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A. L. Rodgers
*University of Cape Town*

D. Jappie
*University of Cape Town*

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# STUDIES ON THE ROLE OF URINARY MACROMOLECULES IN UROLITHIASIS: REVIEW OF METHODOLOGIES AND A PROPOSAL FOR A STANDARD REFERENCE CRYSTALLIZATION SYSTEM

A.L. Rodgers* and D. Jappie

Department of Chemistry, University of Cape Town, Rondebosch, Cape Town, 7700, South Africa

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## Abstract

In this study, urine from a calcium oxalate kidney stone former was ultrafiltered (10 kD cut-off). Crystallization was induced in the ultrafiltrate and retentate fractions as well as in a sample of the whole urine. The progress of crystallization was monitored by Coulter Counter and flow cytometry techniques. (The latter has not been used in studies of the role of urinary macromolecules in urolithiasis). Deposited crystals were examined by scanning electron microscopy. Results indicated that urinary macromolecules in this subject are inhibitors of nucleation and aggregation. These results agree with the findings of some workers but disagree with those of others. Indeed, studies on the role played by urinary macromolecules in promoting or inhibiting urolithiasis have failed to produce consistent findings. Examination of the literature reveals that a wide variety of experimental techniques and crystallization systems have been used in these studies and that this might be the cause of the inconsistencies. Based on reported experiences and those of the present study, a standard reference crystallization system is proposed. The key elements of this system involve the use of real urine, ultrafiltration, continuous crystallizer equipment, Coulter Counter procedures and scanning electron microscopy.

**Key Words**: Urinary macromolecules, crystallization system, ultrafiltration, flow cytometry, calcium oxalate, kidney stones, inhibitors, nucleation, aggregation.

*Address for correspondence:
A.L. Rodgers, address as above.
Telephone number: (27)(21) 650-2572
FAX number: (27)(21) 650-3788

## Introduction

During the past several years many studies have addressed the question of the role played by urinary macromolecules (UMM) in urolithiasis. Particular interest has been focused on their influence on calcium oxalate stone formation. Three general approaches have been adopted. First, workers have investigated the nature and composition of UMM in actual stones and have examined the structure and ultrastructure of such concretions [38, 44, 62]. Second, several studies have attempted to identify differences in the composition and concentration of UMM in the urine of stone formers and normal controls [3, 4, 15, 16, 17, 27, 29, 31, 37, 57, 61]. Finally, *in vitro* crystallization studies have been conducted in aqueous solutions [1, 12, 20, 33, 34, 36, 39, 43, 56, 58], synthetic urines [9, 10, 18, 32, 40, 42, 45, 63] and real urines [2, 5, 6, 11, 21, 24, 47, 48, 50, 51, 55, 59, 60] in the presence or absence of various UMM which have either been added to or removed from the test sample.

Studies employing the first approach are in agreement that hyaluronic acid and heparan sulphate are the main glycosaminoglycans in the matrix component of urinary calculi while chondroitin sulphate is absent [38, 44]. This has been interpreted as indicating that the former are promoters [38] while the latter is an inhibitor of stone formation [38].

Results obtained in studies employing the second approach are inconsistent. Some studies have reported significant differences between stone formers and normals with respect to UMM excretions or concentrations or composition [15, 17, 31, 37, 61] while others have failed to find any such differences [4, 16, 27, 29, 57].

With regard to the third approach, the literature abounds with numerous examples of apparently conflicting findings, and it is in this area of investigation that we wish to focus attention. Some studies have found that UMM are inhibitors of calcium oxalate "growth" [1, 2, 5, 6, 12, 34, 36, 39, 56] - a term used synonymously with "formation" and "precipitation" - while others have found them to be promoters [24, 48, 50, 51]. On the

