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An Optical Micromethod for the Determination of Relative Crystallisation Rates of Calcium Oxalate in Gels: Method and Preliminary Results

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Summary. This paper describes a new, highly efficient micromethod for the determination of relative crystallisation rates of calcium oxalate (CaOx). Crystallisation is performed in the upper layer of a gel (bactoagar, agarose) which contains one component (oxalate) of the sparingly soluble salt. Precipitation is started by pipetting Ca^{++} containing solutions (in the presence and absence of crystallisation inhibitors) onto the gel. The process is followed quantitatively as a function of time by means of vertical light path photometry carrying out quasi-simultaneous multideterminations within a 50-fold multicuvette. The test volume is 0.1 ml. The method is suitable for large scale determinations. About 50 single crystallisation kinetics can be measured within 5–15 min. Testing three known inhibitors of CaOx crystal formation, relative inhibitory activities were obtained with standard errors of 1%–4%.

Key words: Calcium oxalate, Crystal growth, Kinetics, Micromethod, Inhibitors.

Introduction

Although there exist numerous procedures to follow kinetically the crystallisation process of calcium oxalate (= CaOx), e.g. [1–10], none of these methods seems to be suitable for efficient application in routine urine measurements or even to large scale determinations of crystal growth kinetics. Such investigations, however, would be of appreciable importance with respect to diagnosis and therapy of CaOx urolithiasis. With the exception of the method described in [10], inhibitory activities of urines can only be assessed in rather diluted samples.

In order to overcome these difficulties, we have developed a micromethod which allows large scale determinations of relative crystallisation rates, i.e. of the ability of a large number of solutions to form CaOx crystals under certain experimental conditions [11].

The method is described in detail and discussed. Quantitative photometric measurements of crystallisation rates are supplemented by morphologic investigations of the crystals formed.

Materials and Methods

Chemicals and Solutions

Doubly distilled water was used in all experiments. Agarose and bactoagar (Difco-Laboratories, Detroit, USA) were of research grade. Ethylenediamine-N,N,N′,N′-tetramethylene phosphonic acid ($C_6H_{20}N_2O_{12}P_4 \cdot 1.5H_2O$) was prepared according to [12]. Sodium polyphosphate (mean chain length of $n = 40$) was prepared at the Department of Chemistry of the University of Jena. All other substances were of analytical grade. The artificial urine (= standard solution) used for precipitation experiments had the following composition: NaCl = 110, KCl = 20, NH_4Cl = 35, $KH_2PO_4 \cdot 3H_2O$ = 15, Na_2SO_4 = 20, citric acid = 2.5, $MgCl_2 \cdot 6H_2O$ = 3, $CaCl_2$ = 4, NaN_3 = 0.5 mmol/l; pH = 6.4. Stock solutions without $CaCl_2$ (containing double concentrations) were stored frozen. Standard solution and test solutions were prepared by diluting the stock solution (1:2) and simultaneous addition of 4 mmol/l $CaCl_2$ and 0.001–0.1 mmol/l of inhibitor substances.

Method for the Determination of Relative Crystallisation Rates in Gels (Gel Crystallisation Method; GCM)

Principle of Measurement. From two lattice ions forming a sparingly soluble precipitation one (Ox) is dissolved as a suitable compound in a transparent layer of a gel. The gel is located in multiple wells of a multicuvette (microplate). Solutions containing the other ion, in the absence (standard solution) and presence of crystallisation effectors (test solutions), are pipetted onto the gel. The subsequent change of the optical properties of the gel layer, caused by precipitation of the crystals at the border gel/solution is followed by vertical light path photometry as a function of time. The principle is shown schematically in Fig. 1.

Experimental Procedure and Apparatus

0.1 ml of an aqueous hot solution (50 °C–60 °C) containing 0.5 g agarose or bactoagar, respectively, per 100 g, 5 mmol/l ammonium

