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Calcium oxalate crystallization kinetics at different concentrations of human and artificial urine, with a constant calcium to oxalate ratio

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Abstract The effect of in vitro dilution of artificial urine or human urine on the crystallization of calcium oxalate was examined in a mixed suspension, mixed product removal crystallization system. Direct growth inhibition by components of artificial urine was not significant and supersaturation was the dominant factor in determining crystal nucleation and growth rates. Dilution of human urine caused a decrease in crystal growth rate that was independent of the input calcium and oxalate concentrations, suggesting that dilution of growth inhibitors could be physiologically more important than any reduction in supersaturation. This loss of growth inhibition was counteracted by a reduction in nucleation promotion, with the net effect that the mass of crystals declined. Correlation of crystallization measurements with urinary concentration (osmotic pressure) confirmed these observations, with a negative relationship for growth rate and a positive relationship for nucleation rate and suspension density. Increasing the concentration of urine shifts the crystallization balance from low nucleation/high growth to high nucleation/low growth. Calcium oxalate crystalluria in healthy urine is therefore less likely at early stages of urine development in the nephron and the likelihood can be further reduced by increased fluid output. Our results suggest that lowering the heterogeneous nucleation activity by dilution is more than sufficient to override the loss of growth inhibition.

Key words Calcium · Oxalate · Calcium oxalate crystallization · MSMPR · Urolithiasis · Urine volume

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Introduction

There is wide agreement that the single most effective means to prevent renal stone formation is to increase one's fluid output [2–4, 13, 17, 20]. The primary purpose of this is to reduce the concentration, and thus the supersaturation, of stone-forming salts, but a possibly negative (and as yet unquantified) by-product will be to reduce the concentration and activity of crystallization inhibitors [2, 16, 17]. The importance of urine concentration with regard to its ability to support calcium oxalate crystallization is also relevant to intra-nephron crystallization, where the composition is being altered by secretion and reabsorption, particularly of water. Recent work has suggested that dilute urine or specific formulations reproducing the ionic composition of proximal or distal tubules might be more relevant for experimental studies than urine that has passed through the bladder [7, 8, 12]. With these points in mind, the aim of the series of experiments we report here was to investigate the significance of urine concentration on calcium oxalate crystal growth and nucleation and to try to distinguish between effects due to the major urinary electrolytes, to calcium and oxalate and to other urinary components, especially urinary macromolecular substances (UMMS). These results should be viewed in the context of similar experiments using fundamentally different methods.

In seeded, batch crystallizer systems, inhibition of calcium oxalate crystal growth has often been shown to be related to the amount of urine included. Usually this has been performed with urine diluted to about 1–10% of its initial concentration [14, 18, 21], which has the advantage that the calcium and oxalate concentrations of the metastable starting conditions are not unduly perturbed by the contribution from the urine. The inhibition/urine concentration relationship can be used to quantify the crystal growth inhibitory activity [8]. Using a similar method but with 5–100% urine, Baumann et al. [1] also showed growth inhibition was related to urine

concentration and this was relatively insensitive to the calcium and oxalate concentrations in the urine employed. When unseeded systems are used both nucleation and growth effects will contribute to the crystallization; if the observations are made in the early phase then nucleation effects will dominate, whereas growth will be more important as the crystallization develops. Using a method in which crystals are counted 2 min after oxalate additions [23], the calcium oxalate crystallization risk was found to be positively correlated with increasing concentration (measured by conductivity) of urine samples [24]. When the mass of precipitate after a 33 min crystallization period was measured there was a strong positive correlation between the concentration of urine samples (creatinine concentration or osmotic pressure) and the degree of crystallization inhibition [19]. The apparent paradox between these two reports – that the urinary concentration is related both to its crystallization risk and to its inhibitory power – can be understood in terms of nucleation promoters (most likely to dominate in the first case) and growth inhibitors (most relevant in the second). In vivo and in vitro dilution of urine were found to raise the metastable limit for calcium oxalate crystallization (i.e. to lower the propensity for nucleation) [14]. Using concentrated urine, frozen and thawed to induce crystallization, both nucleation-promoting and growth-inhibiting activities were shown to be present in the macromolecular fraction of human urine [6].