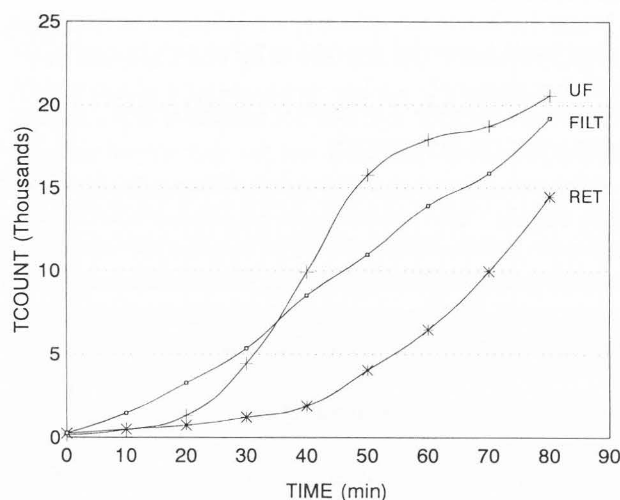



**Figure 1.** Coulter Counter plots of particle number versus (*vs*) time for FILT, UF and RET fractions.

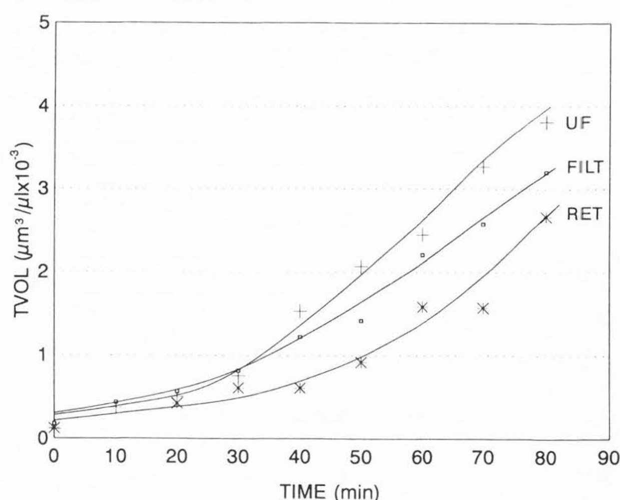



**Figure 2.** Coulter Counter plots of particle volume *vs* time for FILT, UF and RET fractions.

other hand, several workers have conducted sophisticated experiments in which they have been able to be more specific about the aforementioned crystallization and precipitation processes. These studies have shown that UMM are inhibitors of calcium oxalate crystal growth [9, 20, 21, 42, 43, 45, 52, 59, 60, 63] and inhibitors of aggregation [10, 11, 19, 26, 33, 45, 52, 55, 58] but are promoters of calcium oxalate nucleation [9, 21, 42, 45, 47, 59, 60, 63]. As might be expected, there is not much consensus in these findings either. For example, in contrast to some of the previously mentioned studies, Edyvane and co-workers showed that UMM (MW > 10 kD) did not affect the calcium oxalate metastable limit and concluded that therefore they had no effect on nucleation [11] while Gill and associates found that UMM (MW > 12 kD) inhibited nucleation [18]. Similarly, Gjaldbaek [19] failed to demonstrate any inhibition by chondroitin sulphate while Kohri *et al.* [32], in their studies of three UMM, found them to be inhibitors of nucleation and promoters of growth. In a more recent study, Kavanagh and co-workers found that heparin and hyaluronic acid significantly reduced nucleation rates but increased growth rates [30].

The complexity of UMM inhibitor/promoter activity is highlighted by studies which have shown that different UMM fractions might have different functions [2, 51]. The concept of synergism too has been raised by some workers [2, 21, 52]. Alternatively, UMM are often considered as though they were a single substance without reference to the fact that the term includes glycosaminoglycans, Tamm-Horsfall proteins and many other substances, each of which may have its own inhibitory or promotory effects upon crystal formation. Others have drawn attention to more fundamental issues concerning, for example, the possible modification of UMM structure and hence activity as a result of the particular isolation or concentration procedure employed [2, 34].

Many aspects of the relationship between UMM and calcium oxalate stone formation remain ill-defined and unresolved. The present paper reviews methodologies and approaches that have been employed in studies of UMM and describes a new and powerful technique, flow cytometry, that might prove useful in elucidating the role of UMM in urolithiasis. In addition, an attempt is made in this paper to identify, on theoretical grounds, why conflicting results have been reported so frequently in the past and to propose a standardized system that investigators might consider implementing.

## Materials and Methods

A single 24-hour urine (pH 5.8) was collected from a male idiopathic kidney stone former of age 24 years. The specimen was stored in a glass bottle at 4°C during the collection period. No preservative was present. Crystals, cellular debris and proteinaceous material were removed by sieving (74 µm) and filtration {Sartorius cellulose acetate 0.45 µm filter (Sartorius AG, Goettingen, Germany)}, although it is recognized that material lost in this way may include substances on which macromolecules may be adsorbed. Henceforth, this urine fraction will be referred to as FILT.

Aliquots of the FILT fraction were then ultrafiltered using an Amicon stirred ultrafiltration cell (Amicon Corp., Danvers, MA, USA) containing a Diaflo Type YM10 membrane (Amicon) with a molecular weight cut-off of 10 kD. A pressure of 3.7 atm (2812 torr) was maintained within the cell by nitrogen gas. Two further fractions, ultrafiltrate and retentate (henceforth, referred to as UF and RET), were obtained from this procedure.

