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Problems related to the measurement of crystallization conditions in whole urine

Report from a workshop held on November 25, 1988 in Basle

At the first workshop published in Urological Research 1988 16:137–142, 7 different test systems to measure crystallization of stone salts were presented and compared. At this second workshop organized again by J. M. Baumann, Biel and F. Hering and G. Rutishauser, Basel, general problems of the measurement of crystallization conditions in urine were discussed. The different aspects were introduced by short papers and the discussions are summarized and commented upon at the end of this report.

Studies of urinary calcium oxalate crystallization in clinical research

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Many sophisticated technics for measuring the crystallization of calcium oxalate have been developed. However, only a part of them are applicable to measurement in whole urine and suitable for clinical research.

Current methods used on whole urine: Calcium oxalte crystallization is induced in urine by the addition of sodium oxalate alone [1, 5, 10, 11, 14] or in combination with the addition of a constant amount of calcium oxalate seeds [3, 10]. One method induces crystallization by the addition of seeds alone [6]. Most test systems are based on endpoint measurements and determine the upper limit of metastable supersaturation with respect to calcium oxalate. Urine is titrated by oxalate until precipitation or crystal growth starts. Crystallization is monitored: a) continuously by turb idimetry [5] or nephelometry [1], b) intermittently by a Coulter Counter [14] or isotopically [6], c) at the end of the crystallization process by a Coulter Counter [11] or chemical analysis of calcium and oxalate after removal of crystals [3, 10]. The amount of sodium oxalate necessary to induce calcium oxalate precipitation is either directly used as a measure of urinary stability [5] or its reciprocal value, which is a measure for the urinary crystallization risk [14]. These procedures enable one to avoid the difficulties of measuring urinary oxalate. Other groups calculate formation products for calcium oxalate [3, 10, 12]. In three test systems efforts were made to determine growth rates of crystals by repeated examination of urine at fixed time intervals [6, 10, 11].

Importance and evaluation of precipitation tests: The critical oxalate addition necessary to induce crystallization at short incubation times (under one hour) is in the order of mmol/l and thus generally much higher than oxalate concentrations spontaneously found in urine [1, 5, 11, 14]. At such high supersaturations the effect of inhibitors with high potency but small urinary concentration can be lost [2]. Theoretically, nucleation occurs when a critical supersaturation (upper limit of metastability or formation product) is obtained, which is different for each solid to be formed. However, a minimal crystal number has to be formed and the crystals must grow to a minimal size until nucleation can be detected by current methods. Both processes are governed by supersaturation and time. Therefore, the critical oxalate addition for detectable precipitation decreases with increasing incubation time [2].. The formation product is not only influenced by the incubation time but also by nucleators and inhibitors. Precipitation tests in urine always measure the combination of these two effects [10]. If results were expres-

sed in terms of critical oxalate addition necessary to induce precipitation, no differences between stone formers and controls were found [5, 14]. The results mainly reflected the state of the initial urinary supersaturation with respect to calcium oxalate [14]. However, when the results were expressed as critical oxalate concentrations [7] or as formation products [10, 12], they were lower in stone formers compared to healthy controls. The decreased formation product found in urine of stone formers seems to be the effect of nucleators mainly, since it was often lower than that found in inhibitor free control solutions [10]. Growth rates of precipitated crystals did not show differences in urine of stone formers and healthy controls [12]. But growth rates significantly increased after centrifugation and filtration of urine, a procedure that removes inhibitory macro-molecules [13].

Importance and evaluation of seed tests: If crystallization is induced by calcium oxalate seeds, the effect of inhibitors is measured especially [3, 9]. The inhibitor potential against crystal growth is generally high in urine. The growth of seeds in the concentration range of spontaneous precipitation (0.02 mg/ml) can only be induced by the raising of urinary supersaturation to values rarely found in urine [4]. When this critical supersaturation was taken as a measure of urinary inhibitor activity no significant differences were found between stone formers and controls [4]. However, crystal growth rates measured at higher seed concentrations (0.5 mg/ml) were slightly increased in 24 hour urines of some sub-groups of stone formers compared to healthy controls [10]. When growth rates were determined in concentrated morning urine with seed concentrations of 0.2 mg/ml, marked differences were found between patients and controls [6]. The examination of selected urine portions during the day may therefore be more relevant for stone research than the examination of 24 hour urine.

