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METHODS FOR THE STUDY OF CALCIUM OXALATE CRYSTALLISATION AND THEIR APPLICATION TO UROLITHIASIS RESEARCH

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Abstract

Many methods have been used to study calcium oxalate crystallisation. Most can be characterised by changes in supersaturation during the experiment, which may increase, remain constant or decay. Their ability to quantify various aspects of crystallisation often reflects the extent to which nucleation, growth and aggregation can be measured independently, when two or three of these processes may be occurring simultaneously.

The mixed suspension, mixed product removal technique reaches a steady state supersaturation, is a good model for intrarenal crystallisation and allows both growth and nucleation rates to be measured. Using 92% urine and comparing control urines with samples from recurrent stone formers no difference in growth rates was found but the controls had higher nucleation rates ($p=0.003$) and lower supersaturations ($p=0.001$). In parallel crystallisers running simultaneously, heparin or hyaluronic acid addition to 92% urine was studied. Both macromolecules increased growth rates, decreased nucleation rates and increased supersaturation ($p<0.05$).

The steady state supersaturation achieved in this system may be an important determinant of stone forming potential. The ability to reach a lower urinary supersaturation by increased nucleation may be a crucial protective factor distinguishing non stone formers from stone formers.

KEY WORDS: Calcium oxalate, crystallisation, urolithiasis, crystal nucleation, crystal growth.

Introduction

Many different methods have been used to study calcium oxalate crystallisation, some differing in technical detail and others at a more fundamental level. Alternative approaches enable different types of measurements to be made which may be most appropriate for particular applications to urolithiasis research; on the other hand, lack of standardisation of methods used may be at least partly responsible for contradictory results reported from apparently similar experiments (Ryall et al., 1986a).

In order to appreciate the potential of different methods, it is important to remember the underlying processes involved in stone formation. These essentials have been well described (Finlayson, 1978; Ryall, 1989) as crystal nucleation, crystal growth (by precipitation of fresh crystalline material from the surrounding liquor) and crystal aggregation. Inhibition or promotion of any of these processes in urine will reduce or enhance the likelihood of stone formation and have therefore become the focus of many *in vitro* urolithiasis studies (Fleisch, 1978). Supersaturation is a prerequisite for crystallisation. Two states of supersaturation have been identified, a metastable state where crystallisation does not occur unless perturbed by the addition of seed crystals and an unstable state where crystallisation occurs spontaneously. A metastable solution of calcium oxalate can be forced into an unstable state by the addition of more calcium or oxalate in the form of dissolved salts.

This paper will review the more commonly used *in vitro* techniques, discuss the principles by which they operate, their strengths and weaknesses and identify their role in the study of calcium oxalate crystallisation and stone formation. It will also include some new results obtained with a continuous flow method, modified to be applicable to nearly whole urine.

Review of Methods

Background Comments

Methods for studying calcium oxalate crystallisation can broadly be divided into three main types, depending on how the supersaturation changes during the course of the experiment (Fig. 1). Firstly, the supersaturation may be allowed to decay; here crystallisation is induced and allowed to proceed without any further additions and the supersaturation of the solution decreases as calcium oxalate crystallises. Secondly, the supersaturation may be increased. In this approach, initially stable solutions are concentrated to induce crystallisation, with the aim of reproducing the way the kidney brings about supersaturation. In the third approach, constant supersaturation is maintained. Calcium and oxalate are continuously added, again with the intention of generating a better model of intrarenal crystallisation than supersaturation decay systems.

Each of these three distinct approaches can be applied in many different ways with different aims. Crystallisation may be allowed to occur from simple salt solutions, complex mixtures which mimic urine or urine itself. When urine is included it may be very dilute, undiluted or concentrated. The objectives of these experiments are to better understand the physico-chemical processes, to study the effects of certain well defined chemical components and to investigate the influence of urine derived macromolecules and/or to compare urines from different patients. A possible application which has not been widely used is to examine the value of potential therapeutic agents *in vitro* with the urine of individual stone forming patients in order to identify those most likely to benefit from a particular treatment regime. This could be particularly fruitful bearing in mind that any prophylactic regime may have to be pursued for many years. After all, the purpose of these experimental studies is to prevent stone disease.

A variety of parameters may be measured by different techniques, for example, the metastable limit (onset of nucleation), nucleation rate, growth of crystal mass as a whole or distinct from nucleation, growth of crystal size which may include aggregation effects, kinetic rate constants, inhibitor/promoter activities and Langmuir adsorption isotherms. Apparently similar parameters measured by different methods may yield different results and have quite distinct meanings. On the other hand, different groups have used different nomenclature and formulations to express equivalent or similar parameters.

Supersaturation Decay Systems

Seeded Crystalliser Systems (Fig. 1, curve a)

General comments. One of the most common approaches to the study of calcium oxalate crystallisation uses a metastable supersaturated solution of calcium oxalate in which crystallisation is initiated by the introduction of calcium oxalate monohydrate seed crystals. The crystallisation process can be monitored by observing the size and number distribution of the particles in suspension (Robertson & Peacock, 1972; Robertson et al., 1973) or by following either calcium (Meyer & Smith, 1975a) or oxalate (Ito & Coe, 1977; Ligabue et al., 1979) depletion from solution or calcium or oxalate incorporation into the solid phase (Will et al., 1983). Radioactive calcium or oxalate or conventional chemical analysis may be used or the reaction can be followed by conductivity (Nancollas & Gardner, 1974), calcium ion electrode (Baumann et al., 1990) or absorbance due to oxalate at 214nm (Nakagawa et al., 1987).

In such seeded crystalliser systems, nucleation should not occur, while both crystal growth and aggregation may be taking place simultaneously. When the particle size and number distribution is monitored then both crystal growth (precipitation of fresh solid phase) and aggregation will be reflected in the results, but when calcium or oxalate removal from the solution or incorporation into the solid phase is followed, only crystal growth is usually considered.

A frequent aim of crystallisation studies is to quantify the ability of urine or particular components to inhibit some aspect of the crystallisation process. This is often achieved by comparing results under test conditions (R_{-test}) with a control experiment (R_{-ctl}) and defining the % inhibition as

$$I\% = (1-(R_{-test}/R_{-ctl})) \times 100 \quad (1)$$

or

$$I\% = ((R_{-ctl}-R_{-test})/R_{-ctl}) \times 100 \quad (2)$$

It should be noted that equations 1 and 2 are equivalent.

Growth and aggregation considered as a combined process. When particle size analysis of seeded crystalliser systems was first applied in the context of kidney stone research, no attempt to distinguish growth and aggregation processes was made. Instead these were considered as combined effects by measuring the increase in volume of only those particles greater than 20μm, 4 hours after seeding. Solutions containing test inhibitors (Robertson et al., 1973), 5% urine (Robertson & Peacock, 1972) or 1% urine (Robertson et al., 1976) were then compared against a saline control, allowing the degree of inhibition, I%, to be calculated.

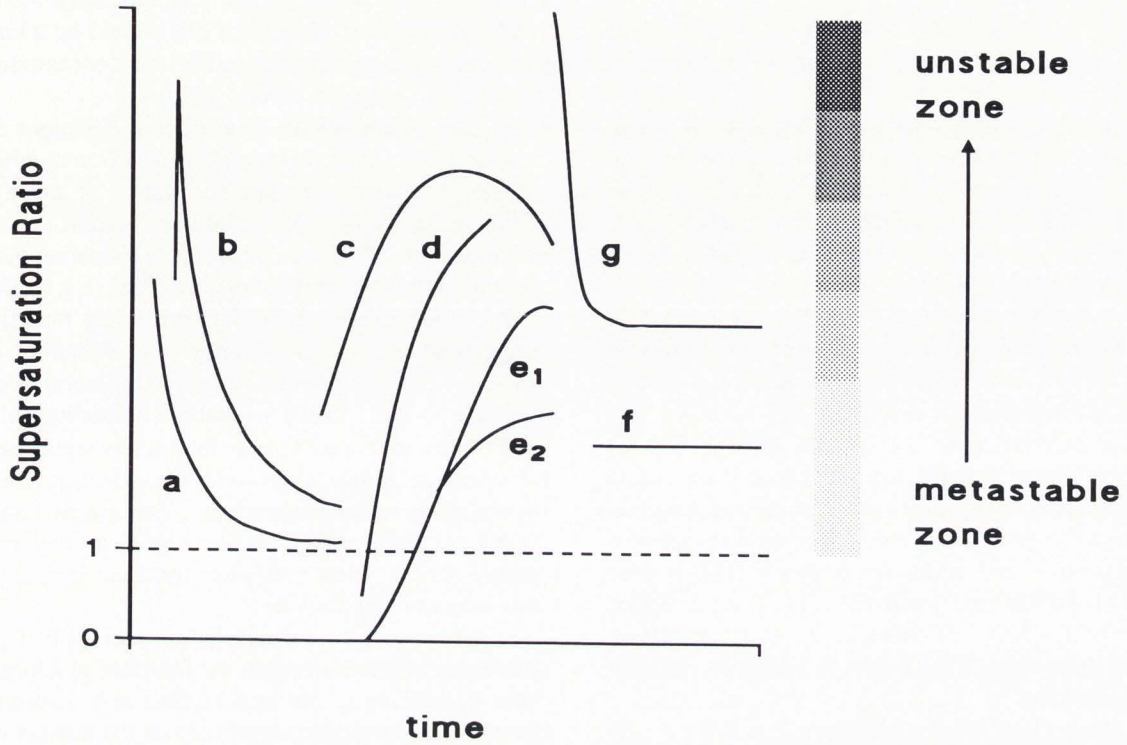

Figure 1. Diagrammatic representation of supersaturation changes that could occur in different methods for studying calcium oxalate crystallisation; **a**, seeded, metastable system with supersaturation decay; **b**, supersaturation raised above the metastable limit and allowed to decay; **c**, supersaturation increased by evaporation; **d**, supersaturation increased by reverse osmosis; **e**, supersaturation increased by diffusion (e_1, unseeded, e_2, seeded); **f**, constant composition system; **g**, mixed suspension, mixed product removal method. The grey scale represents the ill defined boundary between metastable and unstable supersaturation.

