, slopes may be calculated by linear regression analysis. In other cases, like hyperbolic or sigmoidal shapes, a five-parametric nonlinear

Figure 2: Computer controlled microphotometric system for multiple determinations of crystal growth rates in gel matrices (Gel Crystallization Method; GCM) comprising the following parts: Inverted microscope IM 35 with photometer and scanning stage adapted to microplates, stabilized power supply, electronic control unit, video camera (Carl Zeiss, W.-Germany) and computer HP 9816 S.

fit corresponding to Equation 1 could be successfully applied.

$$I(t) = K_1 + (K_2-K_1)*1/((t_e-K_3)/(t-K_3))^{K4} + K_5^{K4} \quad (1)$$

where: I(t)...measuring light intensity; t...time of actual measurement; t_e...time of final measurement; K_n...coefficients to be calculated by regression analysis (Schalk and Achilles, unpublished results).

Materials

For the determination of crystal growth rates of CaOx, the gel phase consisted of 0.5% (w/w) agar-agar, 2 mM sodium oxalate, 1 mM sodium azide, 0.5% (w/w) glycerol, and about 0.05 mM $CaCl_2$. It was prepared corresponding to the following prescription: 250 mg of dry gel substance (Ca-free agar-agar, preferably "high gel strength" or "for electrophoresis", SERVA, Heidelberg, Germany), 250 mg glycerol, 0.05 mmol sodium azide, and 0.1 mmol sodium oxalate were completely dissolved at 98°C in water (final volume: 50 ml). To produce seed crystals, the solution was cooled down to 60°C and a bolus of 0.5 ml 5 mM $CaCl_2$ was added during stirring the solution on a magnetic stirrer (1500 rpm). Subsequently, portions of 0.1 ml/cavity of the hot sol were quickly pipetted (diluter Tecan 450) into the wells of prewarmed microtiter plates (polystyrene; flat bottom) and cooled off to room temperature. Plates were stored in moist chambers and could be used up to 6 weeks.

A solution of the following composition was used as current measuring standard (total concentrations in parentheses given in mM): Na(131), K(40), NH_4(25), Ca(4.0), Mg(3.0), phosphate(20), sulfate(15), citrate(2.0), chloride(149), urea(250), pH 6.0. This was also employed

as a reference solution ('artificial urine') in a series of experiments to measure effects on crystal growth. Though its composition does not correspond to the mean values of 24-h collections of a well-defined population of volunteers, concentrations are in the physiological or pathophysiological range. The quantity of its crystal growth parameter Vcr (=1) reflects conditions relevant to urine of CaOx stone formers. As a rule, substances to be tested with respect to their effects on Vcr(CaOx) had been dissolved in this solution. Principally, measurements in undiluted native urine were performed after thawing of frozen samples and centrifugation (Achilles and Ulshöfer, 1986). All measurements were carried out at controlled room temperature (23±1 °C).

Statistical Evaluation

Statistical results mentioned in this paper predominantly refer to the significances of differences of parameters. Principally, they were calculated as follows. Distributions of paired or unpaired data were evaluated using normality tests. Corresponding to the distributions assessed (normal, symmetrical, others) appropriate tests of significance were chosen (paired t-test, Wilcoxon rank test, or sign-test for paired data, and expanded t-test, Mann-Whitney test or median test for unpaired data).

Results

Methodological Results

The empirical parameter Vcr determined by this method reflects the thermodynamic as well as kinetic effects of the constituents of a solution on crystal growth within the gel matrix. Provided that a certain limit of supersaturation is not exceeded, the seeded gel matrix ensures the measurement of pure crystal growth. Simultaneous nucleation may be unambigeously excluded if crystallization experiments are carried out within the limits of metastability of the system under regard. Metastability limits may be estimated by precipitation experiments in an unseeded gel using corresponding supersaturations (Burk and Achilles, unpublished findings).

Seed crystals of CaOx were produced by pre-precipitation in the sol state in order to provide a regular distribution of crystalline particles within the gel matrix. Predominantly, they consisted of monoclinic, coffin-shaped calcium oxalate monohydrate and some of them grew up to a maximal size of 5 µm. From optical microscopy, minor formation of calcium oxalate dihydrate could not be excluded. The major part of seeds had a submicroscopic size, which could be derived from the number of countable crystals before and after precipitation from Ca-containing solutions or urine. Figure 3 shows a series of typical images of growing CaOx crystals as observed with the dark-field mode of microphotometric measurement (scattered light).

Apart from the thermodynamic and kinetic effects of solutes referred to above, the absolute slopes of crystal growth curves depend on a number of conditions, e.g., diffusion coefficients, temperature, number and size of seed crystals. Therefore, a strictly standardized procedure is necessary in order to prepare the gel matrix. In particular, this refers to the critical generation of seed crystals.

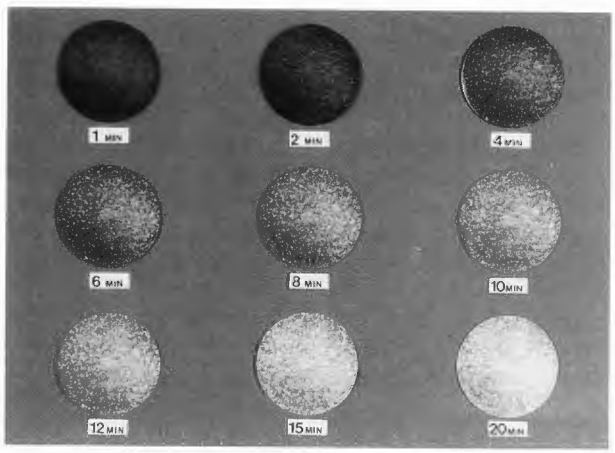

Figure 3: Scattered light image (x8) of CaOx crystals growing in gel of a single cavity of a microtiter plate at different times of reaction (standard conditions; dark field mode of measurement).

However, despite some differences of slopes of measuring curves between series, slopes referred to that of a defined standard gave highly reproducible results. The imprecision (coefficient of variation within a series) of the crystal growth parameter Vcr at standard conditions is <3% for single determinations, and <2% for mean values from four-fold determinations as they were usually carried out. As an example, Figure 4 demonstrates 44 original crystal growth curves of CaOx and four water blanks resulting from simultaneous multiple measurement with the standard solution mentioned before. Intensity of scattered light (as arbitrary units), arising from the dark field mode of measurement, is plotted as a function of measuring time. An additional example of measurements is shown in Figure 5. The crystal growth curves plotted here correspond to a simultaneous multiple measurement in 48 positions of a microplate using artificial urines with varying total calcium.

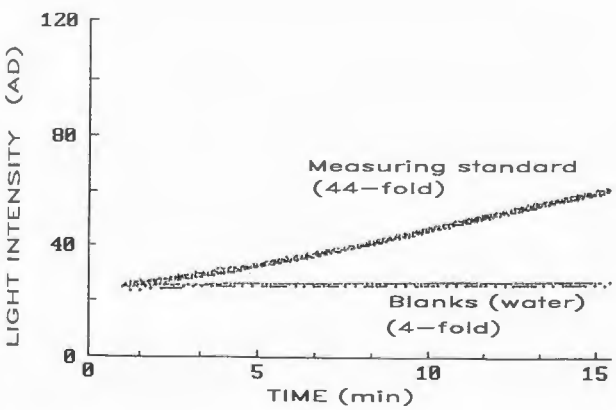

Figure 4: GCM. Typical graphical protocol of 44 simultaneously registered crystal growth curves of CaOx from the same standard solution and 4 blank values (water) as used to control the measuring quality of gel plates.

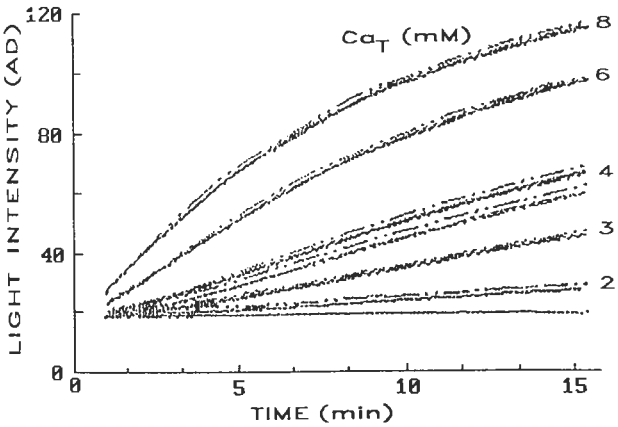

Figure 5: GCM. Typical curves of crystal growth of CaOx (AD-units vs. measuring time) as obtained from artificial urine samples: 4-fold determinations in solutions with different total calcium concentration (Ca_T: 2-8 mM). Gelphase: 0.5% agar-agar; 2 mM Na oxalate; 0.05 mM $CaCl_2$. Mode of measurement: dark field.

The efficiency of the method is high. Applying the actual procedure, 40 crystal growth curves may be registered simultaneously by 15 cycles of measurements, corresponding to at least 120 kinetic determinations per hour. As a rule, a gel volume of 100 µl and a measuring volume of 200 µl of solution were used per well of a microplate. For these reasons, the GCM is suitable for large-scale measurements, and may be applied clinically (Achilles and Ulshöfer, 1986; Achilles, 1989).