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Measurement of urinary supersaturation, creation of standard supersaturations*

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Supersaturation is the sine qua non for formation and growth of crystals and urinary stones. It might be expressed in many different ways (Table 1); saturation

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Table 1. Descriptions of state of saturation

	Value at equilibrium
• Finlayson [1]	
$RS = \frac{AP}{Ksp}$	1
• Nancollas [2]	
$S = \frac{AP^{1/n}}{Ksp^{1/n}}$	1
• Robertson [3]	
$\log_{10}$ (rel. supersat.) scale $= \log_{10} \frac{AP}{Ksp}$	0
• Werness [4]	
$S = \frac{AP\text{-}Ksp}{Ksp}$	0
• Pak [5]	
$APR = \frac{APi}{APe}$	1
$CPR = \frac{CPi}{CPe}$	1
• Tiselius [6]	
$AP(CaOx)\text{-index} = \frac{k \times 3.8 \times Ca^{0.71} \times Ox}{Mg^{0.14} \times Cit^{0.10} \times Vol^{1.2}}$	

RS = relative supersaturation; AP = activity product; Ksp = thermodynamic solubility product; S = saturation; n = number of ions in the formula for the mineral; APR = activity product ratio; APi = initial activity product; APe = activity product after equilibration; CPR = concentration product ratio; CPi = initial concentration product; CPe = concentration product after equilibration

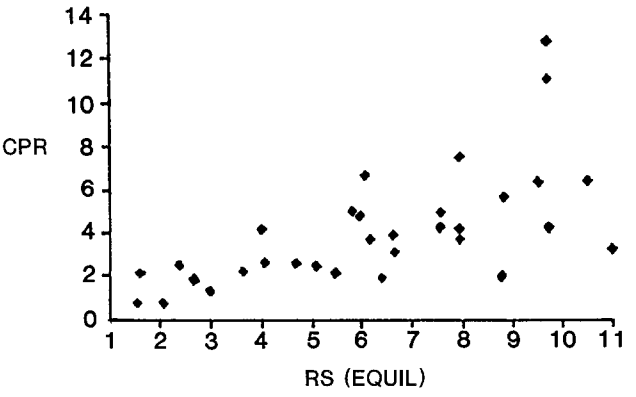

Fig. 1. RS vs CPR with respect to calcium oxalate monohydrate in stone former urine. $y = 0.09 + 0.64\ x$; $r = 0.64$, $P < 0.001$, $N = 32$

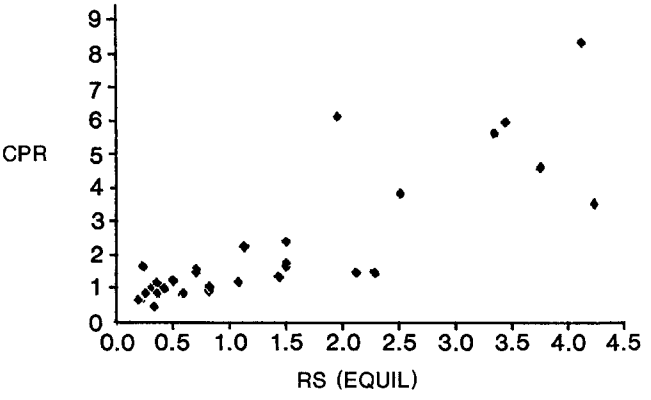

Fig. 2. RS vs CPR with respect to brushite. $y = 0.33 + 1.33\ x$; $r = 0.83$, $P < 0.001$, $N = 31$

is indicated by some methods as 1 and by others as 0. All those methods give useful estimates of the state of saturation. A comparison of CPR with RS in whole urine portions yielded a satisfactory fit (Figs. 1 and 2). The CPR gave somewhat higher values with respect to brushite (slope 1.33) and lower values with respect to calcium oxalate monohydrate (slope 0.64). Determinations of APR and CPR require the equipment for equilibration experiments [5], activity products are calculated either from data found with ion specific electrodes or from the chemical analysis of constituent ions in the solution.