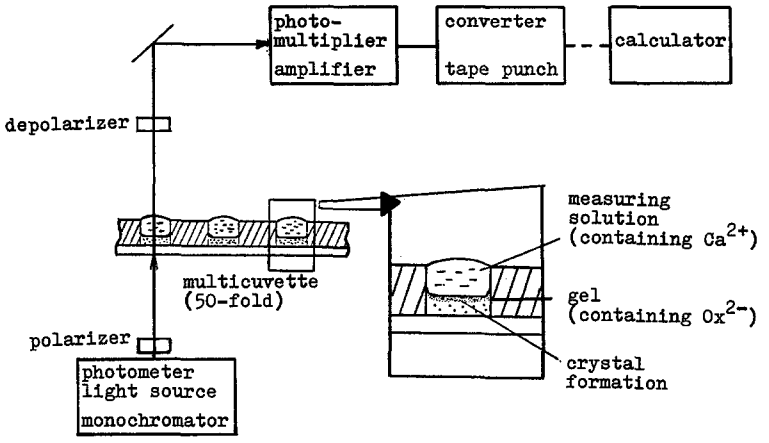

Fig. 1. Scheme of experimental measurement of relative crystallisation rates in gel systems

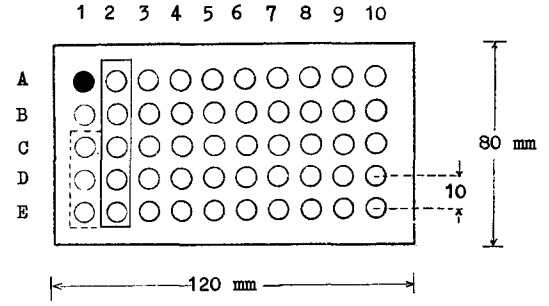

Fig. 2. Scheme of the multicuvette and of sample pipetting. Well dimensions: 7 (diameter) and 5 mm (depth). *A1:* 100% absorbance value, *B1:* turbidity standard/optical rotation standard (for internal standardisation), *C1–E1:* blank samples (gel + water), *A2–E2:* Ca^{++} standard samples (gel + artificial urine), *A3–E10:* test samples (gel + artificial urine + crystallisation inhibitors)

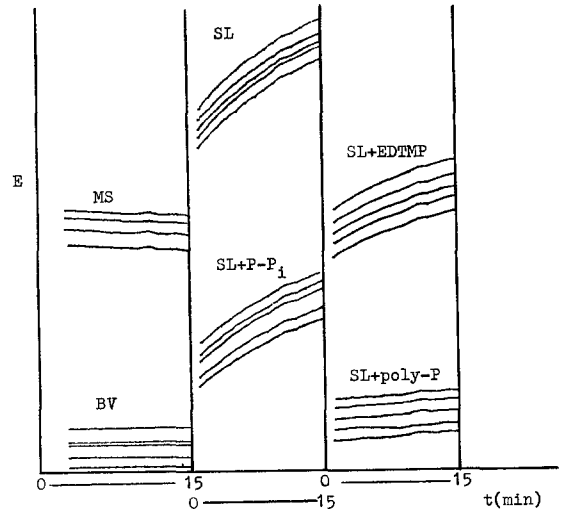

Fig. 3. Kinetic curves from (nephelometric) normal light intensity measurements of CaOx crystallisation in bactoagar gel. Extinction (= *E*) is plotted as a function of time (= *t*). For better distinction, single curves are shifted arbitrarily against each other by the plot programme. Units for *E* are therefore omitted. The Ca^{++} standard solution described in Methods (= *SL*) was used in the absence and presence of crystallisation inhibitors (10^{-4} mol/l) as indicated at the curves. *MS* = measuring standard; *BV* = blank values; *P-P_i* = inorganic pyrophosphate; *EDTMP* = ethylenediamine-N,N,N′,N′-tretramethylene phosphonic acid, poly-P = polyphosphate

oxalate and 0.05 mmol/l $CaCl_2$ were pipetted into each of 48 wells of a 50-fold multicuvette. After cooling to room temperature a gel layer of 1–2 mm thickness was formed in each well. Subsequently, 48 100-µl-samples of a) test solution, b) standard solutions, and c) water (blank values) were pipetted simultaneously into the wells of the plate according to the scheme given in Fig. 2. One well of the multicuvette was covered by a small black plate for measuring the 100% absorbance value, and one contained a small turbid polystyrene plate used as measuring standard in normal and polarised light intensity measurements, respectively. Simultaneous pipetting was achieved using a 50-fold multipipette [13]. Measurement of extinction was started not later than 1 min after pipetting the samples onto the gel. The apparatus used was a vertical light-path photometer as described in [13, 14]. The equipment was coupled with a digital voltmeter and a tape puncher. The samples were measured one after another by sliding the horizontally positioned mulicuvette well by well through the light beam of the photometer (wavelength 365 nm). The measuring cycle was repeated every minute up to 15 times. Data were recorded on punch tapes which were subsequently processed off-line in the calculator HP 9820 A.