In some parallel experiments, we tested the influence of different gel matrices (agar-agar; various sorts of agarose, 0.3-1.0 g/100ml; and gelatine 5g/100 ml) on Vcr using artificial and native urine samples. Relative crystal growth rates referring to the same solutions but different gels agreed quite well ($r>0.98$) (Achilles and Ulshöfer, 1986).

Apart from the CaOx system dealt with here, the GCM could also be applied to the quantification of growth of other crystal phases. Detailed results on crystallization experiments in gels with octacalcium phosphate and barium sulfate will be published elsewhere.

Effects of Calcium, Magnesium, Citrate and pH

Urinary calcium, magnesium, citrate, and hydrogen ion concentrations are known to affect the crystal growth of CaOx in different ways. Optimal alterations of the output of these constituents in urine are aimed by several prophylactic measures in order to prevent recurrent stone formation. However, their interrelationship with respect to the net effect on the crystal growth rate of CaOx has not been investigated yet.

Starting from the standard solution referred to above, we studied the crystal growth rate, Vcr, as a function of total concentrations of calcium (Ca_T), magnesium (Mg_T), citrate (Cit_T), in addition to pH. In a first set of experiments, each parameter was varied in the solution whereas all others were kept constant at their normal values (Achilles and Ulshöfer, 1985 b). The corresponding results are summarized in Figure 6.

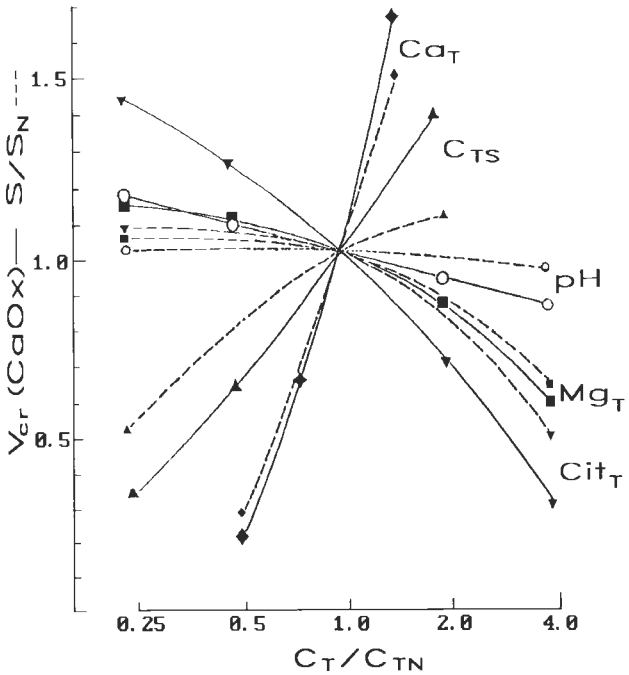

Figure 6: Relative crystal growth rate of CaOx (Vcr(CaOx): ——) and corresponding calculated, normalized supersaturation within the gel phase (S/S_N:---) as a function of different parameters in artificial urine as indicated in the figure. C_T/C_N: normalized, total concentrations of Ca, Mg, and citrate. pH-values at the 5 marked points (from left to right): 5.4, 5.7, 6.0, 6.3 and 6.6. Each variation of a single parameter was carried out keeping all the others at constant values. The curve marked by C_{TS} refers to the simultaneous variation of all constituents of the artificial urine at constant pH (simulation of dilution).

Here, with the exception of pH, independent parameters are expressed in terms of normalized concentrations (C_T/C_N), which seems to be reasonable especially from a physiological point of view. C_N refers to the total concentration of the standard solution of mean (normal) composition as described above. In order to compare the crystal growth rate with the thermodynamic driving force of the process under regard, corresponding normalized supersaturations within the gel phase (S/S_N) were estimated by computer calculation of complex chemical equilibria (Achilles and Ulshöfer, 1985a) corresponding to the following definition (Equation 2):

$$S/S_N = \lg(AP/K_{sp}) / \lg(AP_N/K_{sp}), \qquad (2)$$

where S=supersaturation at actual conditions; S_N=supersaturation at normal conditions, i.e., at concentrations of the standard solution; AP=activity product at actual conditions; AP_N=activity product at normal conditions; K_{sp}=solubility product of CaOx within the gel phase. S/S_N is shown along with Vcr on the ordinate using the same scale (non-dimensional quantities).

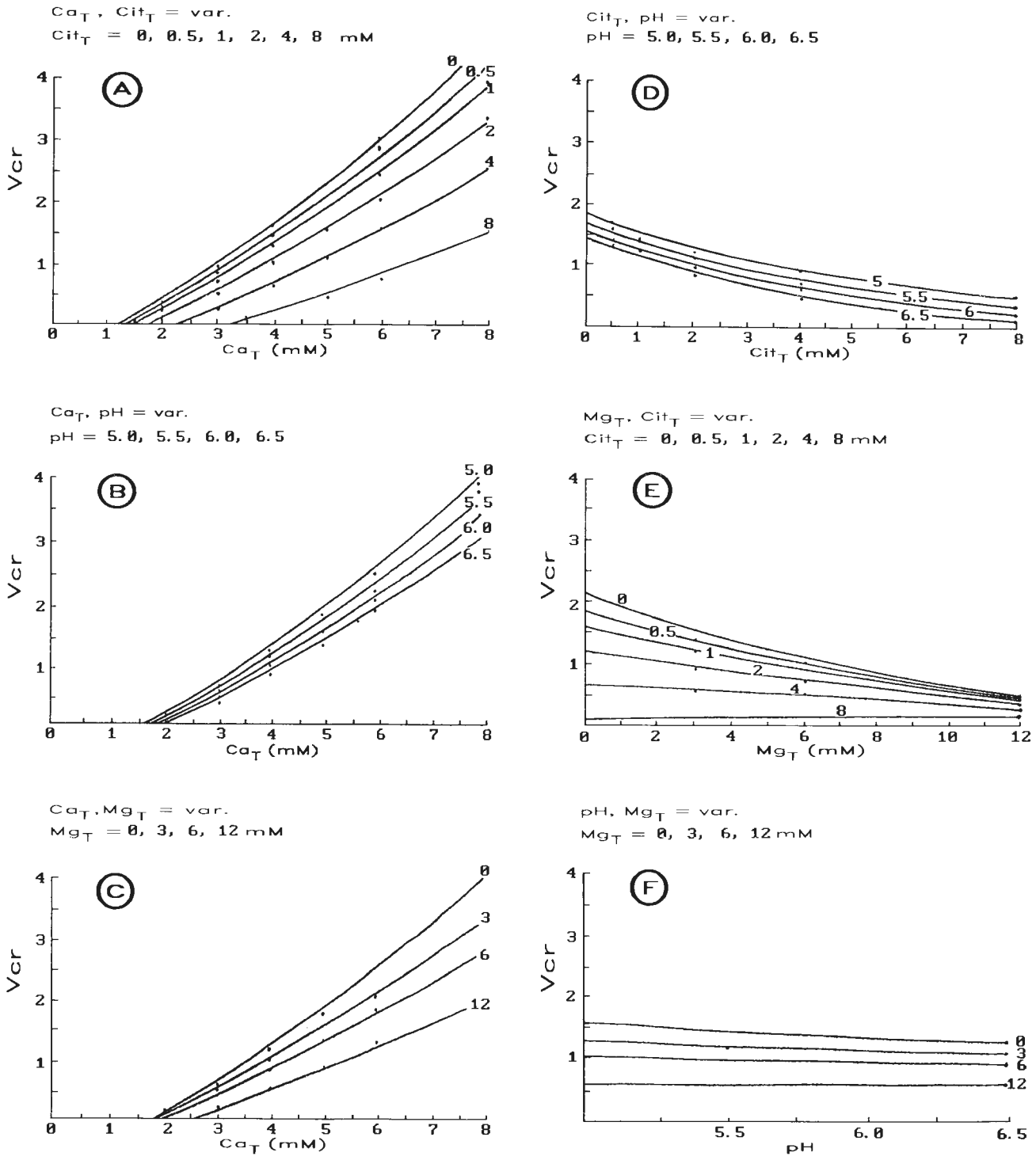

Figure 7: Effects of pH and total concentrations of calcium (Ca_T), magnesium (Mg_T), and citrate (Cit_T) on the relative crystal growth rate of CaOx (Vcr). Variations of parameters were carried out in artificial urine which was basically composed as outlined in 'Materials'. Normal values of the parameters under regard were: Ca_T=4 mM, Mg_T=3 mM, Cit_T=2 mM, pH=6, Vcr=1.0. Corresponding variations are indicated in Figures (a)-(f) as 'var.'. Each parameter was varied while keeping all others at their normal or indicated values. The curves shown in each figure were calculated by non-linear regression analysis from all experimental values corresponding to Equation 3.