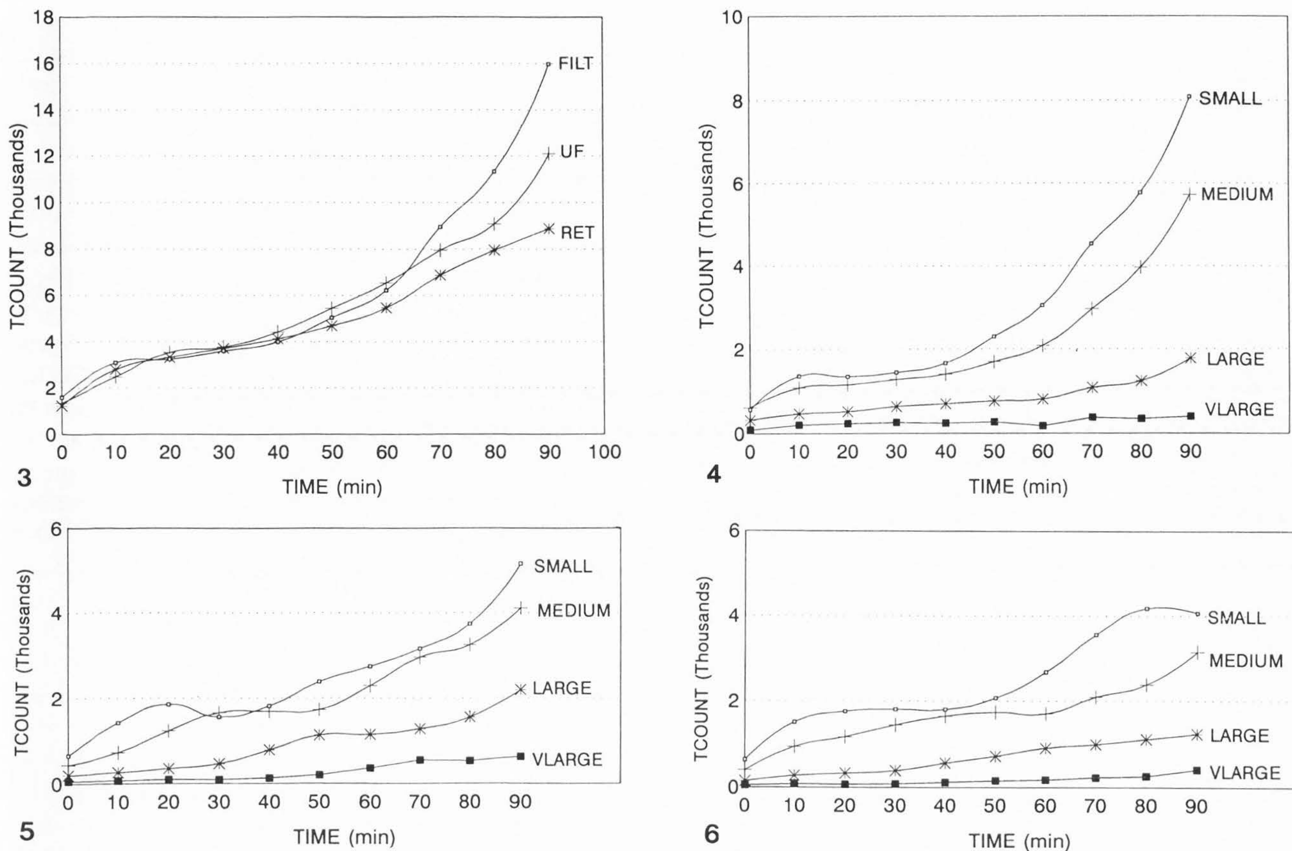



**Figure 3**. Flow cytometer plots of particle number *vs* time for FILT, UF and RET fractions.

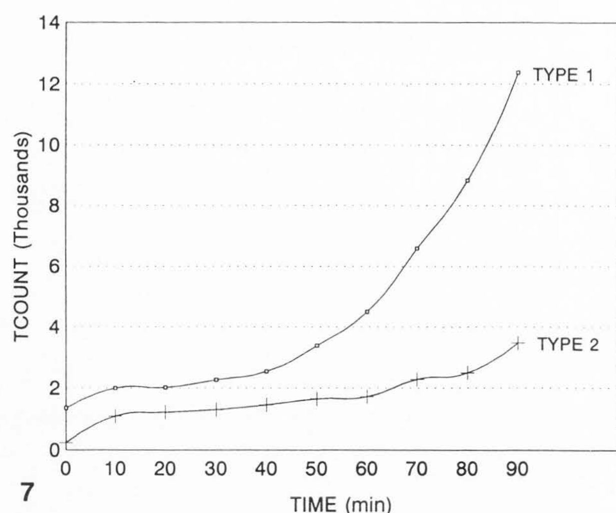
**Figures 4-6**. Flow cytometer plots of particle number *vs* time in four relative size zones in: the FILT fraction (**Fig. 4**); the UF fraction (**Fig. 5**); and the RET fraction (**Fig. 6**).

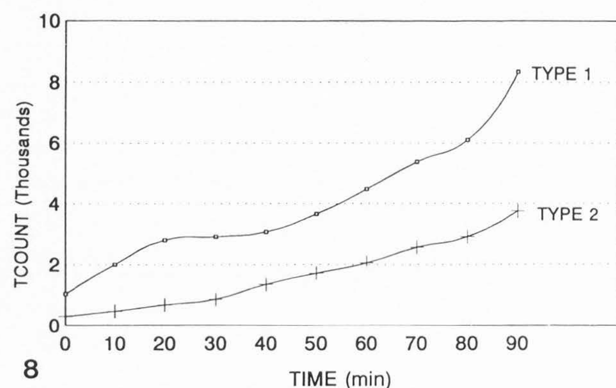
The degree of ultrafiltration was 3-fold. After ultrafiltration was complete, the membrane was examined for solid deposits by optical microscopy.

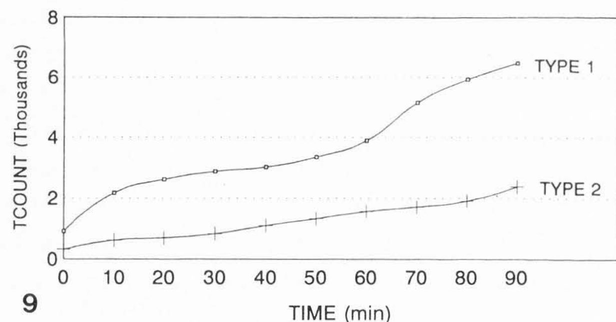
The calcium oxalate limit of metastability in the FILT fraction was determined by titration with aqueous sodium oxalate solution and was defined by the concentration of sodium oxalate which caused a sudden increase in particle number. A flow cytometer (described below) was used for this purpose.

Calcium oxalate crystallization was then induced at 37°C in the fractions FILT, UF and RET, by administration of a dose of aqueous sodium oxalate equivalent to the previously determined metastable limit of the FILT fraction. This amounted to the addition of 1 $cm^3$ of 0.04 M sodium oxalate solution per 100 $cm^3$ of test fraction. Since the dose given to each fraction was identical, their respective responses could be compared effectively.