Growth and aggregation considered as separate and independent processes. Ryall et al. (1981a) showed that a given value of I% could result from very different combinations of growth inhibition and aggregation inhibition. This was achieved by considering growth and aggregation to be independent of each other and treating volume changes as being due to growth and changes in particle numbers as a result of aggregation. They applied this approach to dilute urine (2 to 10%) (Ryall et al., 1981b) and to 1% urine and various inhibitors (Ryall et al., 1981c). They found that the time (t) course of volume changes (ΔV) and particle number changes (ΔN) were non-linear, which led them to criticise the use of an arbitrary fixed time for comparing control and test experiments (Ryall et al., 1981b and 1981c). They overcame this problem by plotting $t/\Delta V$ against t and $t/\Delta N$ against t, which gave straight line plots, from which the initial rates ($\Delta V/t_{t=0}$ and $\Delta N/t_{t=0}$) were found from the reciprocals of the intercepts on the y axes at t=0. Inhibition of growth (I_g%) and aggregation (I_a%) were then calculated by comparing test and control data.

This approach was elaborated upon, to take into account particles growing or aggregating into or out of the size range measured by the particle analyser (Ryall, RG et al., 1986) and has been applied to experiments including 1% urine or inhibitors (Ryall, RL et al., 1986b).

Growth considered as an independent process. Some workers have considered growth and aggregation in seeded systems to be independent of each other and have therefore studied crystal growth kinetics simply by following calcium or oxalate changes. In crystal seeded metastable systems, calcium oxalate crystallisation is

usually found to follow second order reaction kinetics with the rate of reaction being proportional to the square of the driving force for crystallisation (relative supersaturation, RS),

$$-dRS/dt = K(RS-1)^2 \quad (3)$$

where K is a rate constant (Finlayson, 1978; Curreri et al., 1981).

Generally, when working within restricted ranges of conditions, it has been possible to simplify equation (3) to calcium activities (Nancollas & Gardner, 1974; Meyer & Smith 1975a), calcium concentrations (Ito & Coe, 1977) or oxalate concentrations (Ligabue et al., 1979), for example,

$$-dM/dt = K([M_t]-[M_{t=eq}])^2 \quad (4)$$

where $[M_t]$ and $[M_{t=eq}]$ are the solution concentrations or activities of calcium or oxalate at time t and at equilibrium, respectively.

This description of the crystal growth kinetics allows quantitative comparisons of related experiments to be expressed in various ways. A simple approach is to measure the decrease in calcium or oxalate between the initial time and some fixed time ($\Delta[M]_t$). (For example, t=40 minutes, Ito & Coe, 1977; t=2.5 hours, Ligabue et al., 1979; t=4 hours, Koide et al., 1981; t=1 hour, Fellström et al., 1982). The % inhibition can then be calculated as,

$$I\% = (1-(\Delta[M]_{t\_test}/\Delta[M]_{t\_ctl})) \times 100 \quad (5)$$

or when a rate constant (K) is calculated, as,

$$I\% = (1-(K_{-test}/K_{-ctl})) \times 100 \quad (6)$$

In order to calculate the rate constant K (in equation 4), some estimate of $[M_{t=eq}]$ can be made and calcium or oxalate measured at time intervals. K can then be conveniently obtained from the slope of the straight line plot of $1/([M_t]-[M_{t=eq}])$ against t. Meyer & Smith (1975a) and Ito & Coe (1977) measured the solubility product under their experimental conditions by allowing dissolution or crystal growth experiments to reach equilibrium and used the average of these values to calculate $[M_{t=eq}]$. The amount of calcium or oxalate at a fixed time has been taken to approximate to the equilibrium position. (For example, 4 weeks, Gill et al., 1977; 24 hours, Ligabue et al., 1979; 3 hours, Fellström et al., 1982). An alternative is to use nonlinear regression to the [M] versus t data to give estimates of K and $[M_{t=eq}]$ (Nakagawa et al., 1981, 1985). Rather than collect data at many time points, a single time and the equilibrium value can be used to obtain a simplified estimate of K, as,

$$K = \Delta[M_t]/[M_{t=eq}] \quad (7)$$

as before, different methods of estimating $[M_{t=eq}]$ are possible (Gill et al., 1977; Tiselius & Fornander, 1981).

However they are calculated, these rate constants are empirical and will reflect particular experimental conditions such as temperature (usually 37C), ionic strength and the quality and surface area of the seed crystals. If a tested inhibitor significantly associates with calcium or oxalate, then this should be taken into account by using activities rather than concentrations in equation 4 (Meyer & Smith, 1975b).

The % inhibition obtained from equations 5 or 6 can be used to make comparisons of different inhibitors at the particular concentrations tested, or to infer the relative amounts of inhibitor under particular circumstances. This can be useful in following inhibitor activity through a purification procedure (e.g. Ito & Coe, 1977; Nakagawa et al., 1981), examining the effect of some treatment of an inhibitor (e.g. Fellström et al., 1982) or comparing urines at a fixed dilution (e.g. 2%, Ligabue et al., 1979). When comparing different inhibitors and trying to gauge their likely significance *in vivo*, it is important to consider the extent of inhibition over a range of concentrations. The use of Langmuir adsorption isotherms can be helpful in this respect, particularly as this can give some insight into the mechanism of inhibition.

Langmuir adsorption isotherms will apply if growth inhibition is achieved when the inhibitor blocks growth sites by binding to the seed crystals and adsorption of further inhibitor is dependent only on the number of free sites available, in which case simple saturation kinetics will be obeyed,

$$\Theta = k[I]/(1+k[I]) \quad (8)$$

where Θ is the proportion of sites occupied, k is an adsorption coefficient and [I] is the inhibitor concentration. If the crystal growth rate is proportional to the number of free sites $(1-\Theta)$ then

$$[I] = (K_{ctl}-K_{test})/kK_{ctl} + 1 \quad (9)$$

or

$$[I] = (K_{ctl}-K_{test})/kK_{test} \quad (10)$$

and a plot of $K_{ctl}/(K_{ctl}-K_{test})$ against $1/[I]$ (e.g. Meyer & Smith, 1975b; Nakagawa et al., 1981)), $K_{test}/(K_{ctl} - K_{test})$ against $1/[I]$ (e.g. Nakagawa et al., 1985) or $Ln((K_{ctl} - K_{test})/K_{test})$ against Ln[I] (Kok et al., 1986a) will be a straight line with k obtained from the slope (first two plots) or intercept (log plot). A good fit of experimental data to such a line supports the mechanism of inhibition of blocking growth sites by reversible binding and k is an indication of the potency of the inhibitor, being the concentration required to halve the growth rate in the absence of inhibitor. Meyer & Smith (1975b) proposed this method for comparing the inhibition due to different components and for quantifying the inhibition of different urine samples. This would require measuring rate constants (K_{test}) for

a variety of dilutions of urine and has been applied to urine with and without various inhibitors (Werness et al., 1981)

When urine has been studied in seeded crystallisers, it has usually been diluted to between 1% and 10%. This is because of the high inhibitory power of urines in these systems and the requirement to have the initial supersaturation close to control values, which is not simply a matter of matching initial calcium and oxalate concentrations. Baumann et al. (1990) have tried to overcome these problems in a study using whole urine. In order to promote sufficient and rapid crystallisation, they seeded their system with a high seed concentration and followed the reaction with a calcium ion electrode. They evaluated their results as h, the time taken to reduce the calcium ion activity to half the decrease over 20 minutes, by which time the reactions appeared to be complete. The inhibitory effect of urine or inhibitors was calculated as the increase in h compared to a control experiment (h_{test}/h_{ctl}), which appeared not to be strongly dependent on the urinary concentration of calcium or oxalate.