Continuing this study, the dependence of Vcr on its effectors Ca, Mg, Cit, and pH was further determined by their extended variations. The results are summarized graphically in Figure 7(a-f). In contrast to the illustration in Figure 6, absolute quantities of the independent variables were used instead of normalized ones (Achilles et al.,1989b, and unpublished findings).

From these data, an empirical mathematical relationship between Vcr and the effectors under consideration could be derived using a model of non-linear regression analysis (Equation 3) (Achilles et al., 1989b, 1990).

$$Vcr = A/B \qquad (3)$$

with $A = 1 + P_{101}[(Ca_T/Ca_N)-1] + P_{201}[(Cit_T/Cit_N)-1] + P_{301}[(pH/pH_N)-1] + P_{401}[(Mg_T/Mg_N)-1] + P_{231}[(Cit_T/Cit_N)-1]\,[(pH/pH_N)-1] + P_{241}[(Cit_T/Cit_N)-1]\,[(Mg_T/Mg_N)-1] + P_{441}[(Mg_T/Mg_N)-1]^2$

and $B = 1 + P_{102}[(Ca_T/Ca_N)-1] + P_{202}[(Cit_T/Cit_N)-1] + P_{302}[(pH/pH_N)-1] + P_{402}[(Mg_T/Mg_N)-1] + P_{232}[(Cit_T/Cit_N)-1][(pH/pH_N)-1] + P_{242}[(Cit_T/Cit_N)-1][(Mg_T/Mg_N)-1],$

where

P_{101}= 1.780; P_{201}=-0.247; P_{301}=-0.655; P_{401}=-0.041; P_{231}=-0.543; P_{241}= 0.041; P_{441}=-0.021

P_{102}=-0.125; P_{202}= 0.201; P_{302}= 0.792; P_{402}= 0.151; P_{232}= 0.123; P_{242}=-0.036.

Ca_N = 4 mM; Cit_N = 2 mM, pH_N = 6.0, Mg_N = 3 mM.

The symbols used in this equation have the following meaning: P_{xyz}...coefficients mathematically determined; pH...actual pH; pH_N...normal pH; C_T...actual total concentration; C_N...normal total concentration. Equation 3 could be successfully applied to elucidate the effects of an alkali citrate therapy on the crystal growth rate of CaOx in human urine (Achilles et al., 1990).

The results demonstrated above may be summarized and interpreted as follows (Achilles et al., 1989b): Among all the parameters investigated, calcium exerts the most pronounced effect on Vcr. From the relationship $Vcr=f(Ca_T)$ (Figure 7a-c), i.e. from the section point with the Ca_T-axis (Vcr=0), the apparent solubility product of CaOx referring to the gel phase could be derived ($K_{sp}(CaOx,gel)= 3.3x10^{-8}$ mol^2/l^2). It is about one order of magnitude greater than the K_{sp} determined in corresponding aqueous solutions ($2\text{-}5x10^{-9}$ mol^2/l^2; Tomazic and Nancollas, 1979, 1980). Effectors of crystal growth may decrease the slope of the function $Vcr=f(Ca_T)$ and, simultaneously, shift the section point on the Ca_T-axis (Figures 7a-c). While the first effect could be predominantly accounted for by a pure kinetic action, the latter one should refer to a reduction of crystal mass, which , as a rule, might be explained thermodynamically.

Citrate could be shown to decrease the crystal growth of CaOx effectively. As may be derived from Figures 6 and 7a, the effect is of 'mixed' thermodynamic and kinetic origin, i.e., it is caused by ion-pair formation with Ca and inhibition via adsorption onto the crystal surface.

Magnesium affects the crystal growth by two counteracting mechanisms. It reduces the Vcr by ion-pair formation with oxalate and diminishes the inhibiting effect of citrate (and other anions in urine) by corresponding interactions. However, the net effect depends on the actual concentrations of the relevant urinary constituents. Thus, for example at high urinary citrate, increasing Mg may have no remarkable influence on the crystal growth or may even increase Vcr, as demonstrated in Figure 7e.

In the system under regard, increasing pH causes a decline of the Vcr. Predominantly, this effect must be attributed to an indirect mechanism of deprotonation of other effectors of crystal growth. This will be explained later on in this paper.

Simultaneous alterations of different parameters acting in the same direction reducing crystal growth may be very effective with respect to the prevention of stone recurrence. An example will be shown below.

Effects of Major Urinary Constituents (Achilles et al., unpublished findings)

Sodium, potassium, ammonium, chloride, sulfate, and phosphate mainly affect the ionic strength, and thus activity coefficients, of human urine and have some influence on the complex chemical equilibrium. Via these thermodynamic mechanisms, the crystal growth rate of CaOx may be altered. Moreover, direct or indirect kinetic effects of these components on the crystal surface might be of some importance. In order to investigate these phenomena, variations of the ions referred to above were carried out similarly to those described above. The Vcr was determined as a function of these parameters and compared with corresponding degrees of the initial, normalized supersaturation within the gel phase, S/S_N (see Equation 2).

In a first set of experiments, NaCl, KCl and NH_4Cl respectively were added to a simulated urine containing 50 mM of Na(+) as basic (initial) concentration. Increasing the concentrations of the electrolytes added up to 400 mM, the crystal growth rate dropped nearly linearly. The results are demonstrated in Figure 8. A quite parallel course could be found between Vcr and S/S_N. However, the different effects caused by the different electrolytes could not be accounted for by the thermodynamic model used. This issue needs further investigation.

In a second series of experiments, total sulfate was varied in the standard solution from 0 to 55 mM, which caused a decrease of the crystal growth rate from 1.15 to 0.5. Simultaneously, S/S_N dropped from 1.1 to 0.7. It may be concluded that sulfate decreases Vcr predominantly via its ion pair formation with Ca(2+). However, a small additional kinetic effect of inhibition must be taken into account.

In a third set of measurements, Vcr and S/S_N were determined as a function of total phosphate (0-40 mM) and pH (5-6.5). Again, corresponding variations were carried out at standard conditions. As may be seen from Figure 9, both parameters cause a considerable decrease of the crystal growth rate while, in parallel, there is only a relatively small

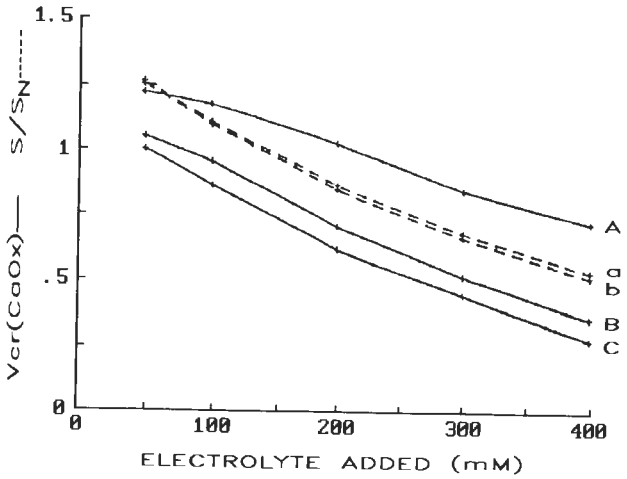

Figure 8: Effects of different electrolytes on the relative crystal growth rate of CaOx, Vcr, in artificial urine. The measuring parameter Vcr (——) is compared to the calculated normalized supersaturation of CaOx within the gel phase, S/S_N (---). Total concentrations of NaCl, KCl, and NH_4Cl were added to artificial urine (pH 6.0) with an initial Na_T of 50 mM. The letters at the curves correspond to the addition of the following electrolytes: A=NaCl, B=KCl, C=NH_4Cl (Vcr); a=KCl,NH_4Cl, b=NaCl (S/S_N).

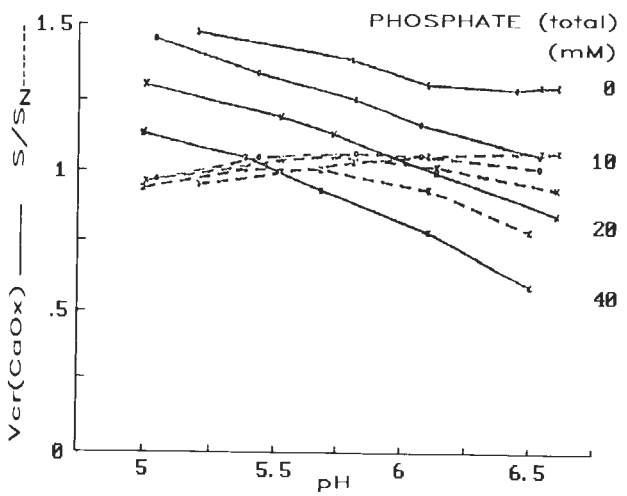

Figure 9: Effects of pH (abscissa) and total phosphate concentration (indicated in the figure) on the relative crystal growth rate of CaOx in artificial urine. The measuring parameter Vcr (——) is compared to the calculated normalized supersaturation of CaOx within the gel , S/S_N (---).

or even negligible change of S/S_N. The following may be concluded from these results: Firstly, phosphate decreases the crystal growth rate of CaOx chiefly by a real kinetic inhibition effect which can be attributed to the species $HPO_4(2-)$ (and $PO_4(3-)$). Secondly, the decreasing effect of pH on Vcr in the system under regard is mostly of indirect kinetic nature. It may be accounted for by the species $HPO_4(2-)$ ($PO_4(3-)$) and Cit(3-). In general, the major urinary constituents regarded here may considerably contribute to the crystal growth of CaOx in human urine via different mechanisms.