Two different modes of measurement were performed:

a) Normal light intensity measurement (nephelometric measurement): as a consequence of crystal formation and crystal growth within the upper gel layer (causing its turbidity) initial light intensity (= I_o) is decreased to the measuring intensity (= I_m) by light scattering and absorption. The extinction $E = -\lg (I_m/I_o)$ is followed as a function of time (Fig. 3).

Although extinctions are measured instead of scattered light intensity, as is usual in turbidity measurements, the term "nephelometric" is used synonymously for this mode of measurement.

b) Polarised light intensity measurement: the formation of doubly refracting crystals of calcium oxalate monohydrate (= whewellite) in the gel causes a rotation of linearly polarised light. Thus, starting the measurement with polariser and depolariser positioned to give minimal light transmission, measuring light intensity increases with increasing whewellite crystals in the gel. For standardisation, measuring values (minus blank values) were divided by measuring standard values. Plots of data vs. time are shown in Fig. 4.

Calculation of Relative Crystal Formation Rates and Inhibitory Activities

From the first five points, i.e. the quasi-linear part of the curves E = f(t) and I′ = f(t), initial slopes were calculated by linear regression analyses. The slopes obtained from test solutions ($\Delta E_o/\Delta t_o$; $\Delta I'_o/\Delta t_o$) were referred to the mean initial slope of the Ca^{++} standard

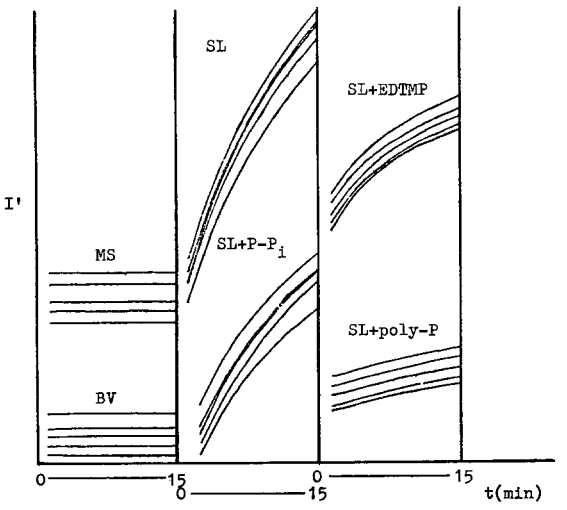

Fig. 4. Kinetic curves from measurements with polarised light of CaOx crystallisation in agarose. Light intensity (arbitrary standardized units) is plotted as a function of time. Single curves are shifted arbitrarily against each other. The solutions used were the same as indicated in Fig. 3

solution ($\Delta E_{os}/\Delta t_{os}$; $\Delta I'_{os}/\Delta t_{os}$). Relative "crystal formation rates" could then be expressed as

$$V_{RC}(\%) = \frac{\Delta E_o}{\Delta t_o} \Big/ \frac{\Delta E_{os}}{\Delta t_{os}} \cdot 100 \quad \text{and}$$

$$V'_{RC}(\%) = \frac{\Delta I'_o}{\Delta t_o} \Big/ \frac{\Delta I'_{os}}{\Delta t_{os}} \cdot 100.$$

Relative inhibitory activities (A_i and A'_i) may then be formulated as $A_i = (100 - V_{RC})$ and $A'_i = (100 - V'_{RC})$.

Microscopic Investigations

CaOx crystal formation was studied by microscopy under the following experimental conditions. Microplates used contained wells of a depth of only 1mm. Agarose gel (30 µl/well) was applied for crystallisation. The gel contained 1ml glycerol per 100 ml to avoid drying up of the thin layer. Standard solution and test solutions (each 30 µl/well) were pipetted onto the gel. After storing the microplate for 1–2 h within a moist chamber, CaOx precipitates were studied within the upper gel layer by means of normal light microscopy and polarisation microscopy (microscope "Ergaval", Jenoptik, Jena, GDR).