The computer program EQUIL [1] gets increasing interest in experimental and clinical urolithiasis research [7, 8]. It allows the calculation of relative supersaturations for calcium oxalate monohydrate, apatite, brushite, struvite, and uric acid. Furthermore, the program yields the ionic concentrations, the ionic strength, and the activity coefficients. The ionic concentrations of the components in the solution are found by the iterative solution to self-consistency of all mass action and mass balance equations. EQUIL starts with a guess of 1/10 of the total concentration as ionic concentration and stops, when none of the estimates change by more than 0.1 percent. With the knowledge of ionic concentration, ionic strength and activity coefficients are calculated, which make possible the calculation of activities. The computer program is expanded continuously with respect to chemical species; in the software version of 1988, 23 components can be entered, and 103 formation constants are used for the calculation.

The high accuracy of the computation with EQUIL could be confirmed in equilibration experiments with a variety of experimental solutions [18]. The behaviour of nucleation and growth of crystals depends on the

relative supersaturation. A well-defined solution without inhibitors is at RS between 1 and 4 metastable with no spontaneous nucleation, but with crystal growth, when seeds are added to it. At RS 4-80 there is predominantly heterogeneous nucleation and at RS over 80 homogeneous nucleation.

It is obvious that results from calculations with EQUIL can only make sense when the chemical analyses of the components in urine or experimental solutions are reliable. Although the state of saturation in urine is governed by an unknown number of components, the constituent ions most important with respect to RS seem to be included in EQUIL [8]. The use of such a computer program also allows the a priori calculation of the composition of a solution at any desired state of saturation. The impact on RS of individual components can be determined by these computations.

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Some methods to monitor crystallization in full-concentration urine

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It is generally accepted that a rational prophylactic therapy for patients with recurrent calcium stone disease should be based on a thorough understanding of the mechanisms leading to crystal formation and subsequently the development of a stone. Therefore considerable efforts have been devoted to develop methods making it possible to measure, in undiluted urine, on a routine basis the crystallization and inhibitory properties of urine.

A system was initially described for measurements of the crystallization risk in only slightly diluted urine in which the increment in oxalate concentration necessary for formation of 100 crystals in the size range 3.5–5 μm was the basis for calculation of the calcium oxalate crystallization risk (CaOx-CR). As could be anticipated there was a fairly good relationship between CaOx-CR and AP(CaOx)-index, an approximate estimate of supersaturation obtained from analysis of calcium, oxalate, citrate, and magnesium. Unfortunately determination of CaOx-CR is a laborious technique, requiring some preparation of the urine. This system measures the combined effects of supersaturation, nucleation, promotion, and growth. However it can be anticipated that the result will be influenced by the initial phase of the crystallization process, which determines whether many small or few large crystals will form. In a situation where many small crystals ($<$ 3.5 μm) initially forms, more oxalate has to be added for these crystals to grow to a size above 3.5 μm. It is therefore likely that subsequent interpretation of crystal size distribution will be difficult.

Addition of different urine constituents and analysis in whole urine systems in terms of nucleation and crystal growth disclosed the most pronounced effects for citrate and magnesium, an effect that at least to a large extent was explained by an effect on AP_{CaOx}, as calculated by the EQUIL II program.

These and other results suggest that the effects of growth inhibitors is high in urine, but when comparison is done between stone formers and normal subjects the differences usually are small. This is exemplified by estimates of CaOx-CR, analysis with seed in diluted urine, and determination of crystallization in dialyzed urine with ^{14}C-oxalate.