Effects of Other Low-molecular Weight Components of Human Urine and Artificial Substances (Achilles et al., unpublished findings)

As will be shown below, crystal growth rates of CaOx measured in natural human urine differ from corresponding values of artificial urines of comparable composition regarding their main components. In order to account for a potential additional inhibiting activity, the following substances occuring in natural human urine were tested: various amino acids and organic acids, bile acids, creatinine, urate, ascorbate, pyrophosphate, and isocitrate. Only a few of them were found to exert significant inhibition within their range of physiological concentration. They are listed in the upper part of Table 1.

Table 1. Influence of urinary constituents and other substances on the relative crystal growth rate of CaOx (Vcr) in artificial urine (standard solution).

Substance	Concentration (mM)	Inhibition (%)
pyrophosphate	0.01 - 0.1	3 - 15 %
isocitrate	0.1 - 1.0	4 - 17 %
hippurate	1.0 - 10	1 - 8 %
glucuronate	1.0 - 10	1 - 9 %
D(-) tartrate	1.0 - 10	9 - 40%
L(+) tartrate	1.0 - 10	7 - 36%
malonate	1.0 - 10	4 - 14%
aspartate	1.0 - 10	0 - 5%
polyphosphate (n=14)	0.01 - 0.1	45 - 100%
phosphocitrate	0.05 - 1.0	22 - 80%
phosphonoformate	0.05 - 1.0	22 - 83%

In a further set of experiments, several artificial substances of potential therapeutical or theoretical interest in the prevention of CaOx urolithiasis were investigated by the GCM (Table 1; lower part). A comparison of the action of citrate, isocitrate, pyrophosphate and phosphocitrate on the Vcr is demonstrated in Figure 10.

Effects of Urinary Macromolecules

Macromolecules have long been supposed to influence crystal growth processes relevant in urinary stone formation (e.g.: Bowyer et al., 1979; Edyvane et al, 1987; Lanzalaco et al., 1988; Robertson and Scurr, 1986; Scurr and Robertson, 1986a,b). However, data in the literature on this topic have been contradictory. Therefore, we tested the macromolecules chondroitin sulfates A,B,C, heparan sulfate, hyaluronic acid and human serum albumin in the standard solution ('artificial urine') mentioned above.

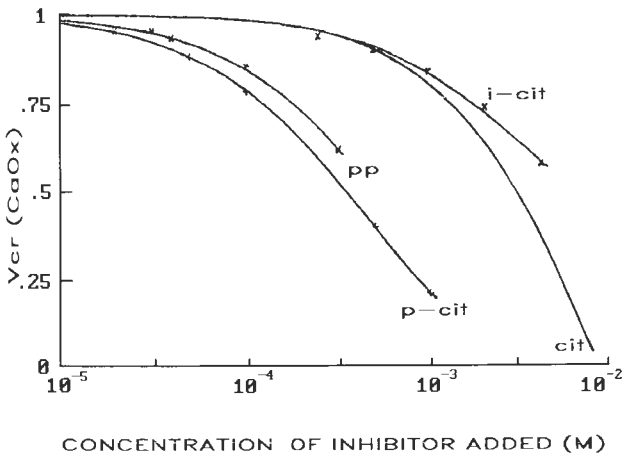

Figure 10: Effects of citrate (cit), isocitrate (i-cit), pyrophosphate (PP), and phosphocitrate (P-cit) on the relative crystal growth rate of CaOx. Concentrations as indicated on the abscissa were added to artificial urine (standard solution).

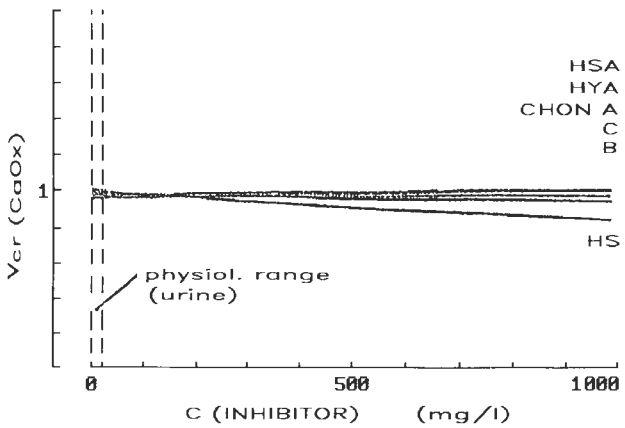

Figure 11: Effect of human serum albumin (HSA), hyaluronic acid (HYA), chondroitin sulfate A,B,C (CHON A,B,C), and heparan sulfate (HS) on the relative crystal growth rate of CaOx in artificial urine (standard solution).

In the concentration range 1-1000 mg/l, most of the substances caused only small effects on Vcr (<5% inhibition) (Figure 11).

In further experiments, Tamm Horsfall Mucoprotein (THMP) and a macromolecular fraction from human urine (MMFU; MW>5000d) were tested at their physiological concentrations in the standard solution. THMP was prepared from pool urine of stone patients corresponding to the prescription of Tamm and Horsfall (1950). Urinary macromolecules were isolated by ultrafiltration from 24-h urine collections of calcium oxalate stone formers and normal controls. For this, low molecular weight components of the native urine samples were washed out and substituted by standard solution. Corresponding to the molecular weight cut-off of the ultrafilter used the fractions contained pools of macromolecules >5000d at their individual, original urinary concentrations. No significant effect on the Vcr of CaOx could be detected with THMP (17-170 mg protein/l). MMFU from natural urine washed into the standard solution decreased the Vcr from 1.0 to 0.97±0.03 (group of 25 recurrent CaOx stone formers) and to 0.95±0.03 (group of 25 normal controls) (Achilles et al., 1989a; and unpublished findings).

Thus, in the model of crystallization under regard and referring to simulated urinary conditions, soluble urinary macromolecules do not exert significant effects on the crystal growth of CaOx in the gel matrix. However, at higher concentrations (>1 g/l), as relevant in stone matrices, they might play a role as inhibitors of crystal growth thus protecting the urinary tract from pathological mineralization.

Measurement of Crystal Growth of CaOx in Natural Urine Samples

The GCM may be directly applied to measure the relative crystal growth rate of CaOx in natural urine samples (Achilles and Ulshöfer, 1986). However, with regard to this application, some principal problems or limitations must be taken into account. As a rule, native urines are metastable solutions with respect to some crystal phases. Thus, especially in 24-h collections, CaOx hydrates, as well as uric acid, may be partially or nearly completely precipitated before measurement. In urinary samples with pH>6.4, calcium phosphate phases and struvite potentially may precipitate, thus decreasing particularly total calcium, which leads to artefacts in the determination of the Vcr of CaOx. Bacterial contamination is especially dangerous in this respect. From a practical point of view, samples of urine can be stored in a frozen state (-20 to -80°C) up to measurement. Then, after thawing, more than 90% of total oxalate is precipitated (Limmer and Achilles, unpublished findings). The small portion of oxalate remaining in solution has practically no effect on the measuring parameter Vcr when determined in the supernatant of the samples after centrifugation. The lack of calcium by precipitation of CaOx may account for a maximum of 10% decrease of the Vcr. Since this error has a similar amount in all samples, especially in intraindividual measurements at different time, it may be neglected for practical purpose. However, urinary samples at pH>6.4 should be checked analytically for the potential loss of Ca by phosphate containing precipitates. Eventually, solids may be dissolved by acidification of the urine, and measurement of crystal growth can be carried out in metastable solutions after adjustment to original pH.

The GCM has been applied to measure the crystal growth parameter Vcr in native urine samples in clinical routine in our laboratory for some years. Results on measurements in urine samples of stone formers and normal controls, as well as on the effects of different therapeutical measures, will be dealt with later on in this paper.

In order to assess whether natural urine samples would contain additional 'inhibitory activity' of CaOx crystal growth compared to artificial urines of comparable known composition, we carried out the following experiment (Limmer and Achilles, unpublished findings). 60 samples of 24-h native urines (NU; supernatants after centrifugation) were analyzed for pH and total concentrations of calcium,

magnesium, sodium, potassium, ammonium, oxalate, sulfate, phosphate, chloride, citrate and isocitrate. Artificial urines (AU) were composed which had the same composition with respect to these constituents analyzed. The Vcr was measured in parallel in all samples under regard. The results are shown in Figure 12. The regression line is described by the relationsship Vcr(AU)= a·Vcr(NU)+b, with a=1.10 and b=0.17. The deviation of the factor a=1.1 of this equation from the theoretical value a=1 may be clearly interpreted by the presence of pyrophosphate, hippurate, glucuronate and macromolecules in the native urines, which were not included in the artificial samples. This may be derived from the results on the effects of these inhibitors in simulated urine as outlined before. However, the constant difference of b=0.17 between Vcr(AU) and Vcr(NU) remains unclear.