Table 1. Reproducibility of quasi-linear initial slopes (obtained by linear regression analysis) from CaOx crystallisation kinetics measured in bactoagar and agarose by two different methods. The coefficients of variation (C.V.) given here refer to measurements within single plates

Gel	Mode of measurement	*n*	C.V. (%)
Bactoagar	Normal light (nephelometric)	10	3– 5
Bactoagar	Polarised light	20	10–12
Agarose	Polarised light	20	4– 6

Representative areas of gel were selected and photographed. The pictures obtained were subjected to semiquantitative evaluation by counting the different particles of CaOx monohydrate and dihydrate produced within a specified area. From some pictures crystal size distributions were estimated by means of image analysis using the system "Quantimet 720" (Cambridge Instruments, UK).

Results

Precision of the Method

The reproducibility of the initial slopes of different crystallisation kinetics are summarised in Table 1. As may be seen from the results, bactoagar is particularly suitable for normal light extinction measurement (turbidity) while agarose should be applied to determinations with polarised light (doubly refracting crystals). Because of unacceptably low extinction values and high relative errors, nephelometric measurements could not be performed in agarose. At the present stage, relative inhibitory activities are reproducible with a standard error of 1%–4% (Table 2).

Efficiency of the Method

Evaluating the kinetic curves by linear regression analysis of the first five couples of light intensity values and time, the measuring time for 45 samples is 5 min. Including the preparation of gel, pipetting procedures and off-line evaluation, in serial determinations up to 100 single crystallisation kinetics can be assessed each hour by one laboratory assistant.

Table 2. Relative inhibitory activities of known inhibitors of calcium oxalate crystallisation measured in bactoagar (normal light, nephelometric) and agarose (polarised light). Standard errors (S.E.) refer to mean values from 4–5 fold determinations in *n* different plates. P-P_i = inorganic pyrophosphate, EDTMP = ethylenediamine-N,N,N′,N′-tetramethylene phosphonic acid, poly-P = polyphosphate (chainlength about 40)

Substance (Inhibitor)	Concentration mol/l	*n*	Relative inhibitory activity (%)	
			Bactoagar (normal light)	Agarose (polarised light)
P-P_i	10^{-4}	6	9.8 ± 1.3	16.7 ± 1.0
EDTMP	10^{-4}	6	21.3 ± 3.8	24.4 ± 3.3
Poly-P	10^{-4}	7	95.2 ± 3.9	95.8 ± 2.6

Table 3. Results of semiquantitative microscopic investigations of CaOx formed in agarose. Counting of bipyramids (weddelite; edge length 3–12 μm) using normal light, and counting of visible light points (preferably whewellite) using polarised light within comparable selected gel areas (size about 0.1 mm^2). s = significance of differences between data from standard solution and inhibitor containing solutions as obtained by the test of Man-Whitney. (+) = $p < 0.05$, (+++) = $p < 0.001$, (–) not significant

Solution + Inhibitor (= 10^{-4} mol/l)	Tetragonal bipyramids		Light points (polarised light)	
	Mean ± S.D. (n = 3)	s	Mean ± S.D. (n = 3)	s
Standard solution (= SL)	31 ± 24		157 ± 57	
SL + P-P_i	28 ± 12	(–)	99 ± 17	(+)
SL + EDTMP	42 ± 27	(–)	100 ± 23	(+)
SL + poly-P	Only oval shape weddelite observable	(+++)	–	(+++)

Relative Inhibitory Activities

Table 2 shows the relative inhibitory activities (A_i, A_i') of three substances which are known to influence the growth and/or nucleation of CaOx crystals in solutions, e.g. [15]. The values measured refer to the standard solution which the substances are dissolved in. The two modes of measurement mentioned above were applied using two different gels. As may be seen from the table, polyphosphate exerts the strongest influence on the crystallisation process of CaOx, while inorganic pyrophosphate is not nearly as effective. The difference between the values A_i and A_i' is only significant in the case of inorganic pyrophosphate, indicating that P-P_i inhibits the formation of whewellite in agarose stronger than that of weddelite in bactoagar.

Microscopic Study of CaOx Precipitates Within the Gel

In order to correlate photometrically measured parameters of CaOx crystallisation with the kind and number of crystals formed in the gel, kinetic measurements were supplemented by microscopic investigations. Qualitative observations show that the specific number of CaOx crystals produced in bactoagar is considerably higher than those in agarose, whereas mean CaOx particle size in bactoagar is appreciably smaller. Furthermore, the formation of CaOx monohydrate is clearly preferred in the agarose medium compared with bactoagar.