Growth and aggregation considered separately but not independently. The studies described above (and many others) have taken the changes in calcium or oxalate during the course of the experiment to be representative of crystal growth only. This assumption has been questioned (Blomen et al., 1983). In a thorough series of experiments Will et al. (1983), Blomen et al. (1983) and Bijvoet et al. (1983) have developed a general equation for calcium oxalate growth in a metastable solution, seeded with calcium oxalate crystals. In their system ^{45}Ca is included in the metastable solution and its incorporation into the solid phase is quantified at intervals and represented by the fractional uptake (U_t) at time t,

$$U_t = ([M_{t=0}]-[M_t])/[M_{t=0}] \quad (11)$$

where $[M_{t=0}]$ is the initial calcium concentration. Second order kinetics were observed as previously, expressed as,

$$dU/dt = U_\infty/t_m((U_\infty-U_t)/U_\infty)^2 \quad (12)$$

where U_∞ is the maximum fractional uptake of calcium that would occur for a particular experiment. The time taken for the uptake to reach 50% of this value is t_m. (This therefore corresponds to the half time, h used by Baumann et al., 1990). U_∞ can be obtained as the reciprocal of the slope of the straight line plot of t/U_t versus t, while t_m is found from the intercept on the t/U_t axis (t_m/U_∞) (Will et al., 1983). If U_∞ is comparable to $[M_{t=eq}]$ in the same way as U_t and $[M_t]$ are related by equation 14, then the term $((U_\infty-U_t)/U_\infty)^2$ in equation 12 is proportional to $([M_t]-[M_{t=eq}])^2$ in equation 4 and U_∞/t_m is an empirical rate constant related to K. In the following two papers of this series (Blomen et al., 1983 & Bijvoet et al., 1983), the relationships between U_∞/t_m and the crystal seed concentration, the initial supersaturation, ionic strength and calcium to oxalate ratio were explored. This led to the formulation of a comprehensive explanation of the growth rate,

$$dU/dt = k_A s[U_\infty] \times ([Ca_{t=0}]/[Ca_{t=eq}]+[Ox_{t=0}]/[Ox_{t=eq}])^2((U_\infty-U_t)/U_\infty)^2 \quad (13)$$

where s is the crystal seed concentration, k_A is the crystal growth constant and $[U_\infty]$ is maximum fractional uptake predicted from the initial and equilibrium concentrations of calcium and oxalate. Considering equations 12 and 13, the empirical rate constant for a particular set of experimental conditions is explained,

$$U_\infty/t_m = k_A s[U_\infty] \times ([Ca_{t=0}]/[Ca_{t=eq}]+[Ox_{t=0}]/[Ox_{t=eq}])^2 \quad (14)$$

and rearranging this k_A is found from equation 15 (Kok et al., 1988).

$$1/k_A = ([U_\infty]st_m/U_\infty) \times ([Ca_{t=0}]/[Ca_{t=eq}]+[Ox_{t=0}]/[Ox_{t=eq}])^2 \quad (15)$$

The equilibrium concentrations ($[Ca_{t=eq}]$,$[Ox_{t=eq}]$) are found from a series of separate experiments which identify the minimum concentration at which ^{45}Ca uptake will occur (Blomen et al., 1983) and can be used to calculate $[U_\infty]$. U_∞ and t_m are obtained from the growth curve as described above (Will et al., 1983). Once k_A is found for test and control conditions the % growth inhibition is readily calculated.

The crucial difference between the approach of this group and previous studies is the distinction they draw between $[U_\infty]$ and U_∞. The difference between $[U_\infty]$ and U_∞ was most pronounced at low seed concentrations and has been attributed to aggregation (Blomen et al., 1983). They were able to quantify this effect by the parameter $[t_m]$, the time when uptake reaches 50% of $[U_\infty]$;

$$[t_m] = t_m([U_\infty]/([U_\infty]-U_\infty)) \quad (16)$$

which increases with inhibition of aggregation.

The suggested mechanism for the influence of aggregation on the initial supersaturation and hence on the growth kinetics by reducing U_∞ was that the solution included within aggregates might not be representative of the bulk solution and some of the crystal growth surfaces would be exposed to lower supersaturations than expected from the calcium and oxalate concentrations (Kok et al., 1988). Some justification for using $[t_m]$ to describe the aggregation during the progress of a crystal growth experiment comes from analysis of particle size distributions in the

same system (Kok et al., 1988). Further support can be seen by comparing Langmuir plots where k_A is used or when I% (as in equation 5) is calculated using the fractional calcium uptake at a fixed time (20min). Using k_A gives a linear plot over a wider range of inhibitor concentrations than does I% (Kok et al., 1986a).

Crystal growth and aggregation have been measured using this method to study the effect of inhibitors (Kok et al., 1988), of urine (20%) (Kok et al., 1986b, 1990a) and effects of dietary changes on urine (Kok et al., 1990b). Some difficulties in interpretation can arise if a compound is an inhibitor of both processes (for example, citrate) because they are not independent. A problem with $[t_m]$ as an indicator of aggregation is that the increase associated with inhibition of aggregation is not linear and $[t_m]$ would tend to become infinite at 100% inhibition of aggregation. Nevertheless, $[t_m]$ adds an extra dimension to the study of calcium oxalate crystallisation in seeded systems.

It is not yet clear how the interpretation of experiments might be affected where the effect of aggregation on crystal growth kinetics was not taken into consideration. Inhibition of aggregation might diminish the apparent growth inhibition estimated by these methods, with any effects expected to be more pronounced at low seed concentrations. The means of determining $[M_{t=eq}]$ can now be seen to be critical. In some cases $[M_{t=eq}]$ appears to correspond to $[U_\infty]$, in others to U_∞ and in others to an average of both. The effect of lack of independence between growth and aggregation may need to be examined on particle size distribution data, where the analysis assumed they were independent.

Concluding comments on seeded, supersaturation decay systems. Crystallisation with supersaturation decay, induced by crystal seeding, has been one of the most widely used techniques for studying calcium oxalate monohydrate crystallisation *in vitro*. It is usually performed in simple buffered saline and the accuracy and reproducibility that can be achieved make it very useful in investigating the interactions between possible inhibitors and the crystallisation processes of growth and aggregation, in the absence of nucleation.

The ability to fit crystallisation data from these experiments to simple equations gives confidence that the extrapolated rate constants and percentage inhibition values relate directly to some well defined physico-chemical process. To take full advantage of this care must be taken to be clear how rate constants and equilibrium values have been calculated or estimated.

The relevance of measurements made in saline or dilute urine to urinary stone formation has been questioned by many authors (Fleisch, 1978; Robertson et al. 1981; Ryall et al., 1986a). As methods have evolved, comparisons with previous studies becomes more difficult. It is unfortunate that similar terminology has sometimes been used to cover different measurements, while different but equivalent expressions of the same formulae may give rise to further confusion.

Non Seeded Crystalliser Systems (Fig. 1, curve b)

General comments. Crystallisation can be induced in simple salt solutions or urine by increasing the initial supersaturation usually by the addition of oxalate in solution. In these systems, nucleation, crystal growth and aggregation may all occur simultaneously as the supersaturation declines to an equilibrium value. The progress of the crystallisation can be followed, as before, by various means, such as measurement of calcium by specific ion electrode, calcium or oxalate determinations chemically or with radioactive tracers (Gill et al., 1977; Rose, 1975; Garti et al., 1980; Koide et al., 1981; Sarig et al., 1982), particle counting and sizing (Robertson et al., 1981; Ryall et al., 1985; Tiselius, 1985), microscopic evaluation of crystals (Grases et al., 1988), nephelometry (Sutor et al., 1979; Grases et al., 1988) or visual estimation of turbidity (Kohri et al., 1991).

The main problem with these methods is that interpretation of the measurements is more difficult than with seeded systems. This is because aggregation and growth will be dependent on the crystal surface area (which will increase as a result of nucleation and growth and decrease due to aggregation) and all three processes will depend, to differing degrees, on the declining supersaturation. In practice it is not possible to distinguish rigorously between these different aspects of crystallisation and attempts are not usually made to measure physico-chemically meaningful rate constants. As a consequence, the choice of experimental conditions is often arbitrary and direct comparisons between different methods can not necessarily be expected to be valid.

At the supersaturations usually found in urine, precipitation of calcium oxalate on surfaces and particles unavoidably present in the crystalliser (heterogeneous nucleation) is likely to predominate over direct formation of calcium oxalate nuclei (homogeneous nucleation) (Finlayson, 1978). When whole urine is used, even if filtered, a highly variable concentration of heterogeneous nucleators are likely to be introduced.

Metastable limits and nucleation tendency. The supersaturation at which spontaneous nucleation and

growth occur may be found by increasing the concentration of oxalate (Pak & Holt, 1976; Ryall et al., 1985; Tiselius, 1985) or oxalate and calcium (Gill et al., 1977; Kohri, 1991). This supersaturation is usually referred to as the metastable limit or as a formation product. This concept does not have a strong theoretical foundation (Finlayson, 1978) because it can not be equated to an exact calcium and oxalate activity product. Any estimate will depend on the method used to detect significant crystallisation and the induction time allowed for this precipitation to begin.

The initial burst of crystallisation will produce particles too small for detection directly, or for separation and quantification by chemical means. Detection of the onset of crystallisation is thus limited by the sensitivity of the method and will only be triggered when crystals have also grown (and possibly aggregated) sufficiently large to be measured. Therefore, a rapidly growing/aggregating suspension with a low nucleation rate may give a similar metastable limit to a rapidly nucleating, slowly growing/aggregating mixture.