Unfortunately the CaOx-CR-method cannot be performed with isotope technique because of the

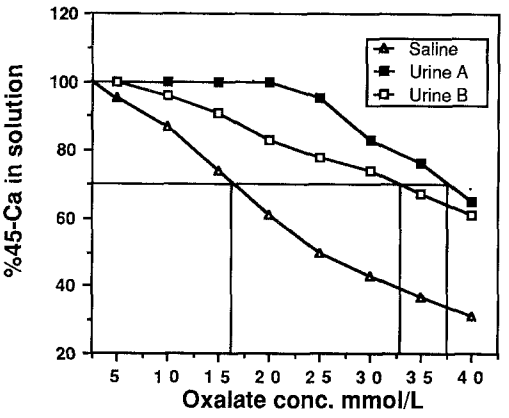

Fig. 1. Crystallization of calcium oxalate (UMM)

variability in specific radioactivity due to different Ca and Ox concentrations.

Inasmuch as the urinary macromolecules apparently play a mayor role in the crystallization events such as crystal growth, maybe even more in aggregation, and possibly also in nucleation we have continued our studies by using dialysed urine. To fractions of dialysed urine sodium oxalate in different concentrations was added together with a standardized amount of $CaCl_2$ labeled with ^{45}Ca. The amount of isotope remaining in solution 30 minutes after oxalate addition was recorded and the curves for saline and urine samples as shown in Fig. 1. The system can be used even with seed crystals and the oxalate concentration corresponding to 70% ^{45}Ca values might be used for description of the crystallization properties.

No large clinical series have so far been completed with this system but evidently urine of different concentrations affect the crystallization differently. Supersaturation of seeded and unseeded dialysed urine results in similar curves of CaOx crystallization but at different levels of supersaturation.

In an attempt to make comparisons more easy, heparin in varying concentrations was added to different systems. The first system studied was that for inhibitory measurements in 1–2% urine concentration. Increased concentrations of heparin resulted in increased inhibitory activity. Heparin added to a system for determination of CaOx-CR had no effect in the presence of urine, but reduced the risk considerably when only saline was used. The presence of high concentrations of heparin also appeared to increase the fraction of small crystals, supporting the view that macromolecules might play a role also in the nucleation process. Heparin with and without seed crystals and with increased oxalate resulted in reduced rates of crystallization. Although heparin is not a natural inhibitor it has pronounced inhibitory properties and can be used for comparison of different systems.

In summary our results with dialysed urine appears attractive to measure problems related to urinary macromolecules. If such systems will be of clinical value can only be decided with large clinical studies.

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Preparation and application of calcium oxalate dihydrate crystal seeds*

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Calcium oxalate dihydrate (COD) is found in approximately 50% of all urinary stones [1] and is the most common form of calcium oxalate crystals in urine of patients with idiopathic calcium urolithiasis [2]. In order to define factors favouring the precipitation of COD, an experimental study was carried out [3].

The artificial reference urine according to Burns and Finlayson [4] was taken as experimental solution and contained 0.1055 M NaCl, 0.0323 M NaH_2PO_4, 0.00321 M $Na_3C_6H_5O_7$, 0.00385 M $MgSO_4$, 0.01695 M Na_2SO_4, and 0.0637 M KCl. NH_4OH or HCl were added for pH 6.5. After preparation of this solution, $CaCl_2$ and $Na_2C_2O_4$ were given to it at different amounts and at different ratios. The mixtures were left for 15 minutes at 22°C or 37°C. The produced crystals were analyzed with light microscopy and scanning electron microscopy.

Precipitation of COD was more likely at 22°C than at 37°C. Its production was favoured at high calcium-to-oxalate concentration ratios and at low relative supersaturations (RS) [5]. Exclusive precipitation of

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COD was observed at 37°C with calcium-to-oxalate ratios between 3 to 5 and with RS values less than 25. At 22°C, COD was found exclusively with ratios between 5 to 8 and RS values less than 49. An increase in RS promoted at both temperatures the precipitation of calcium oxalate monohydrate (COM). The size of the individual COD crystals varied with RS, but it was 10–20 μm in most preparations. The specific surface area was determined by two points BET measurements (Quantasorb, Quantachrome Corp.) which yielded 0.82 m^2/g for COD.