The progress of calcium oxalate crystallization in each fraction was followed by two independent techniques: Coulter Counter and flow cytometry. A Coulter Multisizer (aperture size 140 $\mu$m) and an Epics Flow Cytometer equipped with an argon laser were used for this purpose. Both instruments were obtained from Coulter Electronics Ltd., Luton, England. Alignment and focusing of the sample stream and laser beam (flow cytometer) were performed daily. While the Coulter Counter has been widely used for the measurement of particle size-distribution profiles in many studies involving urinary calcium oxalate crystallization processes, flow cytometry has only recently been used for the first time in this field [49]. Its application in the study of urinary macromolecules has not been previously reported. Briefly, in a flow cytometer, particles in liquid suspension are passed through a laser beam. The scattered light is detected and provides data concerning particle size, number and morphology. Crystallization was monitored for 90 minutes (as advocated by Ryall and coworkers [53]) using both techniques. In addition to Coulter and flow cytometry techniques, scanning electron microscopy (SEM) was performed on the deposited crystals in each fraction. A Leica Cambridge S440 (Leica, Rockleigh, NJ) sigma scanning electron microscope was used for this purpose.




**Figures 7-9**. Flow cytometer plots of number *vs* time for different particle types (i.e., morphologies) in: the FILT fraction (**Fig. 7**); the UF fraction (**Fig. 8**); and the RET fraction (**Fig. 9**).

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## Results

Coulter Counter plots of particle number *vs* time and total particle volume *vs* time for the three fractions are given in Figures 1 and 2 respectively. Figure 3 gives plots of total particle number *vs* time for each fraction as determined by flow cytometry. Each of the three plots shown in Figure 3 can be re-plotted individually to show the distribution of particles in various arbitrarily defined size zones. In the present study, the distribution was divided into four such zones: small, medium, large and very large; these are shown as a function of time in Figures 4, 5 and 6. (Definition of these size zones is based on relative size and not on absolute size [49]. However, zone sizes can be calibrated by using beads of appropriate cross section). As mentioned earlier, flow cytometry can distinguish between different morphologies and can output data concerning the number of particles corresponding to different shapes. Figures 7, 8 and 9 show that two different types of structure were identified in each fraction; the plots show how the number of particles of both types varied with time.

No solid material was detected on the membrane after ultrafiltration. However, SEM of the FILT specimen revealed the presence of only a few calcium oxalate dihydrate (COD) crystals. These were small ($< 5\ \mu m$ cross section) and mainly single, but small aggregates were occasionally observed (Fig. 10). The UF fraction had more crystals than in the FILT sample; these appeared to be slightly larger (Fig. 11). In addition, aggregates were observed more frequently (but not in great numbers), and these were large (Fig. 12). Apatite deposits (Figs. 13 and 14) confirmed by energy dispersive X-ray analysis were also detected. In some cases, embedded COD crystals were observed (Fig. 13). RET fractions did not show any COD crystals.

## Discussion

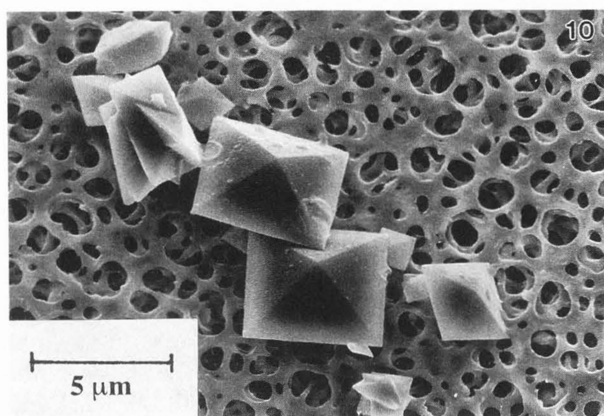
It must surely be a cause of concern and a source of frustration to investigators that studies on the role of UMM in calcium oxalate crystal (and stone) formation have continuously failed to produce consistent results. There are several possible explanations for this lack of reproducibility and agreement.

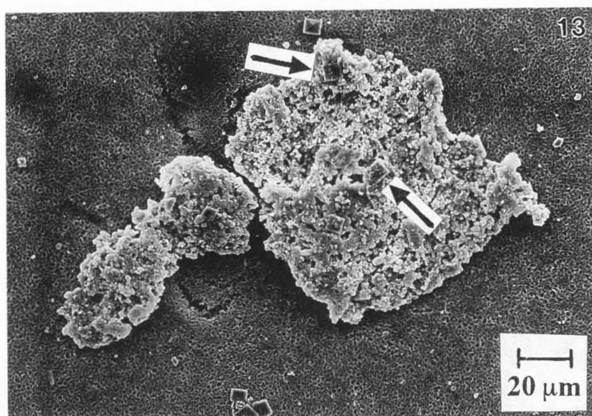
### Macromolecular separation

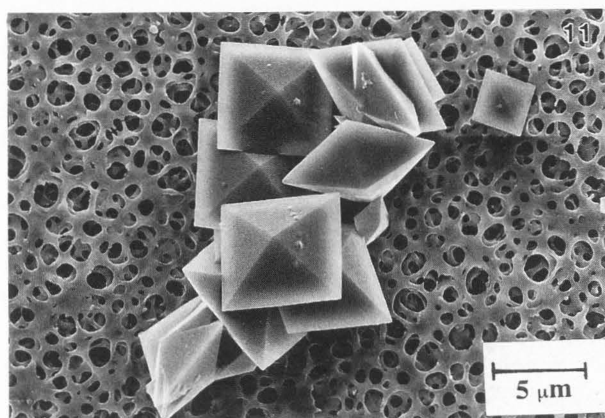
Isolation and separation procedures can induce conformational changes in UMM which will modify their adsorption onto mineral surfaces as well as their binding properties [2, 34]. Among the procedures that have been used frequently are dialysis [1, 6, 18, 20, 36, 56, 58] and ultrafiltration [2, 5, 11, 12, 21, 24, 31, 33, 42, 47, 48, 50, 51, 55, 60, 63]. Bio-Gel filtration, ion exchange chromatography and protein precipitation have also been used [34]. Thus, the role of UMM, as deduced in *in vitro* experiments, may be influenced by the technique used for their original isolation.