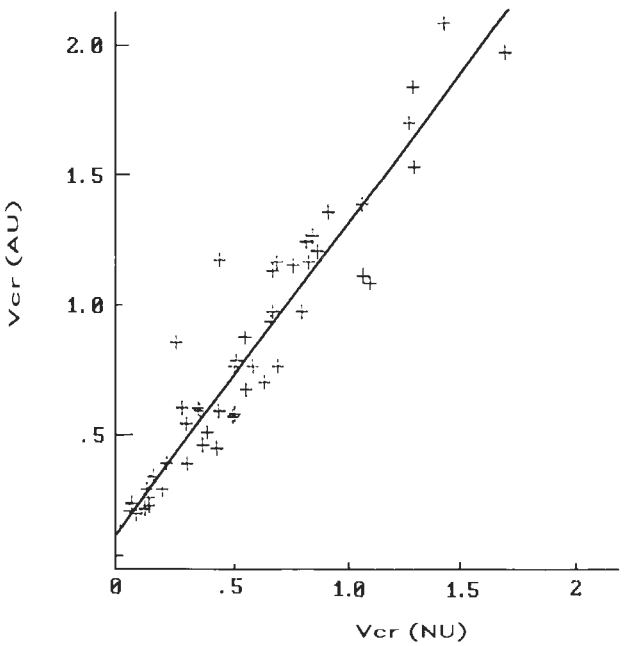

Figure 12: Comparison of crystal growth rates of CaOx, Vcr, measured in 24-h natural urine collections (NU) from unselected stone formers, and in correspondingly composed, artificial urine samples (AU).

Crystal Growth Rates of CaOx in Urine of Recurrent Stone Formers and Normal Controls

In order to evaluate the role of crystal growth inhibition in the genesis of CaOx urolithiasis, we determined the Vcr of CaOx and a number of other parameters in defined urinary fractions of well-matched groups of stone formers and controls (Achilles et al., 1991). The following volunteers were included in the study: 20 male normocalcaemic, recurrent CaOx stone formers (prevalence: more than 2 stones) with normal renal function, aged 40-50 years, and 29 age-matched healthy men as controls. All volunteers were on their usual diet and activities. The collection of urinary fractions was carried out on an outpatient basis in spring time within a period of 45 days in order to avoid seasonal variations of the parameters of interest. From each subject, an 17-h daily urine (d) and a 7-h overnight fraction (n) were collected. The samples were frozen and stored at -70 to -80°C up to further analysis. The crystal growth rate of CaOx, Vcr, was determined in undiluted samples of urine after thawing and centrifugation, at original pH, and after adjustment to pH 5.0. In addition, pH and total concentrations of calcium, magnesium, sodium, potassium, phosphate, sulfate, citrate, isocitrate, creatinine, urate, oxalate and ammonium were determined. From these parameters, saturation ratios (activity product/solubility product) of CaOx, uric acid and brushite were estimated using a corresponding computer program (Achilles and Ulshöfer, 1985a).

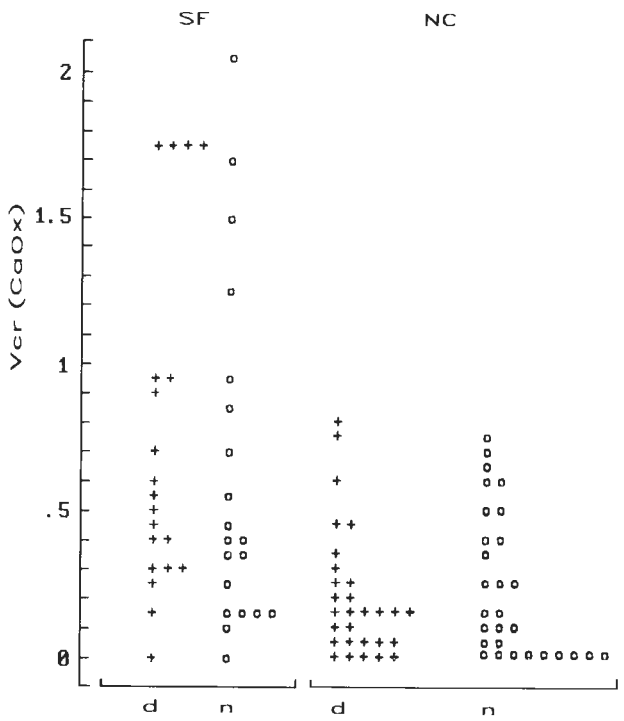

Figure 13: Comparison of crystal growth rates of CaOx, Vcr, in 17-h daily (d) and 7-h nocturnal urine collection (n) of 20 male recurrent CaOx stone formers (SF) and 29 matched normal controls (NC).

Some of the experimental results are summarized in Table 2. In Figure 13, individual measuring values of Vcr in the two urinary fractions from SF and NC are demonstrated. The following may be derived from these data: Among all parameters under investigation, the Vcr determined by the GCM showed the largest difference between SF and NC. Significantly higher concentrations of Ca and lower concentrations of thermodynamic and kinetic effectors of CaOx crystal growth were responsible for the higher crystal growth rates in SF compared to NC. The results demonstrate that the parameter Vcr is clearly indicative of the risk of stone formation and unambigiously superior to calculated saturation ratios (Table 2). However, though the mean values or medians of Vcr differ highly significantly between the corresponding groups, individual values overlap

Table 2. Relative crystal growth rate of CaOx (Vcr), saturation ratio (S[CaOx]), and other parameters in 17-h daily (d) and 7-h nocturnal urinary fractions (n) of 20 male recurrent CaOx stone formers (SF) and 29 normal controls (C).

Parameter	Fr.	Stone Formers (SF)	Controls (NC)	Significance SF/NC	SF d/n	NC d/n
Vcr	d	0.73 ± 0.58	0.21 ± 0.22	***	n.s.	n.s.
	n	0.63 ± 0.58	0.24 ± 0.25	*		
S[Caox]	d	7.31 ± 3.55	4.72 ± 2.25	**	n.s.	n.s.
	n	6.04 ± 3.49	5.40 ± 3.43	n.s.		
pH	d	6.04 ± 0.51	6.06 ± 0.56	n.s.	*	n.s.
	n	5.72 ± 0.56	5.68 ± 0.47	n.s.		
Ca(T)	d	4.81 ± 2.17	3.37 ± 1.66	**	*	n.s.
	n	4.31 ± 2.63	3.55 ± 1.73	n.s.		
Mg(T)	d	3.29 ± 1.39	3.66 ± 1.65	n.s.	n.s.	**
	n	3.47 ± 1.86	4.65 ± 2.04	*		
Na(T)	d	174 ± 54	209 ± 44	*	n.s.	n.s.
	n	158 ± 71	203 ± 62	*		
K(T)	d	46.4 ± 17.1	61.7 ± 19.3	**	**	***
	n	36.7 ± 21.9	47.7 ± 15.8	*		
Ox(T)	d	0.32 ± 0.13	0.34 ± 0.13	n.s.	*	n.s.
	n	0.27 ± 0.10	0.37 ± 0.18	n.s.		
Cit(T)	d	2.33 ± 0.96	2.52 ± 1.05	n.s.	n.s.	n.s.
	n	2.01 ± 1.02	2.40 ± 1.19	n.s.		
i-Cit(T)	d	0.34 ± 0.10	0.44 ± 0.15	*	n.s.	n.s.
	n	0.31 ± 0.13	0.48 ± 0.20	**		
SO_4(T)	d	20.3 ± 6.00	23.1 ± 7.61	n.s.	n.s.	**
	n	20.5 ± 8.07	27.1 ± 8.90	*		
PO_4(T)	d	22.1 ± 7.35	25.0 ± 10.9	n.s.	n.s.	***
	n	22.2 ± 8.59	35.6 ± 13.7	***		

Ca, Mg, Na, K, Ox, Cit, i-Cit, SO_4, PO_4 ...(T) - total concentrations of calcium, magnesium, sodium, potassium, oxalate, citrate, isocitrate, sulfate, and phosphate (given in mM). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ corresponding to appropriate statistical tests.

considerably (Figure 13). This leads to the conclusion that other factors of urine than those which determine the crystal growth should additionally account for the genesis of CaOx stone formation.

Studies on the Efficacy of Therapeutical Measures

All measures of prevention of urolithiasis known up to now principally are aimed at reducing the growth rate of stone-forming crystal phases. This may be achieved by decreasing corresponding supersaturation (thermodynamic effects) or via interactions of effectors on crystal surfaces causing growth inhibition (kinetic effects). Both of them, with respect to CaOx, are reflected by the parameter Vcr (except the influence of urinary oxalate, which must be taken into account in another way). Therefore, the GCM has been applied to study the efficacy of different medications proposed as prophylactic measures in CaOx stone formation. All pilot studies described below were carried out in the same manner. The principal schedule is demonstrated in Figure 14. During a first period (A; without treatment) the 6-8 subjects included in a study were taking their usual diet which was explored in detail using a special questionnaire. At the 2nd and 3rd day of this period they collected a series of urinary fractions. Subsequently, a prescribed dose of a drug preparation was given daily up to the end of period B. In this period (day 9 to 11) the subjects kept the same diet in quality and quantity of nutrition and fluid intake as in period A, and followed the same normal habits. Corresponding urinary fractions in B were collected as mentioned above. The relative crystal growth rate of CaOx and a number of other parameters were determined in all urinary fractions before and during treatment.