The general approach to measuring a metastable limit is to set up aliquots with increasing supersaturation and determine the minimum value at which crystallisation is observed at a fixed time (for example, 3 hours - Pak & Holt (1976); 30 minutes - Ryall et al. (1985); 5 minutes - Kohri et al. (1991) and various times from hours to days - Pak et al. (1975) and Gill et al. (1977). It is to be expected that the measured metastable limit would decrease if the observation time is increased (as shown by Pak et al. (1975) and Gill et al. (1977)).

Tiselius (1985) has used a slightly more complex method of carefully timed addition of oxalate, observation (2 minutes later) and repeating the cycle with further additions of oxalate (5 minutes after the previous addition). This method was applied to filtered urine, diluted to a fixed creatinine concentration (5mM), or to 80% of its original concentration and the calcium oxalate crystallisation risk (CaOx-CR) was defined as the reciprocal of the oxalate concentration required to bring about an initial burst of crystallisation. Baumann (1988) has described a similar method to identify the minimum oxalate concentration required to induce precipitation; an oxalate solution is continually infused into a calcium solution or urine, with the crystallisation followed by nephelometry. Another variation, which takes into account the endogenous urinary calcium by comparison with an artificial urine standard curve, is the oxalate tolerance test (Briellmann et al., 1985; Hering, 1988).

The amount of calcium or oxalate that must be added to reach the metastable limit should depend on the initial supersaturation as well as other factors. It would be expected to decrease with increasing initial supersaturations which is supported by the results of Tiselius (1985) and Ryall et al. (1985 and 1986a). Pak & Holt (1976) take this into account by relating their measured formation product to an empirically determined activity product ratio (an estimate of supersaturation) and call this the Formation Product Ratio (FPR). Ryall et al. (1986a) expressed their results as both the rise in oxalate required to bring about crystallisation and the product of the concentrations of calcium and total oxalate (i.e. endogenous oxalate plus that added).

Measurements of metastable limits as an indicator of nucleation have been used to examine the effects of various additions to simple salt solutions (Pak et al., 1979), artificial urine (Gill et al., 1977), or whole urine (Ryall et al., 1991). Urines from different patients (Pak & Holt, 1976; Pak & Galosy, 1980; Ryall et al., 1985, 1986a; Tiselius, 1985) have been compared and modification of whole urine by ultrafiltration (Edyvane et al., 1987) or urate removal (Grover et al., 1990a) or urate addition (Grover et al., 1990b) have been studied.

Although these methods for studying nucleation tendency are not solely dependent upon nucleation they have the great advantage of being able to be applied simply to whole urine. Without taking into account the initial supersaturation, direct comparisons between samples may not be appropriate and practical variations in the applications of the methods are likely to exert considerable influence.

Crystallisation from above the metastable limit. The effect of inducing crystallisation by rapidly raising the supersaturation and then allowing it to decay may be evaluated by following the time course of events or by taking measurements at some arbitrary fixed time or when crystallisation is almost complete. The difficulty in interpreting these results in terms of the different aspects of crystallisation has meant that the conclusions often drawn are only general. For instance, Garti et al. (1980) compared the time course of calcium activity decay in artificial urine by adding inhibitors and concluded that retardation of crystallisation by these inhibitors is not identical with inhibition of crystallisation, as the same end point could be reached.

The extent of crystallisation at a particular time after initiation of nucleation can be used to give an indication of the crystallisation potential of artificial urine or dilute urine (Rose, 1975; Sutor et al., 1979) and the inhibitory activity characterised by comparison

with control experiments. Sarig et al. (1982) defined a discrimination index (D.I.) as the log of the ratio of calcium activity at two different times (0.5 minutes and 10 minutes),

$$D.I. = Ln([M_{t=0.5}]/[M_{t=10}]) \quad (17)$$

They used this to compare urines diluted to 10% of their initial concentration and to study the effect of urinary ultrafiltration (Azoury et al., 1985). The discrimination index was felt to reflect the inhibitory potential of different samples.

Rather than use a single time or pair of time points, Grases et al. (1988) used the initial rate of increase in turbidity in 89% urine as a measure of crystallisation and Bek-Jensen & Tiselius (1991) used an empirical integral of the decay in oxalate in solution over 30 minutes.

In a method designed to focus upon aggregation, Koide et al. (1981) allowed the crystallisation to continue for a much longer time (24 hours) after which they determined the proportion of the total oxalate precipitated (M_ppt) that was found in particles greater than about 20μm and used this to calculate a non aggregated ratio (NAR),

$$NAR = M\_ppt(>20\mu m)/M\_ppt \quad (18)$$

Ryall et al. (1985) have tried to obtain a better insight into the time course and nature of the non seeded crystallisation in urine by measuring particle size distributions. In this method whole urine is used and the amount of oxalate added to induce crystallisation was 0.3 mM above the previously determined metastable limit. They used the slope of the linear portion of the volume data as a measure of crystal growth rate. This is analogous to the treatment of particle size distribution data from seeded crystalliser experiments (Ryall et al., 1981c), but growth rates from the two systems should not be confused as the non seeded system includes nucleation effects. The changes in particle numbers can not similarly be ascribed simply to aggregation, as had been done before, again because of nucleation.

In a subsequent report Ryall et al. 1986a suggested that the total crystal volume after 90 minutes is another indicator of crystal growth and the peak size of the particle distributions gives an indication of the extent of aggregation. This method has been applied in a number of studies with whole urine (Edyvane et al., 1987; Grover et al., 1990a, 1990b; Ryall et al., 1991) and, in combination with the determination of a metastable limit, it has been possible to draw general conclusions about the effect of an experimental treatment on nucleation, growth and aggregation.

In a non seeded crystallisation experiment the choice of conditions is likely to have a significant effect on the outcome. The nucleation and growth rates will depend on the supersaturation chosen to initiate crystallisation which may in turn indirectly influence the degree of aggregation that occurs. During the initial period, nucleation will dominate over growth and aggregation and this balance will continue to vary as the supersaturation decreases, with aggregation possibly being the predominant process as equilibrium approaches. If the aim of an investigation is to study the effect of components in a well defined medium, non seeded crystallisation experiments have little to offer in comparison with seeded systems. The exception is when a study of nucleation effects is planned. In experiments aimed at the study of whole urine, the approach of Ryall et al. (1985) with a consistent choice of starting supersaturation has much promise.

Increasing Supersaturation Systems

General comments. This group of techniques is distinguished from those already described because the method for increasing the driving force for crystallisation continues to be applied while precipitation is taking place. Of course, when crystals start to form this will tend to reduce the supersaturation and the actual supersaturation during an experiment is unlikely to be well defined.

It could be argued that some of the methods already discussed above should belong in this section as they used intermittent (Tiselius, 1985) or continuous addition of oxalate solution (Baumann, 1988; Briellmann et al., 1985, Hering, 1988). Although some growth and aggregation would be taking place during these additions of oxalate, these studies were concerned mainly with nucleation and were only pursued until the crystals reached the chosen detection limit.

Concentration by Evaporation (Fig. 1, curve c)

Rapid evaporation of urine at 37C to a fixed osmotic pressure (1.2osm/l) has been used to induce crystallisation (Hallson & Rose, 1978) with the sample being examined microscopically or chemically after standing at 37C for one hour after concentration. When calcium oxalate crystals were produced they were mainly the dihydrate form often seen in urine. This method has been used to compare fresh urine from stone formers and control subjects (Hallson & Rose, 1978; Azoury et al., 1987) and can be used to examine effects of ultrafiltration or various additions (Hallson & Rose, 1979; Hallson et al., 1982 & 1983; Rose & Sulaiman, 1982 & 1984a & b; Grover et al., 1990c). The

observed crystalluria may be in response to heterogeneous nucleators or homogeneous nucleation as a result of the increased supersaturation. The change in saturation for each urine will of course vary depending on their initial saturation and osmotic pressure. Although not amenable to rigorous analysis of nucleation, crystal growth or aggregation, the method has two distinct advantages. It can be used with fresh whole urine and the concentration procedure may mimic the water reabsorption that occurs in the upper part of the urinary tract as the urine is formed.

Concentration by Reverse Osmosis (Fig. 1, curve d)

Another method which aims to reproduce intrarenal development of supersaturation is reverse osmosis (Azoury et al., 1986a, b & c). In these studies feed solutions of calcium and oxalate, below supersaturation, were forced at high pressure through hollow fibre reverse osmosis membranes which are permeable only to water. This is a continuous flow procedure and can be so arranged as to have a transit time through the apparatus comparable to transit times through the renal tubules (about 3 minutes). The water reabsorption during this time was about 80% which generated sufficient supersaturation to induce crystallisation, again mainly of the dihydrate form. The crystals produced were counted and sized and examined by scanning electron microscopy and the composition of the output stream analysed. The effects of some inhibitors were examined in this system (Azoury et al., 1986a & b). Apart from some effects on crystal numbers, changes in the hydration habit of the crystals and extent of aggregation were also noted. The main advantage of this technique is that it may provide a good model for generation of supersaturated urine within the kidney, however, it would not be easy to analyse nucleation, growth and aggregation processes separately.