The system we have employed is mixed suspension, mixed product removal (MSMPR) continuous crystallization in which a dynamic equilibrium is obtained and the crystal growth rate and crystal nucleation rate can be measured and from these values the suspension density estimated [8, 10, 22]. This method has advantages over batch crystallization but is not without limitations [9]. The experiments consist of measurements of the equilibrium growth and nucleation rates at different dilutions of human urine (HU) and artificial urine (AU), which differ mainly in the presence and absence of UMMS. We therefore expect to gain information on the effect of urine-concentration and urine-specific crystallization modifiers (especially UMMS) on growth and nucleation.

Materials and methods

Human urine samples were obtained from healthy men or women (six individuals took part) with no history of urolithiasis. Specimens were collected in a pre-warmed Dewar flask, adjusted to pH 6.0 with HCl or NaOH and centrifuged at 3000 *g* for 5 min at 37°C. The urinary calcium concentration was immediately measured by atomic absorption and osmotic pressure by freezing-point depression and the crystallization experiment begun within 30 min of sample collection. The three solutions comprising the artificial urine are described in Table 1.

The crystallization experiments were performed in a pair of identical MSMPR crystallization chambers (20 ml reaction volume, thermostatted at 37°C and continuously stirred: see Nishio et al. [15] for further details). HU or AU Feed Solution 1 (held at 37°C) was pumped into each crystallization chamber at 92% of the total flow rate and Feed Solutions 2 and 3 were each pumped at 4% of total flow rate. When using HU the composition of Feed Solution 2 ($CaCl_2$) was adjusted to take into account the endogenous calcium.

A detailed description of the MSMPR method and the equations governing its behaviour are given by Söhnel and Garside [22]. The residence time (τ) is the average time that crystals remain in the crystallizer and is given by

$$\tau = V/Q \quad (1)$$

where V is the chamber volume and Q is the total flow rate. All experiments reported here were performed with $\tau = 7$ min. After a number of residence times have passed (typically 6–10) the particle size distribution reaches equilibrium. Starting at 8 residence times the particle size distribution in each chamber was measured three times (Elzone 80XY, Particle Data Inc.). Particle number data were collected in 128 size channels, distributed over 8 log scales. When plotted as ln N (where N, no./ml, is the number of crystals per unit volume greater than size L) against size (L), MSMPR data should give a straight line and the growth rate (G, μm/min) and nucleation rate (B_0, no./min/ml) can be calculated from the slope and intercept according to the relationship

$$\ln(N) = \ln(B_0\tau) - L/G\tau \quad (2)$$

After converting the raw data to this form, results from at least 20 size channels, covering a minimum size range of 14.5–21.5 μm but often extending up to 32 μm, were used for linear regression and calculation of the crystallization parameters.

The suspension density (M_T, mol/l) can be estimated from the equation

Table 1 Make-up of artificial urine (relative urine concentration = 1.0) and its composition (mmol/l) in the crystallizer

Input to crystallizer		Within crystallizer	
Feed Solution 1[a]			
NaCl	160.0	Na^+	195.6
KCl	82.1	K^+	81.3
Na_2HPO_4	25.0	NH_4^+	43.5
$(NH_4)_2SO_4$	21.9	Mg^{2+}	3.0
NH_4Cl	3.5	Ca^{2+}	6.0[c]
$MgCl_2$	3.26	Cl^-	244
K_3Citrate	2.17	PO_4^{2-}	23.0
Feed Solution 2 $CaCl_2$	150.0[b]	SO_4^{2-}	20.2
		Citrate^{3-}	2.0
Feed Solution 3 Na_2oxalate	30.0	Oxalate^{2-}	1.2[c]

[a] Adjusted to pH 6.0 with HCl
[b] With human urine this concentration was reduced appropriately to take into account the endogenous urinary calcium
[c] The concentration that would be obtained if no crystallization took place

$$M_T = \pi\rho B_0 G^3 \tau^4 / D \times 10^{-9} \quad (3)$$

where ρ is the crystal density (g/cm^3) and D is the molecular weight of the crystalline species. In practice, for the calculation of suspension density we assumed that only calcium oxalate dihydrate ($\rho = 2.22$ g/cm^3) was precipitated. Apart from this simplification, there are a number of difficulties in applying Eq. (3) as it is very sensitive to small errors in τ and errors in the estimates of B_0 and G can be compounded.