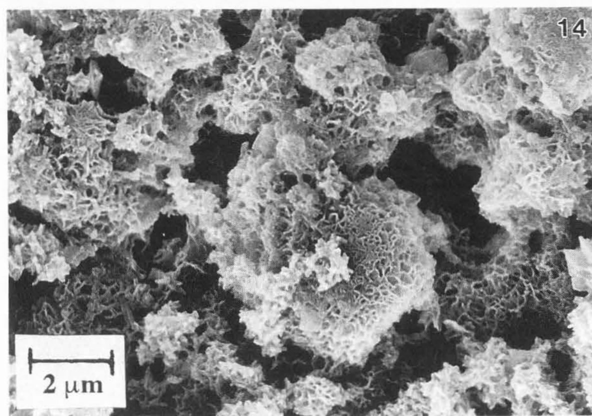
### Nature of the crystallization system

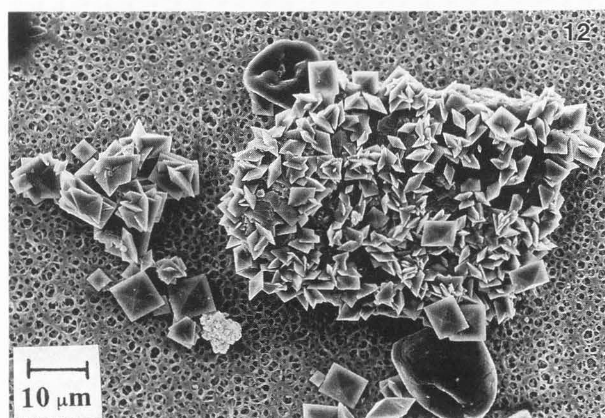
It has been pointed out by other workers that most of the discrepancies concerning the role of UMM are due to the wide variety of crystallization systems that




**Figures 10-14.** Scanning electron micrographs of: small, single and aggregated calcium oxalate dihydrate crystals occasionally observed in the FILT fraction (**Fig. 10**); calcium oxalate dihydrate crystals (**Fig. 11**) and aggregate (**Fig. 12**) observed in the UF fraction; and apatite deposits with embedded calcium oxalate dihydrate crystals (arrows; **Fig. 13**) and apatite deposits (**Fig. 14**) observed in the UF fraction.

have been employed [14, 32]. These include batch [6, 10, 19, 20, 58], seed [3, 12, 19, 33, 36, 43, 52, 63], continuous [9, 32, 42, 45], constant composition [34], rotary evaporation [21, 24, 50, 51, 60], oxalate loading [2, 5, 11, 18, 39, 55, 63], freezing [21, 60], paperwick [56] and vapour diffusion [7] models. Hesse and coworkers [28] have correctly stated that crystallization systems are not identical, and that therefore different results are attributed to the specific characteristics of the experiment. We wish to endorse this view and to elaborate upon it by suggesting that the activity of UMM is highly sensitive to and dependent upon the crystallization milieu in which they are present. This milieu encompasses not only the nature of the crystallization system but also possible additive, competitive or synergistic influences of all UMM components, some of which may be inhibitors and some of which may be promoters. Others may even have a dual inhibitor-promoter capacity depending on experimental conditions [23, 25]. Indeed, the performance of UMM may also be dependent on the presence of other urinary components which themselves may influence the capacity (of UMM) to promote or inhibit the different crystallization processes. Prominent amongst these is monosodium urate which has been shown to reduce the inhibitory potency of certain UMM in inorganic solutions [12, 39, 54]. Thus, conditions operating in a batch crystallizer, for example, will pro-

duce a totally different milieu to that existing in a continuous crystallizer or rotary evaporator. We suggest that the different conditions existing in the different crystallization systems induce a "ripple effect" in which numerous inter-related processes involving inhibition and promotion of nucleation, growth and aggregation interact with each other to finally produce a nett, system-dependent mechanism.

### Detection and monitoring procedures

We believe that interpretation of the role played by UMM in calcium oxalate crystallization depends upon the very method used for the detection and monitoring of the crystallization processes which are actually occurring in the samples under investigation. Detection procedures which have been employed are Coulter Counter techniques [5, 11, 32, 45, 52, 55, 58], radioactive assay [6, 12, 18, 19, 33, 36, 39, 43, 50, 51, 63], optical methods [21, 24, 60], electron microscopy [11, 32, 55], specific ion electrode [2, 34], particle analyzer (electrozone/celloscope counter type) [9, 42], nephelometry [10, 22, 47, 48], colorimetry [20] and fluorimetry [56]. Because these techniques have different sensitivities and detection limits, it is likely that they monitor different stages and aspects of the crystallization processes which are taking place. Therefore, interpretations based on Coulter Counter measurements, for example, may differ substantially from those based on turbidity determinations, even though the same urine specimen and the same method of inducing crystallization are used. Discrepancies in interpretation are further compounded by confusion with respect to the crystallization mechanism(s) (nucleation, growth or aggregation) that is (are) actually being measured by the particular detection process being used.

In the present study, calcium oxalate crystallization was induced in different macromolecular fractions of the same urine and was monitored by two independent techniques: Coulter Counter and flow cytometry. While the former has been widely used for such studies, the latter has only recently been implemented in the investigation of urinary calcium oxalate crystallization [49]. Its application in the study of UMM has not been reported previously.