Reproducible production of pure COD crystal seeds is possible in artificial urine. The in vitro finding of increased COM precipitation with increasing RS and decreasing calcium-to-oxalate ratio compares well with the clinical experience, that patients with hyperoxaluria exhibit COM as predominant urinary crystal [2]. For experiments with COD crystal seeds, two features must be taken into account, i.e. higher solubility than COM and transformation to COM. Based on the data from Lepage and Tawashi [6], the calculation of RS with EQUIL [5] yields a value of 1.96 for COD at equilibrium. In contrast to this, RS is 1.0 for COM at equilibrium. The transformation from COD to COM, which might take place within hours, can be explained by dissolution of COD and secondary nucleation of COM [6, 7].

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Problems with kinetic crystallization tests in whole urine

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Measurement of the rate of calcium oxalate (CaOx) crystallization in whole urine samples is highly desirable for the evaluation and follow-up of patients with recurrent calcium stone disease. The explanation for the fact that such methods usually are not available on a routine basis should be sought in a number of analytical problems.

Determination of isotope remaining in solution at different times of the crystallization process only to a limited extent can be applied. This is attributable to the large variation in specific radioactivity resulting from variable Ca and Ox concentrations. The results with crystal counting therefore might be more reproducible, save for the problems associated with measurement of crystals with a diameter below 2–3 μm. Additional problems might occur with impurities in the sample.

Preparation of urine appears to be one critical step in the analytical process inasmuch as undissolved crystals might both give a falsely low level of supersaturation and bind inhibitory and/or promoting substances. Acidification in order to dissolve CaOx and calcium phosphate crystals might have a negative influence on different urinary macromolecules. Furthermore it is difficult to predict the effect caused by precipitated uric acid in the acid urine. According to recent observations centrifugation, filtration and even sieving of whole urine significantly can reduce the concentrations of macromolecules important for the crystallization process.

In order to get reproducible results a number of analytical variables need to be strictly controlled. It thus is important to maintain a constant stirring rate and temperature. The pH is of great importance for the crystallization kinetics as is the way in which the sample is brought to supersaturation. Bad quality of the glass-ware as well as the possible presence of impurities might result in heterogenous crystallization. The size and number of the initially formed crystals in a seed-free system might have a significant effect on the subsequent process.

Another as yet not fully understood process is the variable lag phase seen in seed-free systems, probably a reflection of the balance between supersaturation and promotion/inhibition. The mechanisms determining whether CaOx monohydrate or dihydrate will form are also less well-known.

Of major concern in all analytical systems dealing with crystallization properties in whole urine is how different the crystallization properties are between proximal (tubular, pelvic) and distal (bladder) urine.

In order to develop useful methods to analyze crystallization properties in a clinically useful way it is important to standardize several different variables. It is thus very important to decide on the duration of the collection period and when during the 24 h period this collection should be performed. How should urine be stored during this period and until the time of analysis? Inasmuch as contamination with bacteria makes the sample useless, a preservative probably is necessary, a substance that not in any way does affect the crystallization. How should urine best be prepared: by sieving, filtration, or centrifugation? Is whole urine, ultrafiltered urine, or dialysed urine the best sample?

At present there is not full information to provide an answer to these questions. However, in our department an experimental program is carried out with night-urine collected between 22.00 and 6.00 h, under normal dietary and drinking conditions. Sodium azide is used as preservative. The sample is brought to the laboratory within 2 h after collection for pH-determination. One aliquot is used for analysis of urine variables necessary for calculation of AP(CaOx)- and AP(CaP)-index. The rest of the sample is divided in two parts, one for determination of CaOx-CR, and one for kinetic and crystal size analysis following dialysis. The results of this series of analyses will show whether such a program might be of clinical value.