One of the pair of crystallization chambers served as a control and received the three feed solutions without further dilution (relative urine concentration = 1.0). Dilution experiments were performed with dilution by the appropriate addition of purified water to urine only (Feed Solution 1 or HU) or dilution of urine, calcium and oxalate (Feed Solution 1 or HU, Feed Solutions 2 and 3). All experiments were therefore carried out at a fixed calcium to oxalate ratio of 5:1 The dilutions used are shown in Table 2. The calcium oxalate supersaturations for the mixed feed solutions of AU were calculated by Equil2 [25], assuming no crystallization (Table 2). Because of the contribution of Feed Solutions 2 and 3, the actual HU concentration at a relative urine concentration of 1.0 was 92%.

Each dilution was repeated four times using freshly prepared AU or fresh HU on each occasion. The results are expressed as the mean difference between control and diluted crystallization chambers. Statistical analysis was by analysis of variance (ANOVA) of the paired differences. The significance of individual relative urine concentrations can be judged by comparison of the 95% confidence limits of the mean difference to zero. Control data (undiluted) with AU showed no significant differences across all experiments ($P > 0.05$, by ANOVA) and in all cases there was no significant difference between the experimental and control chambers at a relative concentration of 1.0 ($P > 0.05$).

Results

The mean paired growth rate differences from the four dilution experiments are presented in Fig. 1. When HU was diluted the growth rate increased and this response was quantitatively the same whether calcium and oxalate were diluted or kept constant. Using AU, growth rates decreased with increasing dilution and this was more pronounced when calcium and oxalate, as well as the urine, were diluted.

The mean paired nucleation rate differences are shown in Fig. 2. When HU was diluted the nucleation rate decreased, and this was a little more pronounced when calcium and oxalate were also diluted than when they were kept constant. When AU, but not calcium and oxalate, were diluted, the nucleation rate increased. When calcium and oxalate, as well as AU, were diluted nucleation rates appeared not to change, but these estimates became difficult to quantify accurately and reproducibly. This is because smaller and fewer crystals were being produced that did not span such a wide range of size channels as was usually the case and hence the regression to the particle size data was dominated by a few small size ranges, giving added uncertainty over the estimation of the intercept.

Table 2 The urine concentrations used in the dilution experiments and the calcium oxalate supersaturation being fed into the crystallizer when used with artificial urine

Relative urine concentration	Supersaturation	
	Calcium and oxalate diluted	Calcium and oxalate constant
1.0	39.7	39.7
0.75		44.5
0.67	24.3	
0.50	18.2	57.7
0.33	12.4	
0.25		80.4
0.0		123.0

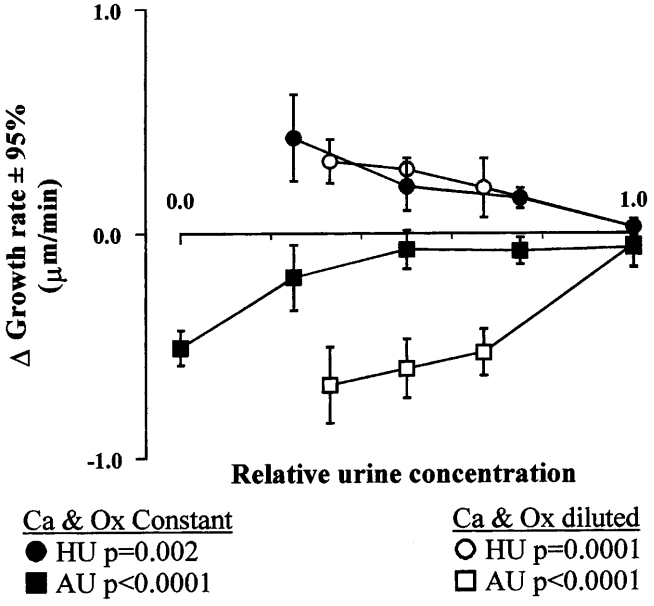

Fig. 1 Mean paired differences in growth rates (±95% confidence limits). *HU* human urine, *AU* artificial urine, *Ca* calcium, *Ox* oxalate