Figure 1 shows that after 90 minutes, the number of particles formed in the different fractions are in the sequence UF > FILT > RET. This demonstrates that nucleation occurs to a lesser extent in the presence of UMM than in its absence. A possible inference is that UMM are inhibitors of calcium oxalate nucleation. This contradicts findings of an earlier study of ours which was conducted under similar conditions except that turbidimetry and Malvern particle size techniques were employed [47]. In that study, UMM were found to be promoters of calcium oxalate nucleation. The different conclusions drawn in the two studies are due to different techniques used to monitor the crystallization processes. These techniques are based on different sensing principles, and thus, data acquired by each will have different significance and may elicit different interpretations.

Figure 2 shows that the final crystal volumes are in the same sequence as crystal numbers. Thus, the volume of crystals formed in UF is greater than that formed in other fractions. There are three mechanisms by which this could occur. First, it could arise simply from the presence of the largest number of particles in this fraction (as shown in Fig. 1). This is consistent with our suggestion that UMM are nucleation inhibitors. Second, larger crystal volumes could occur in UF if bigger particles were present in this fraction. This implies that UMM could be inhibitors of growth.

Third, when crystal aggregation occurs with accompanying entrapment of solution, "particle" volume will increase. In such an event, the smaller particle volumes observed in the presence of UMM (FILT and RET) suggest that UMM may be inhibitors of crystal aggregation.

Thus, Coulter Counter data, for this particular specimen, yield several possibilities for the role of UMM. The data suggest that UMM are probably nucleation inhibitors, but that they may also be growth and/or aggregation inhibitors.

Flow cytometry data for total particle numbers in the three fractions (Fig. 3) show the sequence FILT > UF > RET. It is apparent that FILT and UF occupy different positions in this sequence compared to that observed by Coulter techniques. Nevertheless, in both sequences FILT > RET and UF > RET, indicating that particle numbers are lower in the fraction containing concentrated levels of UMM. We interpret these results as suggesting that UMM in this sample are nucleation inhibitors if they are present in high concentrations. This supports our Coulter data but qualifies concentration as an important factor. The observation, by flow cytometry, that FILT > UF indicates that, in this subject, UMM may be nucleation promoters when present in normal concentrations. As has been mentioned earlier, the role played by UMM as promoters or inhibitors is dependent on concentration. In a previous study involving urine from non-stone formers, we reported that UMM are promoters of nucleation [47]. The present study, involving stone forming urine has confirmed this and shown that, at higher concentrations, inhibition might occur.

Comparison of Figures 4, 5 and 6 shows that the number of "very large" particles in all fractions is negligible. However, "large" particles are deficient in the RET fractions, suggesting that UMM may be inhibitors of growth and/or aggregation. "Small" and "medium"

particles follow a similar sequence which confirms this effect. However, the inhibitory effect might only occur in the concentrated RET fractions while an entirely different effect might occur when lower concentration levels of UMM are present.

The flow cytometer graphs for particle morphology (Figs. 7, 8 and 9) show that there are significant numbers of two "types" present in all fractions. The existence of a second shape (whatever it might be) is interpreted as being indicative of aggregation. Numbers of the predominant shape (type 1) follow the sequence RET < UF < FILT while numbers of the aggregated shape (type 2) follow the sequence RET < UF = FILT. Thus, aggregation occurs to a lesser extent in RET fractions which have concentrated levels of UMM suggesting that the latter are inhibitors of aggregation. This supports our interpretation of the size distribution data. It may be further argued that since aggregation does not occur to a great extent in RET, higher particle numbers would be expected in this fraction. However, to the contrary, lower numbers are recorded. This observation provides further evidence in support of UMM being inhibitors of nucleation.

Our SEM observations are unable to conclusively demonstrate the role of UMM with respect to nucleation and growth. However, the presence of aggregates in UF samples and their absence in FILT and RET fractions support our Coulter and cytometer data which suggest that UMM are inhibitors of calcium oxalate aggregation. We are unable to explain the presence of apatite in UF samples.

It must be emphasized that it was not our intention in this paper, nor was it an objective of the present study, to make a definitive statement concerning the effect of UMM on different crystallization mechanisms. To do this, an in depth investigation involving many urines would be required. Rather, we simply wish to illustrate that different techniques can lead to different, and sometimes incomplete, conclusions. Indeed, inspection of Figures 1 and 3 shows that interpretation of the kinetic plots at different stages of crystallization might yield different conclusions. We also wish to use the data, obtained from only one patient, to demonstrate that flow cytometry provides very useful information and that it might prove to be a valuable tool in this field, as has been the case with the Coulter Counter.

## Crystallization medium

In the light of the above arguments, it should be obvious that the crystallization medium will be of crucial importance in determining the nature of the crystallization which occurs. Thus, experiments carried out in aqueous solutions or in synthetic urines cannot be compared with each other (unless the chemical composition is constant in all the test solutions) or with those performed in whole urines. Although results obtained from such solutions, i.e., aqueous or synthetic, provide useful information about basic inhibition and promotion mechanisms, they are of limited value since data cannot be extrapolated directly to real urine conditions.

## Hypothetical standard crystallization model

Thus, the problem faced by investigators is that, in order to make a meaningful and valid assessment of their results, comparisons must be effected with other studies in which precisely the same procedures and conditions were operative. Unfortunately, this is not easy to achieve because of the wide diversity of experimental systems that have been employed.

