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Effects of urinary macromolecules on the crystallization of calcium oxalate

H.-G. Tiselius A. M. Fornander, and M. A. Nilsson

Department of Urology, University Hospital, Linköping, Sweden

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Summary. The macromolecular fraction of urine with a molecular weight above 3,000 was isolated by dialysis. In the dialysed urine the rate of calcium oxalate (CaOx) crystallization was reduced both in the presence and absence of CaOx seed crystals. There was a clear relationship between crystallization and the relative concentration of the dialysed urine, with the highest crystallization propensity at the lowest concentration of macromolecules. Dilution of dialysed urine also affected crystal size distribution, with a predominance of small (2.8–4.5 μm) crystals in 100% dialysed urine and of large (5.6–14.0 μm) crystals in 5% dialysed urine. This is consistent with a macromolecular inhibition of both crystal growth and aggregation. Analysis of the crystal size distribution 120 min after supersaturation of whole urine to a level at which approximately 100 crystals in the size interval 3.5–5 μm were detected in a Coulter counter surprisingly disclosed a higher mean crystal volume in urine samples from normal subjects than from stone formers. This gives support to the assumptions that macromolecules might be of importance during the initial phase of CaOx crystallization and that urine from stone formers and normal subjects might be different in this respect.

Key words: Calcium oxalate – Dialysis – Urinary macromolecules – Crystal growth inhibition – Mean crystal volume – Stone formers – Normal subjects

Human urine contains a number of substances known to modify the crystallization of calcium oxalate (CaOx). It has thus been shown that urine, even at low concentrations, has a pronounced inhibitory effect on the rate of calcium oxalate crystal growth and aggregation [8, 16, 23, 28]. There are apparently also potent inhibitors of the heterogeneous crystallization of CaOx on hydroxyapatite [3, 4].

Urine constituents with low as well as high molecular weights contribute to the total inhibitory activity [1, 9, 10, 13, 15, 17]. Several recent studies, however, indicate that the macromolecules are of greater importance in this respect than the small molecules [9, 14, 15, 18, 22, 29]. In addition to the inhibitory effects on crystal growth and aggregation, macromolecules might act as promoters of crystallization [21, 24, 30].

In order to increase our knowledge of the role of macromolecules, we studied how the crystallization of CaOx was affected by different concentrations of dialysed urine.

Materials and methods

Preparation of dialysed urine

A pool of dialysed urine was prepared as follows. Freshly voided urine was centrifuged and the sediment acidified with hydrochloric acid to dissolve crystals of calcium oxalate and calcium phosphate. Following neutralization with sodium hydroxide the dissolved sediment was combined with the supernatant. Urine in 100-ml aliquots was then transferred to Spectrapore no. 3 dialysis tubings (exclusion limit 3,000 daltons). Dialysis was carried out over 5 h in 1,000 ml of deionized water with exchange of water every hour, overnight in another 1,000 ml of deionized water and the next day for 3 h in glass-distilled water and 3 h in 0.15 *M* sodium chloride with exchange of dialysis medium every hour. The pH was adjusted to 5.8 and sodium chloride added to restore the volume of the dialysate to 100 ml.

The dialysed urine was subsequently diluted with 0.15 *M* sodium chloride to give different relative concentrations of macromolecules. Before use in the different crystallization experiments each sample was passed through a Millipore filter with a pore size of 0.22 μm.

Determination of crystal growth in dialysed urine

To 4.5-ml fractions of dialysed urine were added 0.1 ml of 0.05 *M* calcium chloide, 0.1 ml calcium 45 chloride solution and 0.05 ml of a crystal suspension of calcium oxalate monohydrate containing 1 mg crystals per ml. To each sample was further added 0.3 ml of sodium chloride, giving a final volume of 5.15 ml. The supersaturation was established by adding 0.1 ml of sodium oxalate solutions with concentrations in the range of 5–80 m*M*. The oxalate concentration

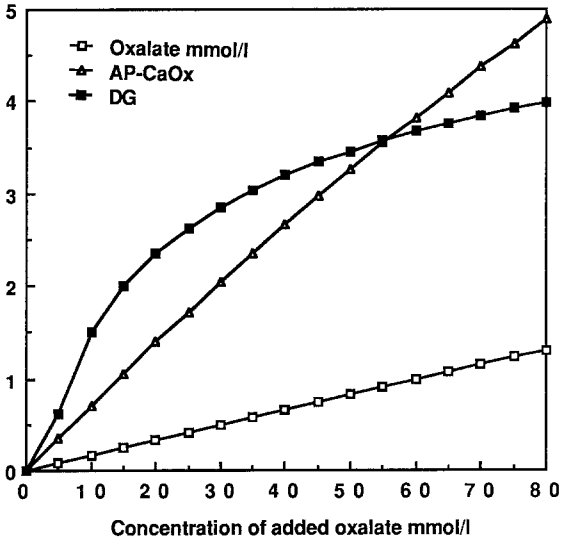

Fig. 1. Oxalate concentration, ion-activity product (*AP*) of calcium oxalate (CaOx) and driving force (*DG*) in the experimental system following addition of sodium oxalate at different concentrations