Supersaturation Increased by Diffusion (Fig. 1, curves e_1 and e_2)

Although not very widely used, it is possible to raise the supersaturation of a test system by allowing calcium or oxalate, or both, to diffuse into it. When this approach has been used, the crystal products have usually been immobilised by forming on glass fibre or in a gel. In the glass fibre method a thread is suspended in a vessel containing urine or buffer and calcium and oxalate allowed to diffuse in via two filter paper wicks. The resulting crystallisation can be quantified by weighing the wick, dissolving the crystal products and measuring calcium or measuring the calcium in solution (Sutor, 1969; Dent & Sutor, 1971; Welshman & McGeown, 1972; Sallis & Lumley, 1979). In these studies, crystallisation was allowed to proceed for many hours and the net result will reflect nucleation, aggregation and growth.

In gel systems, calcium and oxalate can be allowed to diffuse towards each other through an agar gel and crystallisation will occur at the boundary where they meet. This can be quantified by changes in turbidity and inhibitors or urine effects can be examined by permitting them to diffuse into the crystallisation zone (Roehrborn et al., 1986). Another method is to include oxalate and seed crystals in a gel and place the calcium containing solution (which may be urine) above it. Again diffusion will bring about supersaturation and the crystallisation can be followed by the increase in turbidity of the gel (Achilles et al., 1991). These techniques can be easily applied to measure many different samples. The relative diffusion rates of different macromolecular components of the urine may compromise these comparisons. On the other hand, large crystals may form, which, as they are immobilised, could be relevant to mechanisms that enable crystals within the kidney to become sufficiently large to become a clinical problem.

Constant Supersaturation Systems

Constant Composition Crystallisers (Fig. 1, curve f)

Crystallisation of calcium oxalate and stone formation in the kidney takes place in an environment with a relatively constant supersaturation. Tomson & Nancollas (1978) developed a technique to reproduce this *in vitro*, which Sheehan & Nancollas (1980) applied to studies of calcium oxalate monohydrate crystal growth. These experiments are normally set up as for a seeded crystallisation study in a metastable solution, monitored with a calcium specific ion electrode. Small changes in the potential measured by the electrode trigger motorized syringes to add equimolar solutions of calcium chloride and potassium oxalate so that constant calcium and oxalate ionic activities are maintained. The growth rate is found from the rate of addition of the calcium or oxalate solutions, adjusting for volume changes.

The driving force for crystallisation, (the supersaturation), is held constant in this system and growth rates are usually linear for more than 30 minutes, with second order kinetics being obeyed (according to equation 4, with $[M_{t=eq}]$ calculated from the thermodynamic solubility product). As has been found in conventional seeded systems, the growth rate was proportional to the seed crystal concentration

(Sheehan & Nancollas, 1980). Crystallisation of the trihydrate form of calcium oxalate also followed second order kinetics, with a greater rate constant than for the monohydrate (Sheehan & Nancollas, 1984). The inhibitory effect of urine or defined inhibitors could be quantified, as before, by Langmuir adsorption isotherms, leading to the inhibitor concentration (or urinary dilution) required to bring about 50% inhibition (Sheehan & Nancollas, 1980). In a study comparing bladder and kidney urines, and examining the effect of various urinary derived macromolecular fractions, simple percentage inhibitions were presented (Lanzalaco et al., 1988).

The optimal dilution of urine for this method was suggested to be 1% to 2% (Lanzalaco et al., 1982) but changes to the electrode design, later permitted use of whole urine (Nancollas & Gaur, 1984). A 50% dilution of urine reduced the growth rate by 92% of the control rate (Gaur & Nancollas, 1984).

In simple seeded metastable solutions nucleation should not be occurring, but this group have been able to use their system to see if various factors can promote nucleation. In the absence of seed crystals they noted the time before any detectable crystallisation began (the induction time) (White & Nancollas, 1987; Campbell et al., 1989). Aggregation is not considered in this method, although, as has already been discussed, this may have an indirect effect on growth rates.

This constant composition approach has a number of advantages. It is a better model for the renal environment than the systems described above and can be operated at lower supersaturations than would be used in non seeded conventional crystallisation experiments. The constant supersaturation maintains a constant driving force for all calcium oxalate hydrates crystallising. By contrast, as the different hydrates have different solubilities, $[M_t]$-$[M_{t=eq}]$ will not be the same for different hydrates throughout a decaying supersaturation experiment. In some circumstances, dilution of test components during the procedure might be important and the increasing crystal concentration may indirectly effect growth and aggregation.

Mixed Suspension, Mixed Product Removal Continuous Crystallisers (Fig. 1, curve g)

The kidney is a continuous flow system with fresh supersaturated urine being continuously formed and passed out through the renal pelvis some minutes later (Finlayson & Reid, 1978). Finlayson (1972) showed that this could be described as a series of continuous crystallisers. If certain assumptions are upheld, then the crystallisation kinetics of a continuous crystalliser can be estimated from the particle size distribution. For the theoretical analysis to be valid, the system must be well mixed and the outflow must be representative of the remaining suspension. This is known as mixed suspension, mixed product removal (MSMPR). The use of an MSMPR crystalliser to study calcium oxalate crystallisation therefore offers the opportunity of obtaining physico-chemically meaningful data in a crystallizing system which is a better model of kinetics of crystallisation in the urine as it flows through the kidney.

A calcium oxalate MSMPR crystalliser usually consists of a well mixed chamber with two input feed lines and an output line, adjusted to maintain a constant volume. Calcium and oxalate are introduced separately through the feed lines at equal flow rates and after about 8-10 chamber volumes have flowed through the system, equilibrium is achieved with a stable supersaturation and particle size distribution. The average time particles remain in the crystalliser is the residence time (τ),

$$\tau = V/Q \qquad (19)$$

where V is the volume of the chamber and Q is the total flow rate into (or out of) the chamber. If the crystals all grow at the same rate and aggregation is neglected then it can be shown that

$$n = B_0\tau e^{(-L/G\tau)} \qquad (20)$$

or

$$Ln(n) = Ln(B_0\tau)-L/G\tau \qquad (21)$$

where n is the total number of crystals larger than size L, G is the growth rate and B_0 is the nucleation rate (Rodgers & Garside, 1981). With appropriate units, G can be measured as μm/min and B_0 as no./min/ml from the slope and intercept of the graph of Ln(n) against L, according to equation 21. Using the growth and nucleation rates it is possible to calculate the suspension density (M_τ, in mg/l) according to the following equation,

$$M_\tau = \pi\rho B_0 G^3\tau^4 \times 10^{-6} \qquad (22)$$

where ρ is the crystal density (g/cm^3).

There have been various applications of the MSMPR method, often with components being tested in artificial urine (Miller et al., 1977; Randolph & Drach, 1981; Li et al., 1985; Kohri et al., 1988 & 1989; Robertson & Scurr, 1986). It has also been used with dilute urine and macromolecular urinary fractions (Drach et al., 1980 & 1982; Springmann et al., 1986).

MSMPR crystallisers used in urolithiasis research are usually much smaller than their chemical engineering counterparts and typically have volumes of 200ml to 400ml. Bearing in mind that about ten times this volume must pass through until a steady state is obtained, it is clear that only dilute urine could be used

in such a system (Springmann et el. (1986) used 5%). Recently a much smaller crystalliser with a volume of 20ml has been developed (Nishio et al., 1991) and used in a study with artificial urine (Nishio et al., 1990). This system, applied to nearly whole urine (92%), forms the basis of the experimental section of this paper.

The main disadvantage with the MSMPR system is associated with aggregation. If this is significant it could invalidate one of the assumptions underlying the theoretical analysis of the particle size distribution. In practice, if the plot of Ln(n) against L (as in equation 21) is linear over most of the size range, then the calculated growth and nucleation rates are assumed to be realistic. Robertson & Scurr (1986) used the deviation from linearity at the extreme values of L, to estimate aggregation effects. Springmann et al. (1986) added a Couette agglomerator to their system in order to study aggregation. Another problem is the relatively high feed concentrations of calcium and oxalate that must be supplied to the crystalliser. On the other hand, it allows measurement of growth and nucleation rates as independent parameters and the crystallisation takes place in an environment much closer to that of a kidney than is found with other systems.

Concluding Comments on Review Section

Many different methods have been used to study calcium oxalate crystallisation *in vitro*. Some give a general indication of the crystallization potential while others are more specifically aimed at measuring growth, nucleation or aggregation. In supersaturated, crystallising solutions it is not possible to study any one of these processes completely in isolation, although aggregation alone can be studied in a seeded system at equilibrium (Hess et al., 1989).

The variation in response to a particular treatment that might arise in different methods can not easily be predicted from consideration of the technical details. Unless more than one method is included in an investigation then it is always likely to be difficult to see the result of one experiment in perspective against other studies. For instance, Grover et al. (1990c) using two methods concluded that the different results with Tamm-Horsfall mucoprotein, as an effector of crystallisation of calcium oxalate in urine, depended on the method used.