It is clear that a standard reference crystallization model is required. Such a model should define: the method by which the UMM are isolated, concentrated or separated; the crystallization system itself, e.g., continuous crystallizer or rotary evaporator; and a monitoring procedure, e.g., Coulter or specific ion electrode, which allows a distinction to be made between the various crystallization mechanisms which might occur. It is also of fundamental importance to define terminology [41].

Having described the features required of a standard crystallization model, the challenge which must now be addressed concerns identification of the various elements of such a model which best satisfies or meets these requirements. Ideally, individual laboratories should undertake comparative studies in which different techniques for separating UMM, different crystallizers and different crystal monitoring procedures are investigated to establish which of these are most suitable and which combination of these best complement each other. Such an approach would, no doubt, enable an appropriate system to be defined. However, it is unlikely that any single laboratory would be able to undertake studies of this nature because of obvious financial and logistical constraints. Therefore, any proposal for a standard reference model will have to be based on existing knowledge and the documented experience of the many investigators who have used a variety of crystallization systems in their studies of UMM.

Consideration of the literature shows that the most widely used method for the separation of UMM is ultrafiltration [2, 5, 11, 12, 21, 24, 31, 33, 42, 47, 48, 50, 51, 55, 60, 63]. Reservations have been expressed by some authors that this procedure may modify the activity of UMM [2, 34], but this does not appear to have been demonstrated conclusively. Moreover, it has been shown that urinary concentrations of sodium, potassium, calcium, magnesium, phosphate, oxalate, urate, pyrophosphate and citrate are unaltered by this technique [11, 50], and that it does not affect the metastable limit of the

urine [11, 55]. This technique has an advantage over dialysis since it yields two fractions for study, an ultrafiltrate and a concentrated retentate fraction, whereas the latter yields only the retentate fraction. Therefore, we propose that the standard method for separating and concentrating UMM be ultrafiltration.

Choice of the crystallizer itself, i.e., the method by which crystallization is induced, is of paramount importance in establishing a standard reference model. Systems which attempt to simulate *in vivo* conditions and crystallization processes must take precedence over those which are far removed from physiological reality. The mixed suspension, mixed product removal (MSMPR) crystallizer (or continuous crystallizer) first proposed by Finlayson [13] has been widely used in the study of calcium oxalate crystallization processes [8, 35, 46] including those involving UMM [9, 32, 42, 45]. This system reaches a steady state with a constant supersaturation, and because it operates with continuous flow, individual crystals remain in the suspension for a relatively short time, similar to transit times through the kidney [30]. Unlike other methods, it permits the simultaneous but independent measurement of nucleation and growth rates [30]. A detailed description of the theoretical and practical aspects of the MSMPR crystallizer is given by Rodgers and Garside [46].

Although UMM investigations using this type of crystallizer appear to have been limited to synthetic urines [9, 32, 42, 45], a new method using "nearly" whole urine has recently been developed [30]. The effects of heparin and hyaluronic acid were tested successfully in the system. The MSMPR crystallizer is thus an appropriate system for a standard reference crystallization model.

Although we are encouraged by our application of flow cytometry to the study of UMM, we recognize that such a facility is not readily available to all researchers. Thus, of the different techniques used to detect and monitor crystallization, Coulter Counter procedures appear to be the most versatile. Besides providing crystal size and crystal volume distribution profiles, crystal growth and aggregation can be measured, even when both are occurring simultaneously [52]. A further advantage of using a Coulter Counter is its proven compatibility with the MSMPR crystallizer described earlier [8, 40, 45, 46]. Furthermore, by coupling these measurements to SEM investigations [23, 55], a powerful approach for differentiating between nucleation, growth and aggregation is achieved.

Since it is generally agreed that the role of any urinary component in promoting or inhibiting crystal formation is highly dependent on its chemical milieu, studies on the role of UMM should be conducted in whole, real urines and not aqueous or synthetic solutions. Nor, ideally, should filtered or sieved urine be used. Investigators will be aware that crystals in the urine contain many macromolecules both on their surfaces as well as within their own structure. Many of these are crystallization modulators like prothrombin, osteopontin and uromodulin. In addition, when cellular debris is removed, much of the lipids and membranes are lost. Since it is widely accepted that these substances are intimately involved in almost all physiological and pathological calcification processes, their removal depletes the urine of crystallization promoters. Filtration may also remove many urinary macromolecules like Tamm-Horsfall protein, osteopontin and albumin. Thus, urine which has been sieved and filtered cannot be classified as "whole" urine. However, most investigators will agree that "whole" urine clogs up crystallizers and sensitive detectors, many of which use small apertures, e.g., Coulter Counter. Use of "whole" urine is thus practically unfeasible; on the other hand, crystallization experiments using filtered and sieved urine are far more likely to be completed successfully. Thus, sieved and filtered urine would have to be the medium of choice for the standard system. Finally, since the excretion of many urinary components varies diurnally, 24-hour urine specimens should be used in these studies. The pH of the specimen should not be altered as it is a natural determinant property. Experiments should be performed at 37°C.

Of great interest would be a comparative study involving investigation of the same urine by the standardized protocol described in this paper and by other systems. Perhaps an inter-laboratory "quality control" type of exercise could be implemented. Whatever the outcome, unless investigators standardize their whole approach to the investigation of urinary macromolecular activity, discrepancies and lack of consensus on their role in urolithiasis are inevitable.

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## Discussion with Reviewers

**Reviewer I**: What was the reproducibility of the results? For example, are the differences between UF and FILT significant?
**Authors**: The reproducibility of the system was tested by measuring the calcium oxalate crystallization kinetics 6 times in FILT fractions from each of three different urines. The coefficient of variation was 13%. Kinetic data obtained in the experiments involving the urine reported in this paper were tested for significant differences by assuming that particle numbers and volumes have Poisson distributions [64]. All data were significantly different after 90 minutes.