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A new spectrophotometric method for measuring calcium oxalate monohydrate crystal aggregation in the absence of supersaturation: inhibitory effects of urinary glycoproteins

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To date, calcium oxalate monohydrate (COM) crystallization measurements in full urine do not clearly distinguish between nucleation, growth and aggregation of crystals, nor are they able to quantify separately influences of promoters, inhibitors and chelators on these processes. Based on the fact that aggregation in equilibrated suspensions of COM crystals is more prominent at lower stirring rates[1], crystal aggregation in vitro was measured spectrophotometrically without simultaneous nucleation and growth.

Materials and methods: COM crystals were prepared by mixing calcium chloride and sodium oxalate at 4°C, and confirmed as pure COM by infrared spectroscopy. The dry crystals were passed through a 5 μm pore diameter nylon sieve before experiments. The same batch of crystals was used throughout all experiments. COM crystal slurries (0.8 mg/ml) were equilibrated for 16 h under constant fast stirring (1,100 rpm) at 37°C. Crystals were aggregated by slow stirring (500 rpm) in a spectrophotometric cuvette (2 ml) for 180 s; thereafter, spontaneous sedimentation of crystal aggregates was monitored for 300 s. Time course readings of OD_{620} were performed; OD_{620} was linearly correlated to the amount of dry crystals/volume ($P<0.001$). Rate of aggregation was measured by the rate of decrease of turbidity (turbidity slope, T_s) during particle sedimentation, as it reflects terminal particle velocity (v_t). Assuming ideal spheres of radius r, v_t is proportional to r^2. Since the sedimentation distance, L, is known, v_t was derived as L/t, and r was calculated using the formula $v_t=(2/9)(1/\eta)(\delta_p-\delta_1)^t r^2 g$. Scanning electron microscopy (EM) was performed on COM crystal slurries before and after slow stirring, and aggregates ≥ 15 μm and ≥ 20 μm diameter were counted on photographic enlargements on entire EM fields.

Aggregation inhibition by Nephrocalcin (NC) isolated from urines of normal controls (n NC) and calcium oxalate kidney stone formers (sf NC) and by Tamm-Horsfall glycoprotein (THP) from normals as well as by citrate (CIT) was studied at pH 7.2 (10 mM Tris-HCl)/90 mM NaCl. Furthermore, 24 h urines of 5 male controls and 5 males with severe idiopathic calcium oxalate stone disease (> 20 stones) were studied; urines were centrifuged for 15 min at 8,042 × g, and supernatants were diluted to a standard 24 h-urine volume of 2,750 ml (highest volume excreted by 1 of the subjects) by adding buffer solution (pH 7.2/90 mM NaCl). Aggregation inhibition was measured in COM crystal suspensions containing 10 vol% of volume-corrected urines and 90 vol% of buffer solution. All experiments are at least in triplicate, values are mean ± SEM. Percent of crystal aggregation are calculated as $100\times(T_s/T_{sC})$, where T_{sC} is the control (no inhibitor added). Inhibition data are presented in % (100 – % aggregation).

Results: Slow stirring induced crystal aggregation; after slow stirring, T_s was $30 \pm 1 \times 10^{-4}$ s^{-1}, compared to $7 \pm 0.5 \times 10^{-4}$ s^{-1} ($P < 0.001$). Numbers of crystal aggregates ≥ 15 µm on entire EM fields (sample volume 6 µl) were 27.5 ± 0.5 before and 120.5 ± 1.7 after slow stirring ($P < 0.001$); corresponding values for aggregates ≥ 20 µm were 6.5 ± 0.2 and 41.8 ± 0.9, respectively ($P < 0.001$). Calculated average particle radius' were 2.00 µm without and 4.15 µm with previous slow stirring of the COM crystal suspension.