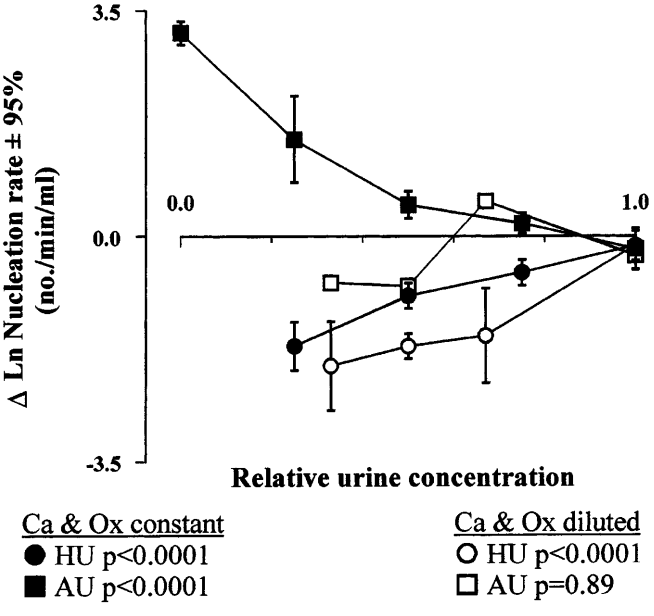

Fig. 2 Mean paired differences in log nucleation rates (±95% confidence limits)

From the growth and nucleation rate data, the suspension density of precipitated calcium oxalate can be calculated (Eq. 3). For AU the control data averaged 0.61 mM (the available maximum was 1.2 mM); with HU there was considerable variation in the suspension density of control samples, the means corresponding to the different relative urine concentrations ranged from 0.34 to 1.03 mM, and the overall average was 0.65 mM. The change in suspension density is shown in Fig. 3. At the lower dilutions of AU, precipitation was almost complete (calcium and oxalate constant) or almost completely absent (calcium and oxalate diluted). This reflects the changes occurring in the supersaturation of the mixed feed solutions being input to the crystallizer (Table 2). With dilution of HU there was a small decline in suspension density when calcium and oxalate were kept constant, but this was only significant at the lowest dilution. There was a more pronounced reduction in suspension density when calcium and oxalate were also diluted. The changes observed in the four dilution experiments are summarized in Table 3.

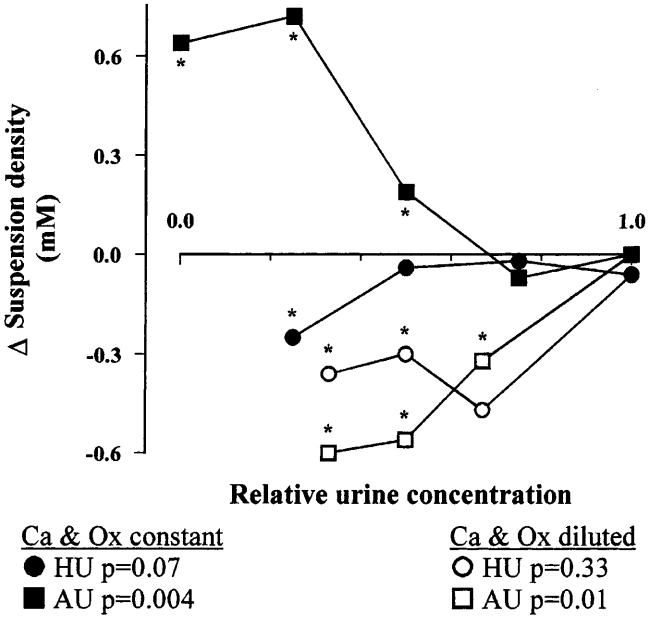

Fig. 3 Mean paired differences in suspension density. Confidence intervals are large and overlapping; they have been omitted for clarity. Points significantly different from zero ($P < 0.05$) are marked with an *asterisk*