**Reviewer I**: What was the osmolality of the urine?
**Authors**: Osmolality was not measured.

**Reviewer I**: The 24-hour collection may not resemble urine as actually passed. What are your comments?
**Authors**: While you are correct in expressing this concern, collection of 24-hour urine specimens for study is a widely practiced protocol. Whatever collection is effected (early morning, random, 12-hour, etc.), limitations will always be present.

**J.P. Kavanagh**: What was the ratio of the volumes of the RET and UF fractions at the end of the ultrafiltration? Any standard protocol for preparation of UMM should include this detail and also define sample collection and pre-treatment.
**Authors**: The UF volume was 120 $cm^3$ while that of the RET was 60 $cm^3$. Since the original volume of FILT in the ultrafiltration cell was 180 $cm^3$, the RET fraction was concentrated by a factor of three. This concentration factor is recommended for the standard protocol as a larger one would be too far removed from physiological conditions while a smaller one might not induce detectable effects. Samples should be collected in glass bottles which have been washed with hydrochloric acid and distilled water. No preservative should be present as it may affect crystallization.

**J.P. Kavanagh**: It is not clear to me if a single experiment was performed for each urinary fraction, with sampling at intervals for analysis by Coulter Counter and flow cytometry, or if separate experiments were performed. Factors other than the measuring technique might also influence the outcome of the crystallization experiments reported. Were there any differences in such details as sample age, vessel geometry or stirring?
**Authors**: A single experiment with sampling at various times was performed. Since only one sample was used, age was not a variable. Vessel geometry and stirring were constant.

**J.P. Kavanagh**: I share the authors' concern about the system dependence of results and the associated difficulties in interpretation that this can lead to, but would a reference crystallization method resolve this difficulty? It is doubtful if any one method would always be appropriate for the questions being asked by different investigators. On the other hand, there could be some merit in being able to compare baseline descriptions of crystallization modifying properties of urines or urinary fractions being tested.
**Authors**: Investigators are using many different systems to answer a set of questions of common interest. Perhaps an inter-laboratory "quality control" series of experiments (as discussed between the reviewer and author) might provide some (consistent) answers.

**S. Ebisuno**: In Figures 7 to 9, two particle crystals show different patterns. However, it is impossible to understand the morphological difference because of lack of microscopy.
**Authors**: The cytometer data only indicate a qualitative difference in particle morphology. In this study, two such shapes were detected; these have been interpreted as being associated with nucleation and aggregation. Although direct correlation with SEM studies was not achieved, examples of both mechanisms were observed using the latter technique.

**S. Ebisuno**: How do you confirm the apatite deposits with embedded calcium oxalate dihydrate crystals in Figure 13?
**Authors**: These deposits were confirmed by energy dispersive X-ray analysis which revealed the presence of Ca and P in the former and Ca in the latter.

**S. Ebisuno**: The inhibitory activity of UMM on calcium oxalate crystal growth and/or aggregation is depend-

ent on ionic strength [65]. Were there any differences in the ionic strengths of the three urinary fractions?
**Authors**: This is a good point. Unfortunately, ionic strengths in the various urinary fractions were not determined.

**R.L. Ryall**: Advances in protein chemistry and immunological techniques have enabled us to study the effects of individual, identified urinary macromolecules on calcium oxalate crystallization. Do the authors feel that useful information will be derived from studies based simply on global removal of all urinary macromolecules?
**Authors**: Removal of all urinary macromolecules will identify the size ranges that are of importance. By careful selection of membranes with different cut-offs, the effective size range can be accurately defined. Perhaps thereafter, immunological techniques should be used to identify the urinary macromolecules within such a range.

**R.L. Ryall**: A call was made 15 years ago (reference 41) for a standard system of nomenclature in urolithiasis research. Though more simple than a standard experimental model, universal use of a reference nomenclature has never been achieved. How feasible would be the implementation of a standard reference model for studying the inhibitory or promotory effects of macromolecules or other agents?
**Authors**: The feasibility is directly dependent on the extent to which investigators are honest enough to recognize that standardization of nomenclature, and a reference model would not only decrease the confusion that exists in this area of stone research but would also clarify the inhibitory and promotory role of urinary macromolecules and other agents.

**R.L. Ryall**: I am most concerned about Figure 12. This micrograph clearly shows the presence of two red blood cells, and this suggests that the patient had haematuria. His urine therefore would have contained any number of additional macromolecules not found in urine under normal conditions. These would have affected the inhibitory potency of the FILT and RET fractions and their activity relative to the UF specimen. The presence of these red blood cells in urine that had been ultrafiltered indicates either that (**a**) the ultrafiltration was incomplete (the membrane may have been defective) or (**b**) the urine sample was later contaminated with blood, the patient's or someone else's. In either case, the validity of the data must be questioned.
**Authors**: The two deposits referred to are not red blood cells. All our urine specimens used in crystallization and other studies are routinely tested for the presence of haematuria [49] using a Boehringer Mannheim Combur 9 test strip (Boehringer, Mannheim, Germany). Urines which test positive are discarded [49]. The urine which was used in the present study was thus free of blood prior to ultrafiltration. We have seen these deposits, the morphology of which closely resembles that of red blood cells, in several of our crystallization experiments using urine from normal and stone-forming subjects. Energy dispersive (spot) X-ray analysis has revealed the presences of Na, K, S and Cl in these deposits. Therefore, it is likely that they are salts which have crystallized during our experiments.

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