The main objectives of *in vitro* calcium oxalate crystallisation studies are to evaluate the physico-chemical response of effectors of crystallisation; to investigate mechanisms of stone formation and the role of proposed effectors; as a diagnostic tool to identify those at high risk of recurrent disease and to test possible treatments aimed at preventing recurrence. When the investigative goals are limited to physico-chemical evaluation of effectors, then seeded crystallisation from a metastable simple salt solution may be most appropriate, with the constant composition approach having some advantages over supersaturation decay methods.

If the study objectives are directed towards stone forming mechanisms, diagnosis or treatment, then the relevance of experiments using dilute urine will always be questionable. Nevertheless, there are methods which employ dilute urine, as well as some which use whole urine, which appear to discriminate between stone formers and non stone formers and may be of diagnostic value, or help in investigation of possible treatments. When the methods used are not good models of stone formation within the kidney then any differences between patient groups must be considered empirical and to be without a strong theoretical and mechanistic foundation.

A good *in vitro* model of stone formation can therefore be seen as crucial for improving our understanding of the significance of the various mechanisms involved and to enable the multitude of crystallisation studies to be put into perspective. No one method answers all the requirements of a good model, is applicable to whole urine and allows quantifiable analysis of nucleation, growth and aggregation. Until such a system is devised, different methods with different advantages and disadvantages will continue to be used. It would be helpful if, in future developments, apparently arbitrary differences were kept to a minimum and if new approaches were compared alongside existing methods.

Experimental Techniques

Introduction

Mixed suspension, mixed product removal (MSMPR) continuous crystallisation has some advantages over other methods commonly applied to studies of calcium oxalate crystallisation. Nucleation rates can be measured as no./min/ml and growth rates as μm/min, independently of each other, but at the same time. The method offers a better representation of the renal environment than other crystallisation techniques, both because it reaches a steady state with a constant supersaturation, and because it operates with continuous flow; thus, individual crystals only remain in the suspension for a relatively short time, not much different from transit times through the kidney.

Applications of the MSMPR approach have previously been limited to artificial urine, with up to 5% urine included (Springmann et al., 1986)). The main reason for using dilute urine is the volume requirements. Most MSMPR crystallisers would need between 2 and 4 litres of undiluted urine. Our small scale crystalliser (Nishio et al., 1991) has a volume of 0.02 litres and hence a total feed solution requirement of about 0.2 litres.

This experimental section describes some experiments to study nucleation and growth rates in fresh, minimally diluted urine (by a factor of 1.09) or to 92% of its initial concentration).

Materials and Methods

Urine samples were obtained from healthy men with no history of urolithiasis and from male recurrent renal stone formers. Specimens (collected in a pre-warmed Dewar flask) were adjusted to pH 6.0 with HCl or NaOH and analysed for calcium and oxalate. They were filtered (8μm cellulose nitrate prefilter (Sartorius Ltd.), 0.45μm membrane filter ((Millipore Ltd.), performed at 37C) or centrifuged (3,000g for 5 minutes at 37C).

This urine (at 37C) was used as one of three feed solutions to the continuous crystalliser, pumped at 92% of the total flow rate. The other two feed solutions were calcium chloride and sodium oxalate, each flowing at 4% of the total flow. Knowing the urinary calcium and oxalate concentrations, it was possible to adjust these two solutions at the start of each experiment to give concentrations in the crystallisation chamber (if no precipitation occurred) of 12mM calcium and 2.4mM oxalate. The experiments were started about 30 minutes after the sample was passed and completed about 1 hour later. The crystalliser chamber (see Nishio et al. (1991) for details) had a volume of 20ml and was kept at 37C. An average residence time (τ) of 6.7 minutes was used.

When equilibrium had been reached the particle size distribution was measured (Elzone 80XY, Particle Data Inc.). Crystal growth rates (G, μm/min) and nucleation rates (B_0, no./min/ml) were calculated from the slope and intercept of the data replotted as Ln no. against size (see equation 21). The suspension density (M_τ, mg/l) was calculated using equation 22 with the crystal density taken to be 2.22g/cm^3. Occasionally the calculated value of M_τ exceeded the amount of starting material, probably because of errors in estimates of τ and B_0. To overcome this problem, calculated values of M_τ were all scaled so that the maximum value encountered would give a supersaturation of 1. Calcium and oxalate solution concentrations were estimated as the difference between their initial concentration and the suspension density, other electrolytes were measured after the crystallisation experiment and the supersaturation calculated using EQUIL2 (Werness et al., 1985). Because of the uncertainty in our estimate of M_τ, we refer to the supersaturation as an index, rather than a ratio.

In a second series of experiments, two crystallisers were run simultaneously with the same urine. Fresh urine from control subjects was obtained and centrifuged as before and divided into two portions. Heparin or hyaluronic acid was added to one aliquot. Each portion was used as a feed solution for one of the crystallisers. Calcium chloride and sodium oxalate solutions were fed to each chamber (adjusted to give initial concentration of 12mM Calcium and 2.4mM oxalate). In these experiments the urinary calcium was taken into account but endogenous oxalate was neglected in making up these feed solutions. Each concentration examined was tested on four different urine samples.

Oxalate, sulphate and phosphate were measured by high performance ion chromatography (HPIC) (Robertson & Scurr, 1984); chloride by HPIC at a lower dilution; calcium and magnesium by atomic absorption; sodium and potassium by atomic emission; citrate by coupled enzyme assay; ammonium by Berthelot's method and osmotic pressure by freezing point depression. Hyaluronic acid and heparin were obtained from the Sigma Chemical company. Molecular weights of 20,000 and 30,000, respectively, were used to calculate concentrations.

The statistical significance of results was assessed by analysis of variance (ANOVA), with repeated measures when appropriate. Logarithmic transformations were used for growth and nucleation rates to give normally distributed data. Including other factors, such as urinary ionic strength, in the statistical treatment of results allows their influence to be taken in account. Multiple linear regression was used to examine which additional factors were significant and independent and should be included. Tukeys critical range test was used to identify significant effects ($p<0.05$) in ANOVAs with multiple groups.

Results

Comparison of control and stone formers urine.

Growth rates were found to be almost the same in control and stone forming groups and they did not differ between centrifuged and filtered urines (Table 1). Nucleation rates were higher in the control samples

Table 1. MSMPR growth rates (μm/min) in 92% urine from controls (Ctl) and stone formers (SF), centrifuged (C) or filtered (F).

	Geometric means (no. of samples)		
	centrifuged	filtered	C and F
controls	0.65 (10)	0.64 (12)	0.64 (22)
stone formers	0.65 (10)	0.68 (11)	0.66 (21)
Ctl and SF	0.64 (20)	0.66 (23)	

	Results from ANOVA	
	F (d.f.)	p
centifuged versus filtered	0.3 (1,39)	>0.1
	0.1 (1,38)*	>0.1
controls versus stone formers	0.2 (1,39)	>0.1
	0.6 (1,38)*	>0.1

*2nd ANOVA adjusts for the initial supersaturation of urine samples.

Table 2. MSMPR nucleation rates $x10^{-3}$ (no./min/ml) in 92% urine from controls (Ctl) and stone formers (SF), centrifuged (C) or filtered (F).

	Geometric means (no. of samples)		
	centrifuged	filtered	C and F
controls	68 (10)	127 (12)	95 (22)
stone formers	34 (10)	53 (11)	43 (21)
Ctl and SF	48 (20)	83 (23)	

	Results from ANOVA	
	F (d.f.)	p
centifuged versus filtered	3.2 (1,39)	0.08
	4.9 (1,38)*	0.03
controls versus stone formers	7.2 (1,39)	0.01
	10.7 (1,38)*	0.003

*2nd ANOVA adjusts for the initial supersaturation of urine samples.

Table 3. MSMPR supersaturation indices in 92% urine from controls (Ctl) and stone formers (SF), centrifuged (C) or filtered (F).

	Means (no. of samples)		
	centrifuged	filtered	C and F
controls	12.4 (10)	9.8 (12)	11.0 (22)
stone formers	15.0 (10)	13.7 (11)	14.5 (21)
Ctl and SF	13.6 (20)	11.7 (23)	

	Results from ANOVA	
	F (d.f.)	p
centifuged versus filtered	6.7 (1,39)	0.013
	6.9 (1,38)*	0.012
controls versus stone formers	18.8 (1,39)	0.002
	12.5 (1,38)*	0.003

*2nd ANOVA adjusts for the ionic strength of urine samples.

compared to the stone formers and higher in samples which were filtered rather than centrifuged (Table 2). The supersaturation within the crystalliser was lower in control samples than in urines from stone formers and lower in filtered urine compared to centrifuged urine (Table 3).