The dilution experiments with HU take no account of the different concentrations of the native urines as passed by the subjects. From consideration of the results presented, one would expect this to be a significant factor; indeed this underlines the requirement to use matched control and experimental samples running in parallel. To examine this, the urinary concentration (expressed as the osmotic pressure) of urines used as controls and experimental samples from the dilution experiment with constant calcium and oxalate were related to the crystallization results. Twenty-eight control urines had a mean osmotic pressure of 486 mosmol/kg H_2O (range, 162–949); 16 diluted experimental samples had a mean (and range) of 279 (97–775) mosmol/kg H_2O (taking into account the dilution applied: see Table 2). There was a significant negative relationship between growth rate and urine osmotic pressure (Fig. 4), a significant positive relationship between the natural log of nucleation rate and urine osmotic pressure (Fig. 5) and a significant positive relationship between the suspension density and urine osmotic pressure (Fig. 6).

Discussion

In an MSMPR system the supersaturation reaches a dynamic equilibrium that is determined by the prevailing growth and nucleation rates, acting upon the input supersaturation conditions within the selected residence time. The supersaturation of the input is primarily dependent on the calcium and oxalate concentrations and decreases as these are diluted; when these are kept constant, and the remaining components are diluted, the input supersaturation rises because of dilution of ions such as citrate and magnesium and ionic strength effects (Table 2). Therefore dilution of all components might be expected to have a different result from dilution of all except calcium and oxalate.

The experiment in which HU, calcium and oxalate were diluted (open circles, Figs. 1–3) is comparable to

Table 3 Summary of changes in input supersaturation (SS), nucleation rate (B_0), growth rate (G) and suspension density (M_T). Arrows show the direction of change i.e., increasing (↑), no change (↔) or decreasing (↓); the number of arrows is indicative of the magnitude of the change (*HU* human urine, *AU* artificial urine, *ns* not significant)

Dilution experiment			Outcome			
Urine	Ca, Ox diluted	Symbol in Figs. 1–3	SS	B_0	G	M_T
HU	Yes	○	↓	↓↓*	↑*	↓ns
AU	Yes	□	↓	↔ns	↓↓*	↓↓*
HU	No	●	↑	↓*	↑*	↔/↓ns
AU	No	■	↑	↑*	↓*	↑↑*

* $P < 0.05$

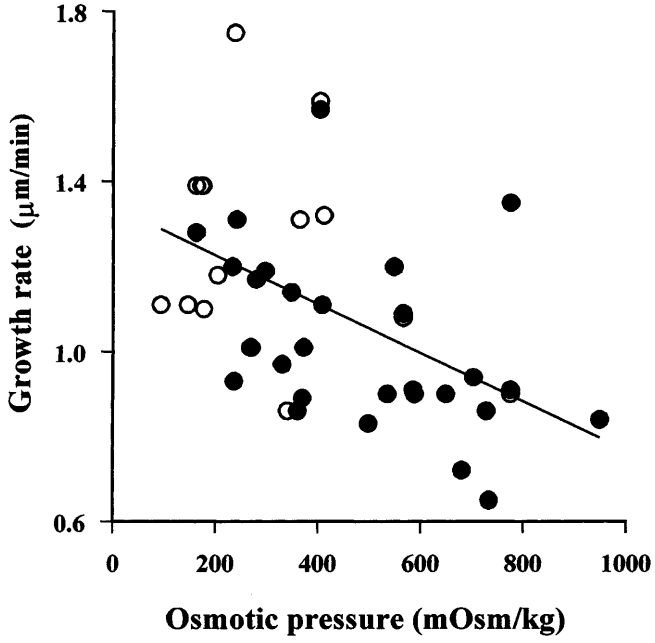

Fig. 4 The relationship between calcium oxalate growth rate and the osmotic pressure of human urine samples (n = 44, r = –0.51, P = 0.0004), at a constant calcium and oxalate load. ● 92% urine, ○ urine further diluted