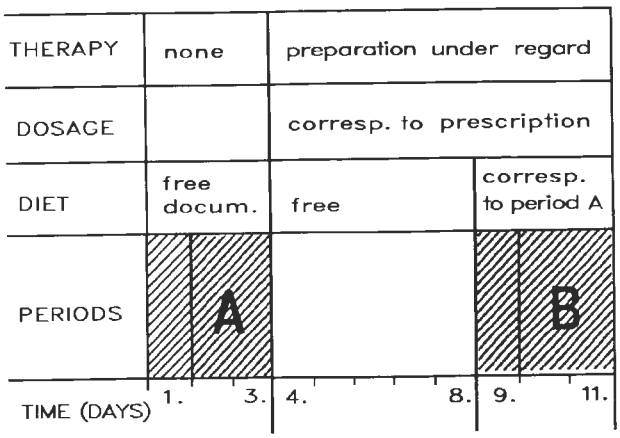

Figure 14: Treatment schedule for in-vivo tests of different preparations with respect to their effects on parameters of crystal formation in urine. A and B: periods of collections of urinary samples.

Sodium Potassium Citrate. Applying a daily dose of 3x11 mmol of this preparation, the mean relative crystal growth rate of CaOx, Vcr, measured in the whole urines of 6 healthy male volunteers could be decreased by about 70% during medication (Achilles et al., 1990). Predominantly, the effect could be accounted for by the alteration of three urinary parameters: decrease of calcium output, increase of citrate, and increase of pH. As could be derived from the relationship $Vcr=f(Ca_T,Mg_T,Cit_T,pH)$ (Equation 3), the change of total Ca during therapy was most effective with respect to the reduction of the crystal growth rate. It caused more than 50% of the total decline of Vcr while simultaneously increasing citrate and pH each accounted for 20-25% of the effect. In contrast to the pronounced in-vivo effect of the preparation on the crystal growth

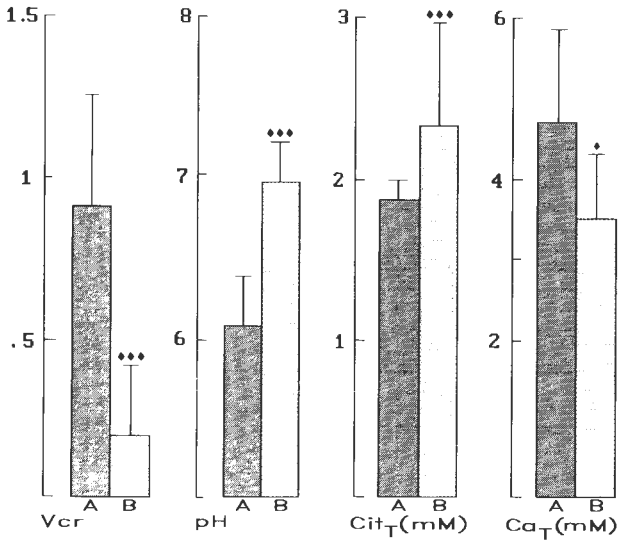

Figure 15: Effect of sodium potassium citrate therapy (daily dose: 2x2.9 g) on the crystal growth rate of CaOx (Vcr) and other parameters in 24-h urinary collections of 6 male healthy volunteers. A: without therapy; B: during therapy. *: $p<0.05$; ***: $p<0.001$.

rate, a decrease of only 30% could be observed for the relative saturation ratio of CaOx. The most important parameters with respect to the formation of CaOx regarded in this study are summarized in Figure 15.

Magnesium Therapy. Since the basic investigations of Hammarsten (1936) on the solubility of CaOx in the presence of Mg(2+), this ion has long been supposed to exert a beneficial effect also in the prevention of CaOx stone formation, e.g. (Johansson et al., 1980). In order to prove this, we carried out an in-vivo study with the following Mg-containing preparations (the quantities of the constituents given here refer to the daily dosage of 12-13 mmol Mg applied in the study): I: 1830 mg Mg citrate, 90 mg Mg laevulinate, 2 mg vitamin B1 nitrate, 3 mg vitamin B2, 3 mg vitamin B6, 200 mg citric acid; II: 1840 mg Mg citrate trihydrate, 800 mg Mg L-hydrogenglutamate, 40 mg Mg nicotate; III: 3074 mg Mg L-aspartate hydrochloride. The evaluation of the most relevant parameters obtained from 24-h urinary collections of 7 male healthy volunteers before and during therapy is demonstrated in Figure 16. The results show that the preparations applied do not decrease the crystal growth parameter Vcr. On the contrary, Mg-aspartate hydrochloride, which shows a marked acidifying effect on urine, enhances the crystal growth rate of CaOx significantly ($p<0.05$). Again, the results may be interpreted by our results described above (effects of calcium, magnesium, citrate and pH; Equation 3). All preparations cause a significant increase of urinary magnesium and, in part (I and II), of citrate. However, the Vcr-decreasing effects of these alterations are compensated, or even overcompensated, by the opposite influence of simultaneous increase of urinary calcium and (with III) decline of pH. Thus, the physico-chemical effect of Mg(2+) on the Vcr known from in-vitro measurements is abolished in-vivo by a contrary physiological mechanism (Tischmann et al., 1987).

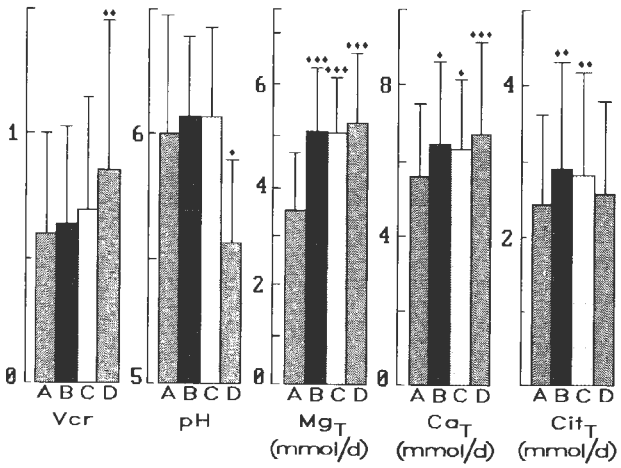

Figure 16: Effect of 3 different magnesium preparations on the crystal growth rate of CaOx (Vcr) and other parameters in 24-h urinary collections of 7 healthy volunteers. Each column corresponds to the following period of collection. A: without application (=control period); B,C,D: during therapy with preparations I, II, and III, respectively. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Fiber Preparation (Farnolith(R)). Dietary fibers have been supposed to be useful in preventing the recurrence of stone formation, e.g. (Griffith et al., 1981; Jarrar et al., 1984). Farnolith(R) (Hesse et al.,1987) is a mixed preparation of wheat and soya brans, with a high content of cellulose, enriched with potassium, magnesium, iron, and zinc. In a study with 7 male healthy volunteers, the preparation was tested in the same manner as described with alkali citrate and magnesium compounds. The daily dose was 2x15g. As the most essential parameter, the relative crystal growth rate of CaOx was determined in 3-h fractional and 24-h urine samples before and during application. No significant decrease of Vcr could be demonstrated administrating the preparation. This lack of a benificial action could be interpreted again by counteracting effects of Vcr-decreasing (i.e., decline of calcium excretion) and increasing factors (i.e., decrease of the crystal growth inhibitors citrate and phosphate) in the urinary samples under investigation (Achilles et al., unpublished results).

Discussion

In this paper, an optical microtechnique has been described which allows the efficient determination of relative growth rates of sparingly soluble crystal phases in gel matrices (Gel Crystallization Method; GCM) (Achilles 1984, 1985, 1987, 1989). It has been predominantly applied to investigate the crystal growth of CaOx and its potential role in the genesis, diagnosis and medical therapy of urinary stone formation (Achilles et al., 1985b, 1986, 1989a, 1989b, 1990).

Compared to conventional methods measuring crystal growth in solutions or suspensions, e.g. (Baumann, 1988; Fleisch, 1985; Meyer and Smith, 1975; Sheehan and Nancollas, 1980; Will et al., 1983) the GCM shows a number of advantages. It is characterized by high efficiency and

small measuring volume. For example, by conventional methods about 10 crystal growth curves may be registered per day using a mean test volume of at least 100 ml. By comparison, applying the GCM 120 growth curves may be measured per hour employing only 0.2 ml solution per test. Irrespective of this features, the reproducibility of the crystal growth parameter Vcr (CV < ±2% at standard conditions) is comparable to or better than that of crystal growth rates obtained by macromethods (Baumann, 1988).