There were factors which our experimental design did not control which might have varied between the different groups and could, perhaps, explain the observed differences in nucleation rates and supersaturation. The urinary osmotic pressure (OP), ionic strength (IS) and the supersaturation of the whole urine samples (S_0) were considered as possible confounding factors. Their relationship to the crystallisation kinetics was investigated. Crystal growth rate was significantly related to S_0 ($p=0.001$), OP ($p=0.040$) and IS ($p=0.041$), but only S_0 had a significant and independent relationship with growth rate. Nucleation rate was significantly related to S_0 only ($p=0.015$). The supersaturation index was significantly related to IS ($p=0.025$) and OP ($p=0.025$), but only significantly and independently related to IS. The significant and independent factor was included in a second ANOVA, allowing its influence to be discounted. This reinforced the conclusions of the first ANOVA (Tables 1-3).

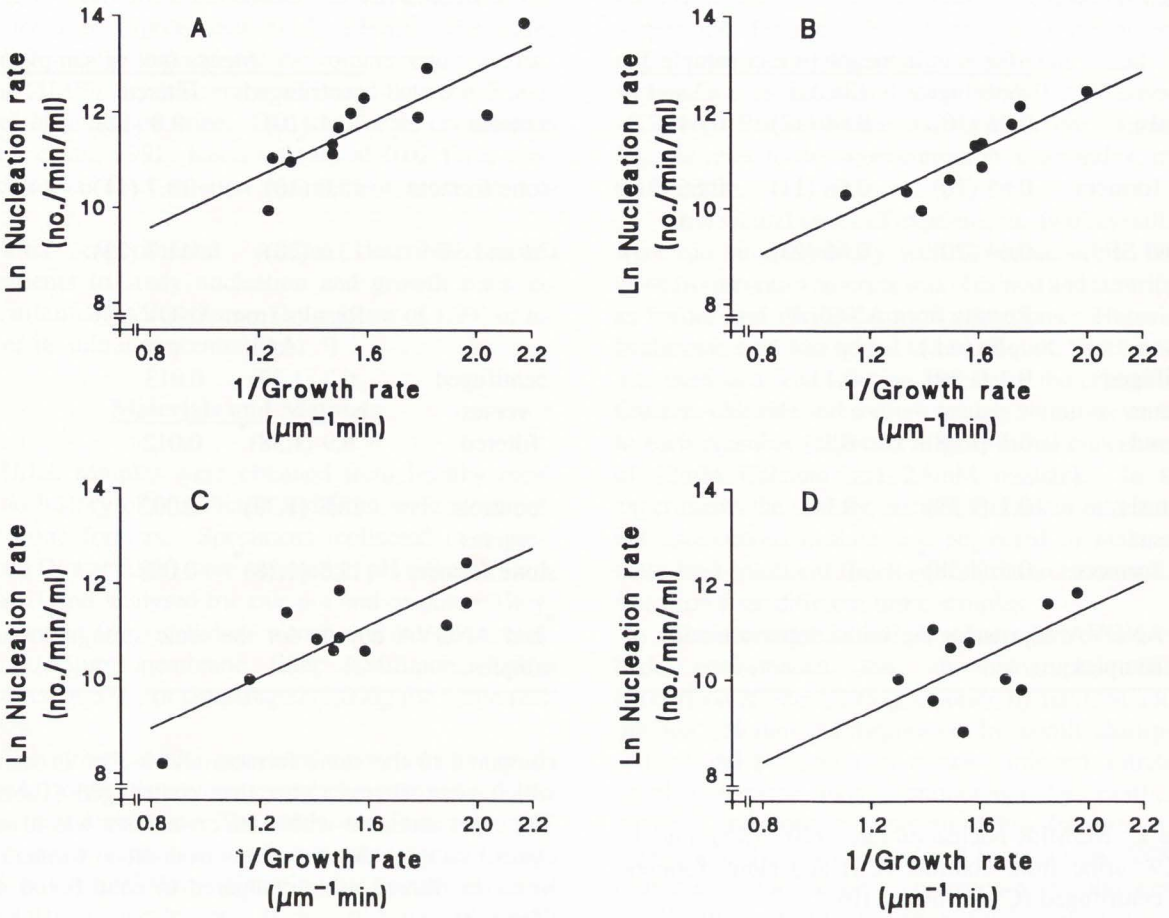

Figure 2. The relationship between nucleation rate and growth rate in 92% urine, shown as Ln nucleation rate against 1/growth rate. The four groups of samples shown are **A**, controls/ filtered; **B**, controls/ centrifuged; **C**, stone formers/ filtered; **D**, stone formers/ centrifuged. The intercepts are **A**, 7.40; **B**, 6.87; **C**, 6.77; **D**, 6.08; the common slope is 2.72.

An inverse relationship between the growth rates and log nucleation rates was noted. Plots of LnB_0 against 1/G were significantly correlated (for all samples, the correlation coefficient r was 0.7). Analysis of variance of the regression data also showed the relationship was highly significant ($p=0.00001$), with the intercepts ($p=0.0006$), but not the slopes ($p>0.05$) of the regression lines being influenced by the type of sample. Figure 2 shows the regression lines for the four groups of samples. Using Tukeys multiple range test, the intercept of the control/ filtered samples was significantly greater than the other three samples and the intercept of the stone formers/ centrifuged urine was significantly lower than the others ($p<0.05$)

Effect of heparin and hyaluronic acid.

Heparin and hyaluronic acid were added at various concentrations to chamber two while chamber one acted as a control in parallel crystalliser experiments. Both additives brought about significant dose dependent decreases in total crystal numbers, nucleation rate and an increase in growth rate (figures 3 and 4). The ANOVA revealed overall significant differences ($p<0.003$) between chamber 1 and 2, for all parameters measured. There was no difference for the control experiment ($p>0.05$), all three concentrations of heparin and the highest two of hyaluronic acid gave significant differences ($p<0.05$). At the concentrations tested, hyaluronic acid was less effective than heparin which increased growth rate and decreased crystal numbers by nearly 100% (figure 4).

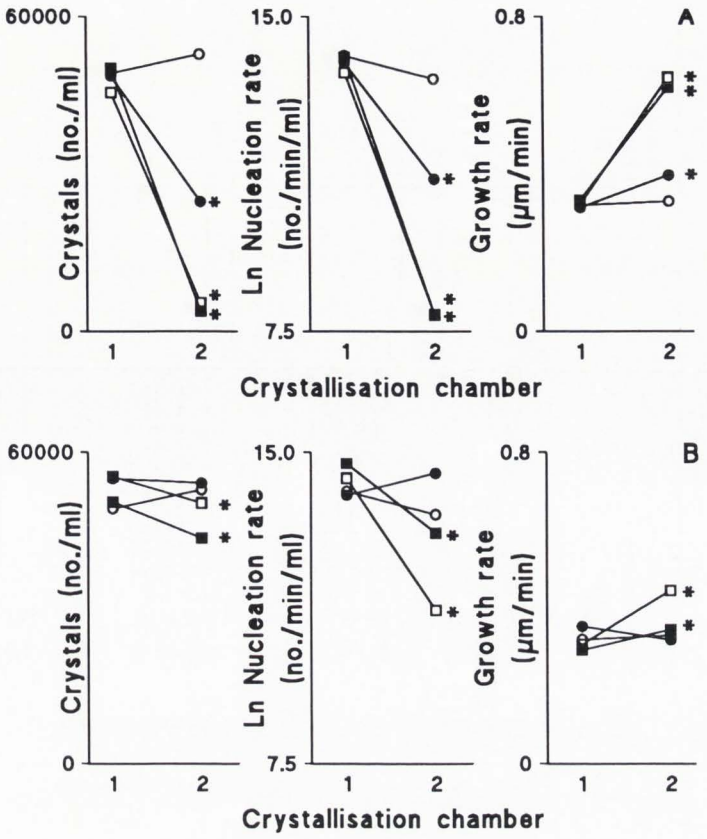

Figure 3. The effect on crystallisation of **A**, heparin and **B**, hyaluronic acid addition to 92% urine in chamber 2, compared to the same urine without additions in chamber 1. ○, 0µM; ●, 1µM; □, 5µM; ■, 10µM. Each point shown is the mean of 4 experiments. * $p<0.05$.

Discussion

These experiments demonstrate that an MSMPR continuous crystalliser system can be applied to nearly whole urine (92%). Some preliminary treatment to remove any crystals or cellular debris that could act as sites for heterogeneous nucleation was considered necessary and the effects of filtration and centrifugation were studied. In order to compare samples from different patients we adjusted all samples to the same starting concentrations of calcium and oxalate. The concentrations which were used were necessarily somewhat higher than physiological, in order to bring about sufficient crystallisation. In the absence of precipitation very high supersaturation would prevail and this provides the thermodynamic driving force for the crystallisation. Nevertheless, the steady state supersaturation actually achieved within the crystalliser was not much higher than is typical of whole urine.

The response of urines to this challenging load of calcium and oxalate differed between controls and recurrent stone formers and also depended to a lesser extent on whether the urines had been pre-treated by filtration or centrifugation. The mean growth rate did not differ between groups but the mean nucleation rate was higher in control samples compared to stone formers urine. The mass of crystalline material is proportional to the product of the cube of the growth rate and the nucleation rate and this was higher, and hence the supersaturation lower, in the control group. This reproduces observations with whole urine in which stone formers have often been reported to have higher supersaturations than controls (Fleisch, 1978; Robertson et al., 1976, 1981).