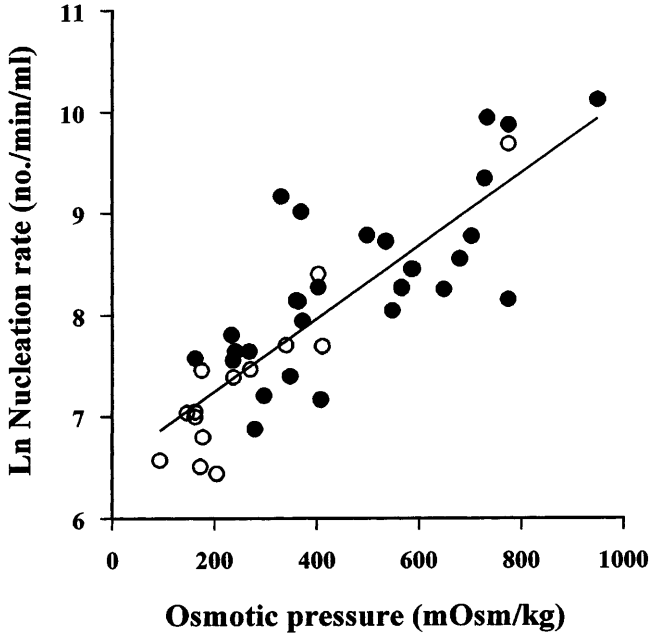

Fig. 5 The relationship between calcium oxalate log nucleation rate and the osmotic pressure of human urine samples (n = 44, r = 0.84, P < 0.0001), at a constant calcium and oxalate load. ● 92% urine, ○ urine further diluted

increasing urine output (reading the graphs from right to left) and is also relevant to changes in crystallization behaviour that might occur as urine is concentrated by water reabsorption during passage through nephrons (reading from left to right). The changes in growth and

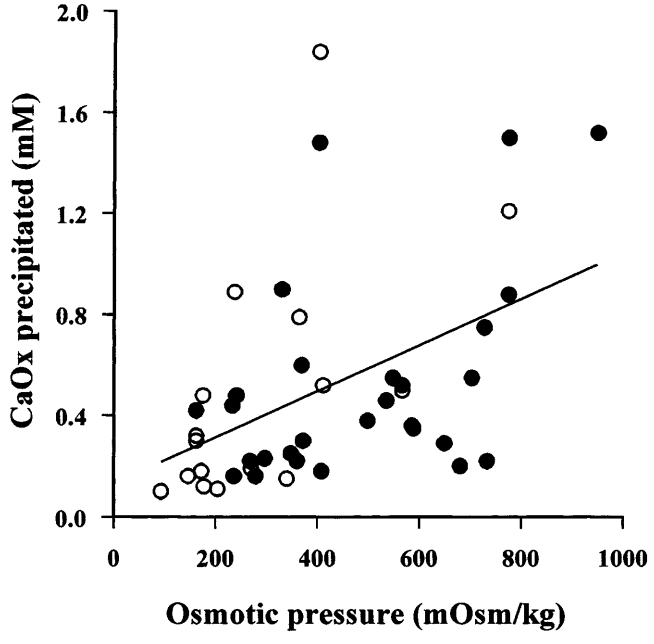

Fig. 6 The relationship between calcium oxalate precipitated and the osmotic pressure of human urine samples (n = 44, r = 0.47, P < 0.0014) at a constant calcium and oxalate load. ● 92% urine, ○ urine further diluted

nucleation rates observed here will be the net result (but not necessarily in a simple additive manner) of effects due to the supersaturation and any direct or indirect effect of crystallization modifiers. The other dilution experiments are an attempt to identify the relative significance of these factors.

The components of AU might affect calcium oxalate crystallization through their influence on the supersaturation or by direct crystal interaction. In both the AU dilution experiments the growth rate was reduced by dilution, showing that any direct growth inhibition (e.g. by citrate or magnesium) was not significant in relation to other changes taking place. In these two experiments the results are comprehensible in relation to the changes in the supersaturation of the input to the crystallizer. When calcium and oxalate were kept constant (filled squares, Figs. 1–3) the input supersaturation rose until, at the lower dilutions, homogeneous nucleation could become relevant (Table 2) and the nucleation rate rose dramatically. At this stage growth rates declined, presumably limited because the amount being precipitated was close to the maximum available oxalate. When calcium and oxalate were diluted (open squares, Figs. 1–3) the input supersaturation was reduced so that, while heterogeneous nucleation could persist, the growth rate was markedly reduced and a negligible amount of precipitation occurred.