A comparison of our model to other ones evaluating crystal growth in gel matrices, e.g. (LeGeros and Morales, 1973; Hunter et al., 1986; Rao et al., 1988; Mandel et al., 1990), is difficult because in none of these techniques growth is induced by seed crystals. Therefore, heterogeneous nucleation by the gel matrix, which need not be taken into account in the GCM, plays a role in these models. Furthermore, times of reaction and observation, as well as number and distribution of crystals are quite different compared to our method.

The GCM has been used to quantify the effects of different parameters on the crystal growth of CaOx under conditions relevant to stone formation. A number of basic results could be obtained with respect to the influence of urinary constituents on the crystal growth of CaOx. The mechanisms underlying the action of relevant parameters like calcium, magnesium, citrate, phosphate, and pH, and their interactions could be clarified and differentiated as being of thermodynamic and/or kinetic nature.

The GCM may be directly applied to natural urine samples. Here, the quantity Vcr reflects the net effect of thermodynamic and kinetic factors of whole urine except oxalate on the crystal growth of CaOx. The comparison of the crystal growth rate of CaOx measured in native urines and correspondingly composed artificial ones revealed a constantly lower Vcr of about 0.17 in the natural samples. Because this can not be caused by growth inhibition, a thermodynamic reason should account for the difference. An unknown binding capacity for Ca(2+), as stated by Hodgkinson (1980), seems to be unlikely. This interpretation would be in contrast to the findings of Daniele and Marangella (1982), who could show a reasonable agreement between free Ca(2+) in urine calculated from complex chemical equilibria and measured electrochemically. Possibly, the partly reduced Vcr in native urine could be accounted for by an influence of (unidentified) urinary constituents on the crystal phase formed. For instance, a preferred formation of COD (and/or COT) from natural urine compared to a prevailing growth of COM from the artificial samples could be responsible for the difference under regard. The effects of substances causing changes of crystal habits or phase transformation are well known (e.g. Akbarieh and Tawashi, 1990; Campbell et al., 1989; Nancollas, 1982). However, the hypothesis has to be proven by further experiments. Apart from the small difference discussed here, the crystal growth rate of CaOx in urine seems to be mainly governed by low molecular weight compounds. In different kinds of experiments, urinary macromolecules could not be found by us to exert remarkable effects on the Vcr under the conditions applied in this study. Our findings are in reasonable agreement with those of Sutor et al. (1979). They demonstrated the anionic urinary constituents citrate, isocitrate and pyrophosphate to be the major inhibitors of CaOx precipitation in their system. Glycosaminoglycans made no contribution to the inhibition of CaOx formation in their assay used. However, these results are in contrast to those of other reports on the role of macromolecules in CaOx crystal growth, e.g. (Edyvane et al., 1987; Lanzalaco et al. 1988). Comparative studies are highly desirable in order to assess the value of different crystal growth models under compatible experimental conditions.

The rationale of any existing prophylaxis in recurrent CaOx urolithiasis is the reduction of the growth rate of CaOx in the patient's urine. As has been mentioned already above, this may be achieved by lowering CaOx supersaturation, e.g., via the reduction of Ca and/or oxalate excretion or increased diuresis, and/or by an increase of urinary inhibitors like citrate or pyrophosphate. Therefore, the Vcr seems to be an ideal parameter to follow up the efficacy of therapeutical measures aimed at reducing the crystal growth of CaOx. As could be shown by the studies described above, the application of alkali citrate was most effective with this respect. The simultaneous decrease of Ca output, and increase of urinary citrate and pH were acting in the same direction of a desired decline of crystal growth (Achilles et al., 1990). These findings explain the beneficial effect of alkali citrates in the prevention of recurrent CaOx stone formation (Pak et al., 1981; Preminger et al., 1985a, 1985b). On the other side, no or even an adverse influence on the parameter Vcr could be detected in human urine applying Mg-containing preparations (Tischmann et al., 1987). The results account for the lack of a beneficial effect in the prevention of CaOx urolithiasis by Mg therapy as demonstrated by Ettinger et al. (1988) in a controlled, double-blind therapy study. Furthermore, it could be shown by our investigation that the counterion of Mg(2+) (e.g., citrate or aspartate hydrochloride) may significantly affect the crystal growth rate in urine.

The rationale for the application of fiber preparations to prevent recurrent CaOx stone formation refers to their Ca-absorbing properties aimed at the reduction of CaOx supersaturation in urine. However, as could be shown by our study with the preparation Farnolith(R), simultaneous alterations of other effectors result in a negligible net effect on the crystal growth of CaOx during therapy. Thus, fiber preparations might be of use only in patients with intestinal Ca hyperabsorption as stated by others (Strohmaier et al., 1989). In conclusion, the parameter Vcr measured by the GCM is a suitable diagnostic mean in order to control the efficacy of therapeutical measures with respect to the crystal growth of CaOx in urine of patients suffering from urolithiasis. It may be clinically applied and is also suitable to select responders from nonresponders, or compliers from noncompliers undergoing a certain prophylactic therapy. With this respect, the Vcr is clearly superior to the calculated supersaturation of CaOx (S) in urine which is often used as a criterion for the risk of stone formation. While S represents only the thermodynamic driving force for crystallization, the growth parameter Vcr, additionally, reflects kinetic effects on the crystal surface.

The application of the GCM to study the crystal growth of CaOx in urinary fractions of the two groups of male recurrent CaOx stone formers and carefully matched

normal controls described above revealed a highly significant difference of the Vcr between the two groups under regard (Achilles et al., 1991). However, the overlap of corresponding individual values also demonstrates that other factors than those which govern the crystal growth rate should additionally be responsible for the genesis of CaOx stone formation. This means that the 'crystallization theory' of stone formation, e.g. (Robertson, 1982) can be confirmed by our study only in part. Increased crystal growth caused by hypercalciuria and other effectors of crystallization can be contributing but not sufficient factors causing urolithiasis. Obviously, phenomena like crystal agglomeration (Edyvane et al., 1987; Kok et al., 1990) and/or adherence (retention) on biological surfaces (Khan et al., 1990; Finlayson, 1982; Riese et al.,1988) must be taken into regard in order to account for the genesis of stone formation. Here, the macromolecular Tamm-Horsfall-Mucoprotein seems to be likely to act as a promotor of CaOx crystal agglomeration (Rose and Sulaiman, 1982; Rose, 1986). Recently, the group of Nancollas (Campbell et al., 1989) has stressed the dual role of polyelectrolytes as mineralization promotors when immobilized on surfaces, and as crystallization inhibitors when present in solution, which should be taken into consideration in further studies on the cause of stone formation. These facts, at least in part, support the 'matrix theory' (Boyce and Garvey, 1956; Khan et al., 1983; Khan and Hacket, 1987; Morse and Resnick, 1988).

With respect to crystal growth of CaOx, the GCM described here seems to simulate physiological conditions of urinary stone formation better than studies carried out in aqueous solution. Regarding the results of Iwata et al. (1985, 1986, 1989), growth of urinary stones occurs in a gel-like state. The gel, which covers the stone mineral as a mucinous layer, is encrusted by further crystalline material thus forming the later stone matrix. Hering et al. (1987) have detected in human kidneys by scanning electron microscopy crystalline particles which were embedded and fixed in mucinous material. Khan and Hackett (1987) could show that even single crystals in human urine are covered by organic material. With this respect, laws of crystallization in gel matrices as detected by the group of Pucar (Srzic et al., 1976) and theoretically interpreted by Nielsen et al. (1977) should also be of relevance with respect to stone growth. Moreover, it should be taken into account that concentrations of macromolecular inhibitors like glycosaminoglycans, in the medium of stone growth, i.e. matrix material, are by some orders of magnitude higher (Fraj, 1989; Nishio et al. 1985; Roberts and Resnick, 1986) than in urinary solution (2-10 mg/l) (Michelacci et al., 1989). At those concentrations (> 1g/l), GAGS could also be found to reduce significantly the crystal growth of CaOx in our gel model. Therefore, it may be concluded that GAGS might protect the urinary tract from pathological mineralization as highly enriched constituents of the stone matrix rather than as soluble compounds.

Regarding the principal ability and limits of the gel crystallization method discussed in this paper, the following has to be mentioned. The measuring parameter of this method (Vcr) is a relative, empirical quantity which has not been related yet to an absolute crystal growth rate. For this reason, basic research must be done in order to interpret theoretically the optical measuring curves, e.g., in terms of particle size and particle distribution within the gel matrix. Scattered light, which is used to detect the growth of crystals, is related to the mean size of particles. Under the conditions applied for CaOx so far, these consist of a mixture of different calcium oxalate hydrates, predominantly COM. In principle, applying polarized light to follow the crystal growth in gels, the birefringent crystal phase COM should be detectable separately. However, this mode of measurement has been tested yet only in preliminary experiments and must be evaluated in detail. Keeping to the conditions of metastability in a gel system used, the parameter Vcr reflects pure crystal growth excluding nucleation and agglomeration. The model is not suitable to evaluate the phenomenon of crystal adhesion or retention which might be of considerable importance in the genesis of urolithiasis. Taking this into account, it seems to be an important task in future to create a model of crystallization which simulates the process of stone formation by combining a flux of urine with a gel matrix in order to observe and to evaluate crystal nucleation, adhesion and agglomeration in addition to growth.