The thermodynamic driving force for each sample tested would be very similar as they all had the same starting concentrations of calcium and oxalate. Their response can be viewed as a balance between the two

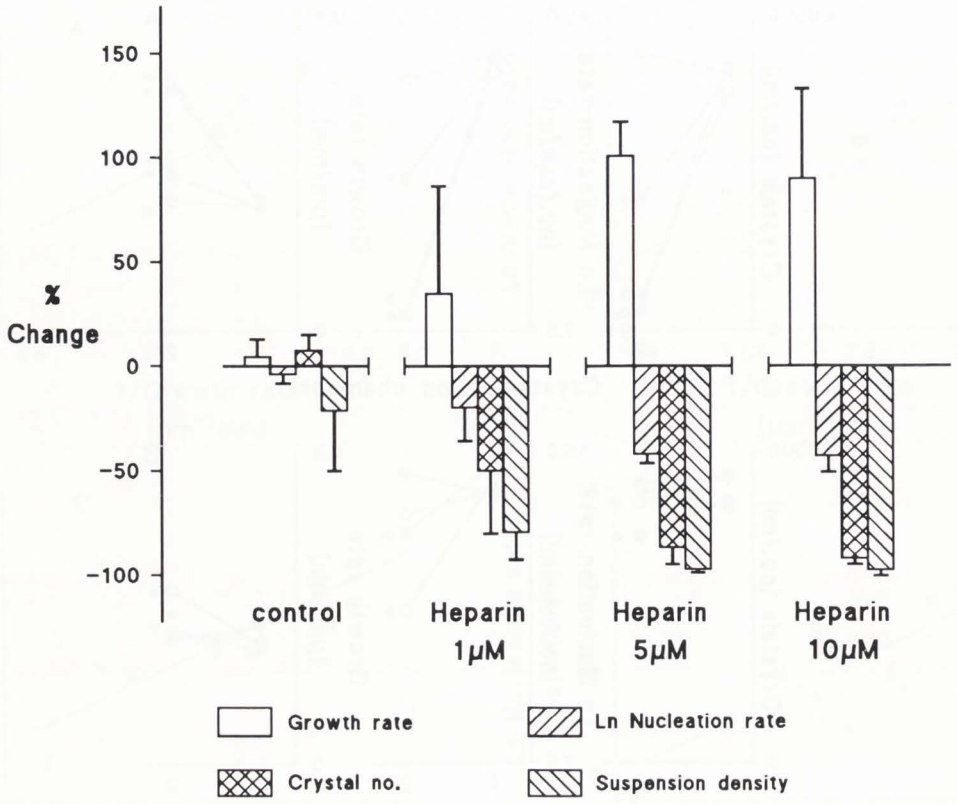

Figure 4. Effects of heparin on crystallisation of calcium oxalate in 92% urine. % change in response, brought about by different concentrations added (means of 4 experiments ± s.d.).

competing mechanisms of supersaturation relief, nucleation and growth. This balance can be seen in the relationship between LnB_0 and 1/G in figure 2. The observation that the slopes of these lines are the same but the intercepts are different helps to explain the difference between the groups of samples. It suggests that, for a given value of G, the different groups have different values of LnB_0 and furthermore, as the log differences in B_0 are the same for all values of G, then the proportional difference in B_0 will also be the same. Taking the nucleation rate of the control/ filtered samples as 100%, then for all values of G, the nucleation rates of the other groups will be about 59% (control/ centrifuged), 53% (stone formers/ filtered) and 27% (stone formers/ centrifuged).

The effect of the higher nucleation rate in the control groups is to bring about a lower steady state supersaturation and this may be a crucial protective factor distinguishing stone formers from controls. Any crystals which become temporarily lodged within the kidney will have a greater chance of growing and developing into a clinically significant stone when growing in a higher supersaturation environment.

The difference between filtered and centrifuged samples is consistent with the widely held belief that macromolecular urinary factors are important determinants of crystallisation *in vivo*. These pre-treatments of the urine are likely to have removed a different proportion of high molecular weight factors such as Tamm-Horsfall mucoprotein.

In the experiments with two crystallisers running in parallel, we have demonstrated an extension of the MSMPR method for examining the effect of potential crystallisation effectors, in nearly whole urine. This avoids the need for comparing results from different urines, which may have widely different compositions. In the particular experiments shown, effective concentrations of heparin and hyaluronic acid were higher than commonly found in other systems. This may be because our experiments already include the majority of endogenous urinary factors and large doses of crystallisation effectors may be needed to bring about

further change. Both heparin and hyaluronic acid seemed to cause a shift in the balance between nucleation and growth in favour of growth, with a net increase in supersaturation.

This method of studying urine in two crystallisers simultaneously might be usefully applied to examine the *in vitro* response of urine from a particular individual to a proposed therapeutic regime. As any prophylactic measures would have to be followed for many years it would be extremely useful to establish in advance the likely efficacy of the treatment.

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Discussion with Reviewers

Reviewer I: How would the authors justify the use of such high initial concentrations of calcium and oxalate in their MSMPR system?

Author: Because the system is unseeded, the initial supersaturation must be above the metastable limit and most MSMPR studies have operated at or above the extreme range of physiologically appropriate calcium and oxalate concentrations. The concentrations used in these experiments were higher than others have worked with because of the inhibitory effect of the high urine concentration. In order to be able to calculate the

growth and nucleation rates, sufficient crystals must be generated over a wide particle size range and this could only be achieved by employing high initial calcium and oxalate concentrations. The steady state solution concentrations would, of course, be very much lower.

Reviewer I: Would not the use of such high concentrations produce too high a slurry density (a) for counting crystal numbers accurately and (b) for the amount of inhibitors and promoters in urine?
Author: The maximum particle concentration generated in the crystallisers was less than 60,000/ml. The orifice tube of the particle sizer had an aperture of 150μm and the probability of there being more than one particle at a time in the critical volume (three times the aperture volume) is <.025, which I feel is an acceptable coincidence level. If urinary inhibitors and promoters are bound to crystal surfaces, then their effective concentration in this system could indeed be significantly reduced. This may explain why responses to heparin and hyaluronic acid was only found at higher concentrations than other workers have observed using different experimental techniques.

Reviewer I: What range of M_τ values does the authors' system produce?
Author: The calculated suspension densities (M_τ) ranged from 22 to 775 mg/l. The distribution of values was highly skewed with few high values and the mode was 183 mg/l.

Reviewer I: Did the authors find any evidence of crystal agglomeration in their studies? If so, how did they handle this mathematically?
Author: Significant agglomeration in the MSMPR system would result in non linear particle size distributions when plotted as Ln(n) against L. In practice we found that good straight lines were always obtained for particles up to 30μm in diameter. Beyond this size, deviations were sometimes noted but these were not very reproducible. Therefore, no attempt was made to handle these mathematically. Scanning electron microscope images of filtered crystal slurries showed well formed calcium oxalate di- and tri- hydrate crystals, with no evidence of extensive aggregation.

B. Hess: At the high concentrations selected, heparin and - to a lesser extent - hyaluronic acid in the author's MSMPR continuous crystallizer system seem to inhibit nucleation rate and to promote crystal growth, which might be taken as an attempt to reduce the high supersaturation with respect to calcium oxalate present in the system. How does this apply to other - naturally occurring - urinary inhibitors when tested under the same conditions?
Author: The promotion of growth rate under these circumstances may well be a response to the inhibition of the nucleation rate, this being the only means of supersaturation relief once the nucleation rate has diminished. We have no data yet on naturally occurring urinary inhibitors but their overall effect in this system would be a combination of any direct effects on nucleation and growth and their response to the supersaturation pressure. Thus a factor which acts as an inhibitor of crystal growth in a seeded method may appear to promote crystal growth in an MSMPR system.

H.-G. Tiselius: A higher nucleation rate was recorded in urine from control subjects. It is suggested that this reflects a protection from forming large crystals and could be a distinguishing factor between stone formers and normal subjects. Can you speculate on how this increased nucleation is achieved? Is this process in any way modified by the urine composition?
Author: It is generally assumed that heterogeneous nucleation dominates over homogeneous nucleation *in vivo* (text reference 21) and this is probably true of our system as well. Therefore, increased nucleation could be achieved either by increasing the number or the characteristics of nucleation catalysts. The difference between filtered and centrifuged urine samples suggests that variation in the concentration of nucleators could be an important factor. The urinary composition might modify heterogeneous nucleator activity by altering the interaction between nucleating crystals and catalyst, which will depend on physico-chemical and structural factors. There was some evidence of an effect of urinary composition in the significant correlation ($p=0.015$) between nucleation rate and the urinary supersaturation before the MSMPR experiment was started.

H.-G. Tiselius: Is there any explanation of the reduced nucleation in urine with additives of heparin and hyaluronic acid?
Author: The experimental data shown here do not give any direct information on the mechanism of nucleation inhibition but adsorption of these macromolecules to the surface of heterogeneous nucleators with reduction in the interaction between the catalytic surface and nucleating calcium oxalate is a plausible explanation.