The main difference between AU and HU is the presence of UMMS in HU. In both the HU experiments dilution caused a decrease in nucleation rate and an increase in growth rate, which is consistent with dilution of nucleation promoters and growth inhibitors.

In comparison with the corresponding experiments with AU these factors lessened the overall response in terms of the mass being precipitated. With a declining input supersaturation (calcium and oxalate diluted) there was a more modest reduction in suspension density with HU than with AU, and with an increasing input supersaturation (calcium and oxalate constant) there was little change or a decline in the suspension density with HU, rather than the increase seen with AU. That the growth rate changes with HU were the same regardless of the input calcium, oxalate and supersaturation (Fig. 1) indicates that growth inhibitors are a major determinant of the crystallization behaviour and that dilution of their activity is not compensated for by the dilution of calcium and oxalate. In a seeded crystallization method using similar proportions of human urine, Baumann et al. [1] also found that crystal growth rate reflected the urine concentration rather than the prevailing calcium and oxalate concentrations. The changes in nucleation rate on dilution of HU counteract the changes in growth rate and these nucleation rate changes were enhanced by a falling rather than rising input supersaturation (Fig. 2, Table 2). The net effect was a significant reduction in the mass precipitated as almost-whole HU was diluted (Fig. 3). These urine samples were provided by a small group of healthy individuals and it is possible that, with others, a different balance between the nucleation-promoting and growth-inhibiting activities might apply, which could lead to different conclusions.

Using the osmotic pressure as a measure of the urinary concentration reinforces the observations with diluted HU. The growth rate was inhibited and the nucleation rate promoted with increasing urine concentrations (Figs. 4, 5) with the overall outcome that the precipitated mass also increased (Fig. 6). This suggests that urinary dilution by increased fluid output will be beneficial, despite an increased growth rate due to loss of growth inhibition. If these results could be extrapolated to events occurring during the passage through the renal tubules, while water is being reabsorbed and the urine concentrated, then it suggests a changing balance in crystallization risk as urine is concentrated, from a situation of low nucleation/high growth to one of high nucleation/low growth. This might discourage the early initiation of crystallization but favour the continued growth of any crystals that do form, particularly if they are not freely flowing. The point of addition of the crystallization modifiers would have important consequences for this interpretation.

The underlying assumption behind the recommendation that stone formers should increase their fluid output is that the consequent reduction in supersaturation will reduce the risk of crystallization and slow the growth of any crystals that do form. The decreased crystal nucleation rate brought about by diluting urine and its associated decrease in suspension density indicates that the risk and extent of calcium oxalate crystalluria can be reduced by increased fluid output.

The changes in growth rate we have observed suggest that enlargement of fixed or freely suspended crystals will be enhanced by urine dilution, despite the reduced supersaturation. In our model we have used high initial oxalate concentrations, which might undermine this conclusion but, at the lowest dilutions used, physiologically normal concentrations were fed into the crystallizer and, because of the precipitation occurring, even less is present in solution. Furthermore, we have not taken into account the role of aggregation inhibitors. Their dilution might be expected to encourage further crystal enlargement. If a nascent stone is fixed in the urinary tract, then increased fluid output might be of little benefit unless the increased flow is sufficient to flush it free before it reaches a critical size. The clinical value of increased urine volume may be greatest as a preventive measure, with little if any impact on pre-existing stones.

The value of increased fluid output in reducing stone recurrence has recently been confirmed in a randomized prospective trial [2], in which the benefits were ascribed to the large drop in urine supersaturation. Our results suggest that a decrease in the concentration of nucleation promoters might have been a major contributory factor.