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Discussion with Reviewers

G. Mandel: Why is your Ksp for CaOx different from that determined by Tomazic and Nancollas ?

Author: The solubility product estimated in this study is an apparent one. It was derived from Vcr = f(Ca_T). Possibly, the extrapolation of this function to Ca_T at Vcr=0, together with the detection limit of the optical method measuring crystal growth, might be responsible, at least in part, for an overestimation of the "Ksp". However, further research has to be done to give a more satisfactory answer to this question.

G. Mandel: The description of Figure 6 is hard to follow. What is the significance of the differences observed between the Vcr and S/S_N curves ? Why are the slopes of the experimental and calculated curves so different for PO_4 in Figure 9 ?
Author: S/S_N has been defined as a term which is under certain conditions compatible with Vcr. With respect to the variation of Ca_T in the standard solution (Vcr=f(Ca_T)) both parameters should have at least two common points: 1. at AP=Ksp --> S/S_N=Vcr=0, and 2. at AP=AP_N --> S/S_N=Vcr=1. Significant differences between the course of S/S_N and Vcr (as seen from Figure 9) give an estimate of the kinetic nature of an effect exerted on the crystal growth rate , i.e., phosphate affects the crystal growth rather via its surface action than by decreasing Ca(2+) via ion pair formation.

S.R. Khan: I would like to see the composition of agar that is being used for making the gel. Is the gel totally inert ? It has often been said that any particulate material can instigate crystal nucleation in a metastable solution. Can the agar used in this study act as a heterogeneous nucleator? Some of the agars may contain macromolecules that can be inhibitory to crystallization.
Author: We used gels like agar-agar, agarose (carbohydrates) and gelatine (protein) to compare crystal growth in different matrices. Concentrations of the gels were very low (e.g., 0.5% for agar), resulting in matrices which were near to the liquid state. Using the same measuring conditions, growth curves obtained with different gels resulted in different absolute quantities of scattered light and increase of light intensity per time. However, the differences disappeared when slopes had been referred to the same standard as done by calculation of the parameter Vcr. Thus, potential effects of the gel matrix on absolute crystal growth rates seem to have no significant relevance in comparative measurement. Heterogeneous nucleation of crystals by the gel matrix can be absolutely excluded as to our conditions of seeded crystal growth. Principally, experiments were performed below the limits of metastability, which had been determined previously in gels without seed crystals.

S.R. Khan: It is pointed out in the manuscript that during urolithiasis crystals grow in a gel-like "mucinous macromolecular" layer. What is the nature of this layer ? What is the thickness of such a layer ? Is the author suggesting that that is the only way for matrix to be incorporated into the stone ?
Author: As to the growth of stone mineral in a gel-like state I refer to the findings of Dr.Iwata from Japan. His group interprets radially oriented crystal growth in stones as a process which can only take place in a gel-like medium. The mucinous macromolecular layer covering a stone must have a composition which is very similar to or even like that of the stone matrix. Its thickness may be between 0.1 and some micrometers. This can be estimated from SEM results on microcrystals in human kidneys as shown by the group of Dr. Hering from Switzerland. It is in a good agreement with the thickness of the layer which may be derived from OM and SEM photographs of stone material by Dr.Iwata (personal communication). I do not know whether the growth of crystalline material within this gel-like state is the only way for matrix to be incorporated into the stone. However, it seems to be a very obvious mechanism.

S.R. Khan: I would like the author to comment about the porosity of the gel. Would urinary macromolecules, some of which can be pretty large, be able to travel through the gel?
Author: Referring to data given by Henisch et al., the large pores of light gels as we use them should exceed 5 µm in diameter. The mean diameter of macromolecules of up to 10^5 d can be estimated to be about three orders of magnitude smaller. Corresponding to a personal communication of Dr.Pucar from Zagreb, who has done a lot of work on crystallization in gels, molecules of a molecular mass of up to 10^6 d should be freely diffusible in those light gels. However, large aggregates of THMP as they are formed at high ionic strength should be excluded from diffusion into the gel matrix.

S.R. Khan: Whole urine is generally frozen and then thawed for the determination of Vcr. What would be the effect of freezing and thawing on the macromolecules ?
Author: We have not tested this up to now. However, because macromolecules of urine could not be found to affect the crystal growth of Caox significantly in the model, I suppose that potential alterations of this fraction by freezing and thawing should not be of remarkable influence on the measuring parameter Vcr, too.

H. Iwata: Urinary macromolecules, especially glycosaminoglycans, are believed to inhibit the crystal growth mainly by covering the crystal surface, thereby blocking the growth site (kinetic effect in your words). I wonder if this inhibitory effect of glycosaminoglycans may be underestimated in the gel system because of the retarded diffusion of the macromolecules through the gel ?
Author: At first I should like to refer to my answer to a similar question of Dr.Kahn regarding the effect of macromolecules in gels. With the exception of very large aggregates like polymeric THMP, retarded diffusion of urinary macromolecules through the gel should not be responsible for an underestimated effect of inhibition. I would rather suppose that the seed crystals within the gel are connected to structures of the matrix. Thus, they may not, or only in part, be accessible to larger molecules. Further work has to be done on this problem.

R. Tawashi and M. Akbarieh: Your figures show the growth rate between 0 to 15 minutes. This period is too short for

the growth studies especially if the author is planning for a future model for stone formation. What happens if growth is extended over days ?
Author: I agree with the reviewers that a longer period of crystal growth and observation is desirable for future models of stone formation. However, the experimental conditions in the model presented here were optimized with respect to the detection limit and sensitivity of the optical mode of measurement in order to gain sufficiently reproducible results (slopes of crystal growth curves). The rather short periods of growth and observation are a consequence of this optimization which leads to the high efficiency of the method. However, the influence of relevant effectors on crystal growth may be clearly recognized under these conditions. If growth is extended over days the crystal image is governed by equilibrium conditions rather than by kinetic ones. The measuring signal reaches a maximum which is difficult to interpret because of superimposing crystals within the gel.

R. Tawashi and M.Akbarieh: If figure 16 is true regarding the Vcr, what is the author's response to the many clinical reports published on the beneficial effects of Mg(2+) in urolithiasis ?
Author: As far as I know, with respect to the in-vivo effect of magnesium preparations on CaOx stone recurrence, there are only one or two corresponding clinical studies which may be denoted as being reliable, because they followed a controlled, double-blind protocol. Referring to these results (Ettinger et al.), no beneficial effect of magnesium has been proved. This is in agreement with our findings.

G.Mandel: The observation that natural urine had a Vcr 0.17 below that of synthetic solutions was attributed to possible changes in CaOx crystal diamorphs. This possibility can easily be tested by taking scanning micrographs of the crystalline products formed under the various conditions.
Author: Up to now we have not used SEM in parallel to kinetic experiments because the method is not available in our laboratory and because we have focussed our work to kinetics at first. However, I agree with you that a supplementation of micrographs could be fruitful in order to clarify some open questions in the paper. I shall take the comment into regard in our future work.

G.Mandel: The author has stated that "the Vcr is clearly superior to the calculated supersaturation of CaOx ... as a criterion for the risk of stone formation." However, Figure 13 shows that only 7(d) or 6(n) of 20 stone formers have Vcr values outside the range of stone formers. Since these Vcr values are very significantly different from the non-formers, the statistics are skewed. The wide variation in Vcr values for stone formers is also evident in Table 2. The standard deviations in Vcr values for stone formers are 79% (d) and 92%(n) of the actual Vcr value, whereas the standard deviations in S for the same stone formers are only 48% (d) and 58% (n). This would suggest very wide variation in Vcr values. This would further suggest that the number of stone formers having a Vcr value within the range of non-formers is greater than the number of stone formers having a S values within the range S for non-formers.
Author: There are a series of reasons to state that the parameter Vcr is superior to the calculated supersaturation of CaOx. a) The ratios of the means of Vcr in SF and NC (Table 2) were 0.73/0.21=3.5 ($p<0.001$) in daily urine and 0.63/0.24=2.6 ($p<0.05$) in overnight collections. Comparative values for S(CaOx) were 7.31/4.72=1.5 ($p<0.01$) and 1.1 (n.s.). The skewness of the distribution of Vcr has been regarded in the statistics. b) The effect of therapeutic measures, e.g. with alkali citrate, could be clearly detected by the crystal growth (decrease of more than 70% during therapy). In comparison, S(CaOx) dropped only by 33% (Achilles et al. 1990: Urol.Res. 18,1-6). c) The crystal growth rate may be measured very efficiently by a single analytical procedure. The calculation of S(CaOx) requires the determination of 8-10 different analytical parameters. d) Direct (e.g. by pyrophosphate) or indirect (e.g. by pH) kinetic effects may only be detected by crystal growth rates.

G.Mandel: Could you provide additional data on the number of seed crystals per unit area or volume ?
Author: We have tried to make an estimate of the number of seeds per volume by several techniques. However, it has not been possible up to now to obtain reliable results. Probably, image analysis of SEM-images may provide the desired quantity.