The effect of inhibitors on CaOx crystallisation in agarose was studied semiquantitatively by counting the different particles formed within a specified area of the upper gel layer. The results for the standard solution (= artifical urine) in the presence and absence of the substances indicated are summarised in Table 3. As can be derived from the data, all three inhibitors reduce the specific number of whewellite particles formed, while only polyphosphate shows also a striking effect with regard to weddelite bipyramid formation. Preliminary results obtained by means of image analysis (not shown here) indicate that the inhibitors mentioned are able to change also CaOx particle size distribution in agarose. Final quantification of this effect, however, needs further study.

Discussion

The method for the determination of relative crystallisation rates described here (gel crystallisation method "GCM") shows a number of advantages compared with conventional methods. The principle of crystallisation in gels combined with a device of vertical light-path photometry for quasi-simultaneous multideterminations allows the performance of kinetic microdeterminations with a very high efficiency. More than 500 single crystallisation kinetics can be assessed per day using only 100 μl solution per test. By comparison, conventional procedures are not able to perform more than 10–20 kinetics per day. As a rule, they need test volumes of at least 10–200 ml, e.g. [4, 5, 8]. Due to its high efficiency the method described here is suitable in particular for large scale determinations of crystallisation inhibitory activities. This should be especially valuable for routine clinical measurements. Furthermore, large numbers of pharmacologically interesting substances can be tested with respect to the possibility of their being inhibitors of crystal growth or nucleation.

The precision of the A_i values determined here is satisfying with regard to kinetic methods and permits also the assessment of relatively low inhibitory activities (< 5%).

The ability of the "GCM" to follow the crystallisation process of CaOx by normal and polarised light photometry probably offers the possibility of differentiating between the effects of inhibitors on the crystallisation of both CaOx monohydrate and dihydrate. Hitherto this has not been possible by any other method described. As can be derived from Table 2, P-P_i seems to inhibit the formation of whewellite in agarose more strongly than that of weddelite in bactoagar, while the inhibitory activity of EDTMP and poly-P are practically the same as determined by both methods of measurement.

Relative crystallisation rates, or inhibitory activities as they are defined here, cannot be related to the terms pro-

posed in [16] for standardised nomenclature. Our data are valid only under specified conditions (kind of gel, mode of measurement, temperature, concentrations, and standard solution applied). Nevertheless, their usefulness for comparative measurements is without any doubt.

The data obtained by the "GCM" result from the formation of CaOx within the gel including nucleation as well as crystal growth. Measuring overall changes of light intensity, the partition of these different processes in whole crystal production cannot be assessed. Therefore, direct observation of the crystal formation is necessary to give further insight into the process. As a first approximation, microscopic studies of the CaOx formed in the gel were carried out, particularly in agarose. As may be seen from the results, the crystallisation process is strongly influenced by the kind of gel. While bactoagar is especially suitable for nephelometric measurements due to the smaller crystals formed, agarose has proved to be very useful for polarised light measurements. Furthermore, in contrast to bactoagar, agarose is a suitable medium with respect to microscopic studies. From these studies it follows that the inhibitors may not only effect the rate of crystal formation (nucleation and growth) but also the kind, number and size distribution of the crystals produced. It has been suggested several times in the literature, e.g. [17], that the latter factors could play an important role in calcium stone formation. Microscopic investigations of gel crystallisation could therefore be a useful supplement to the kinetic measurements described above.

Until now the "GCM" has not been applied to determinations in urine samples. However, this should be easily possible using diluted urines (1 : 50), as conventionally practised. In this case, like in the application of low inhibitor concentrations, rate-determining free Ca^{++} concentrations of standard solutions would not be changed significantly after addition of the sample. However, the assessment of inhibitory activities of CaOx crystallisation related to a certain standard should also be realisable in undiluted urine. Due to the different free Ca^{++} concentrations in the samples under consideration, an overall tendency of the urine to form CaOx crystals would be assessed by such test. This could be a valuable measure in evaluating risk situations for CaOx formation in urine and should be suitable for clinical routine measurements as well as in animal experiments.

Most probably, the gel crystallisation method is not only restricted to CaOx. It should also be applicable to the study of precipitation of other substances sparingly soluble in aqueous solutions. Thus, the procedure could be of importance in studying processes relevant to biological mineralisation, in pharmacology, as well as industry.

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