Glycoproteins inhibited COM crystal aggregation in a concentration dependent manner. THP was the strongest inhibitor; at 2×10^{-7} M, aggregation inhibition was 93%, at 2×10^{-9} M still 34%. At 2×10^{-7} M, n NC inhibited by 90%, sf NC by 32%; inhibition by n NC was lost below 1×10^{-8} M. Under the saturated conditions of this system, CIT at micromolar as well as at millimolar concentrations failed to inhibit COM crystal aggregation. Inhibition by 10 vol% of urines from normal controls was 43.5 ± 1.9% vs. 31.6 ± 3.4% by stone-former urines ($P < 0.01$)

Conclusions: 1. COM crystal aggregation in vitro can be quantified by the rate of decrease of OD_{620} during spontaneous sedimentation of equilibrated crystals aggregated by slow stirring (500 rpm) at 37°C. 2. The method allows to measure COM crystal aggregation inhibition by isolated urinary glycoproteins; it can be used to identify functionally abnormal urinary aggregation inhibitors. 3. The method can be used to measure COM crystal aggregation inhibition by 10 vol% urine; urines of severe calcium oxalate stone formers are less inhibitory than urines from normals ($P < 0.01$).

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Summary and conclusions of discussions

The participant of the workshop agreed that the study of crystallization processes in whole urine is of the upmost importance in stone research. Major problems arise from the control of the test conditions and from the controversy regarding which units do best express results.

Urine collection and conservation

Difficulties already begin to arise with the collection and conservation of urine. Selected urine portions during the day may be more conclusive for stone formation than 24 hour urines. In most studies urine was immediately frozen after collection. This procedure allows one to perform multiple and time consuming tests. However, freezing enhances crystallization and care has to be taken not to loose inhibitors by centrifugation and filtration. Acidification or other procedures to dissolve crystals may alter urinary macromolecules. Several authors use thymol, sodium azide, boric acide or neomycin to prevent bacterial growth. However, the influence of these substances on crystallization processes is only partially known. Studies to determine optimal modes of urine collection and conservation are therefore urgent.

Measurement of urinary saturation and expression of results

To obtain reproducible results, the test conditions have to be strictly controlled. One of the most important factors is urinary supersaturation. Urinary supersaturation can be measured either by equilibration experiments or by computation of the results of extensive chemical analyses. Urinary inhibitors make it difficult to reach the true balance between stone forming salts and urine. Computer calculation using the EQUIL-Program, which is generally available now, therefore seems to be the most accurate method of determining the state of urinary saturation, provided that chemical analyses are correct. Determination of urinary oxalate is still most difficult. The state of urinary saturation can be expressed in at least 7 different ways. Confronted with this confusion in terminology, the workshop felt that results might best be expressed in terms of

ionic activities or activity products. In order to get comparable values of crystal growth rates, these values also have to be calculated from ionic activities.

Monitoring crystallization processes

Crystallization processes can be monitored in urine chemically, isotopically, optically or by a Coulter Counter. Coulter Counter analysis is laborious and can not measure crystal sizes under 2 μm. Optical technics require relatively high crystal densities. Isotopical methods are limited in urine by a large variation in specific radioactivity resulting from variable calcium and oxalate concentrations. Chemical analysis or ion selective electrodes, the latter allowing continous measurements, therefore seem to be prefered methods for monitoring the crystallization processes in urine.

Preparation of crystal seeds

Crystallization processes can be induced by increasing urinary supersaturation or by the addition of crystal seeds. At this workshop methods for preparing calcium oxalate dihydrate crystals were presented. However, for reasons of instability of these crystals, calcium oxalate monohydrate will be used further more in most crystallization tests. The audience could not decide whether calcium oxalate monohydrate too should be prepared by special technics or whether commercially available crystals should be used. But there was an agreement that the quality of seeds should always be chequed by the measurement of growth rates under standard conditions.

Special test systems

In most test systems endpoint measurements have been performed with sufficient reproducibility. Kinetic crystallization tests in whole urine pose several problems which have been pointed out in a special paper published in this report. A clinically practical method for determining crystal aggregation in whole urine must be found. At the workshop a new spectrometric method for measuring crystal aggregation was presented. However, like other approaches, it is based on saturated solutions with respect to calcium oxalate, a state rarely found in urine.