References

1. Baumann JM, Ackermann D, Affolter B (1990) Rapid method of measuring the inhibition of calcium-oxalate monohydrate growth in urine. Urol Res 18:219
2. Borghi L, Meschi T, Amato F, Briganti A, Navarini A, Giannini A (1996) Urinary volume, water and recurrences in idiopathic calcium nephrolithiasis: a 5-year randomized prospective study. J Urol 155:839
3. Consensus conference (1988) Prevention and treatment of kidney stones. JAMA 260:977
4. Curhan GC, Willett WC, Speizer FE, Stampfer MJ (1998) Beverage use and risk for kidney stones in women. Ann Intern Med 128:534
5. Embon OM, Rose GA, Rosenbaum T (1990) Chronic dehydration stone disease. Br J Urol 66:357
6. Gohel MD, Shum DKY, Li MK (1993) The dual effect of urinary macromolecules on the crystallization of calcium oxalate endogenous in urine. Urol Res 20:13
7. Höjgaard I, Fornander A-M, Nilsson M-A, Tiselius H-G (1998) Crystallization during volume reduction of solutions with an ion-composition corresponding to that in the distal tubuli. In: Höjgaard I. Crystallisation of calcium phosphate and calcium oxalate in solutions simulating the composition at different levels of the nephron. Linköping University medical dissertation no. 551. Department of Biomedicine and Surgery, University Hospital, S-581 85 Linköping, Sweden, Paper II, pp 111–141
8. Kavanagh JP (1992) Methods for the study of calcium oxalate crystallisation and their application to urolithiasis research. Scanning Microsc 6:685
9. Kavanagh JP (1995) The kidney as a mixed suspension, mixed product removal (MSMPR) crystallization chamber. In: Rao PN, Kavanagh JP, Tiselius H-G (eds) Urolithiasis: consensus and controversies. Proceedings of the fifth European Urolithiasis Symposium. Published by PN Rao and JP Kavanagh, Lithotriptor Unit, Manchester, pp 89–93
10. Kavanagh JP (1995) Calcium oxalate crystallization in vitro. In: Khan SR (ed) Calcium oxalate in biological systems. CRC Press, Bora Raton, pp 1–21

11. Kok DJ (1997) Intratubular crystallization events. World J Urol 15:219
12. Lupták J, Bek-Jensen H, Fornander A-M, Höjgaard I, Nilsson M-A, Tiselius H-G (1994) Crystallization of calcium oxalate and calcium phosphate at supersaturation levels corresponding to those in different parts of the nephron. Scanning Microsc 8:47
13. McCormack M, Dessureault J, Guitard M (1991) The urine specific gravity dipstick: a useful tool to increase fluid intake in stone forming patients. J Urol 146:1475
14. Meyer JJ, Smith LH (1975) Growth of calcium oxalate crystals. II. Inhibition by natural urinary crystal growth inhibitors. Invest Urol 13:36
15. Nishio S, Kavanagh JP, Garside J (1991) A small-scale continuous mixed suspension mixed product removal crystallizer. Chem Eng Sci 46:709
16. Pak CYC, Sakhaee K, Crowther C, Brinkley L (1980) Evidence justifying a high fluid intake in treatment of nephrolithiasis. Ann Intern Med 93:36
17. Parivar F, Low RK, Stoller ML (1996) The influence of diet on urinary stone disease. J Urol 155:432
18. Robertson WG, Peacock M (1972) Calcium oxalate crystalluria and inhibitors of crystallization in recurrent stone formers. Clin Sci 43:499
19. Rose MB (1975) Renal stone formation. The inhibitory effect of urine on calcium oxalate precipitation. Invest Urol 12:428
20. Ryall RL (1989) The formation and investigation of urinary calculi. Clin Biochem Rev 10:149
21. Ryall RL, Bagley CJ, Marshall VR (1981) Independent assessment of the growth and aggregation of calcium oxalate crystals in vitro. Clin Chim Acta 18:401
22. Söhnel O, Garside J (1992) Precipitation: basic principles and industrial applications. Butterworth-Heinemann, Oxford, pp 119–124 and 217–221
23. Tiselius H-G (1985) Measurement of the risk of calcium oxalate crystallization in urine. Urol Res 13:297
24. Tiselius H-G (1992) Calcium oxalate crystallization properties in urine with different specific electrical conductivities. J Urol 148:990
25. Werness PG, Brown CM, Smith LH, Finlayson B (1985) Equil2: a basic computer program for the calculation of urinary saturation. J Urol 134:1242