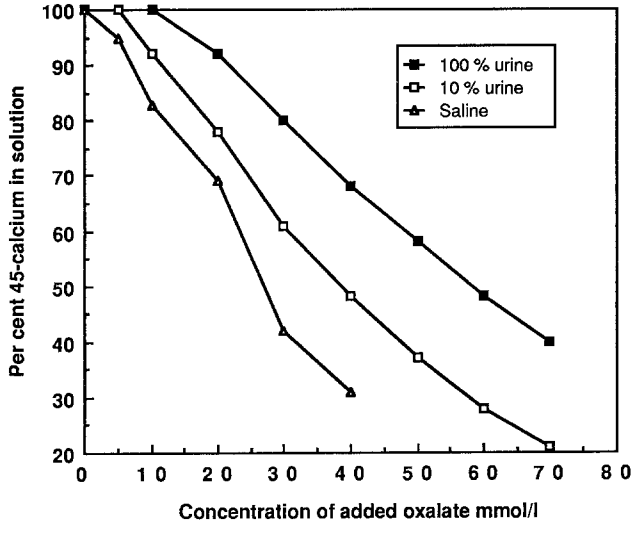

Fig. 2. Percentage of ^{45}Ca (*Percent 45-calcium*) remaining in solution in dialysed urine, in the same urine diluted to 10% and 0.15 *M* sodium chloride in the presence of calcium oxalate seed crystals following addition of sodium oxalate at different concentrations

in each sample, the ion-activity product of CaOx and the driving force (DG) at different concentrations of added oxalate are shown in Fig. 1. The ion activities and the DGs were calculated by means of the EQUIL 2 program [31].

Crystallization was determined by measuring the amount of isotope remaining in solution after 30 min. The principles of the experimental procedure were similar to those previously presented by Nicar et al. [20] and Baumann et al. [3].

Determination of crystallization in dialysed urine

The crystallization of CaOx in the absence of seed crystals was measured in samples of dialysed urine and 0.15 *M* sodium chloride in the same way as above but with the addition of 0.05 ml sodium chloride solutions instead of the seed crystal suspension.

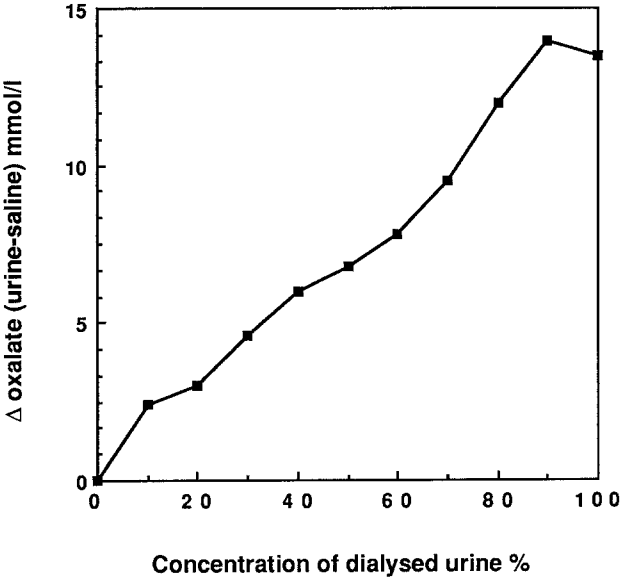

Fig. 3. Differences in concentration of addded sodium oxalate corresponding to precipitation of 30% of ^{45}Ca between dialysed urine at various concentrations and sodium chloride, 30 min after supersaturation of the samples. Seed crystals of calcium oxalate were added

Determination of crystal size distribution in dialysed urine

Three different samples of dialysed urine (A, B and C) in concentrations of 100% , 25% and 5% and with a pH of 5.8 were used for analysis of crystal size distribution following supersaturation. After pH adjustment each sample was passed through a Millipore filter with a pore size of 0.22 µm. To 80 ml of sample A were added 6 ml of 0.1 *M* calcium chloride, 4 ml of 0.01 *M* sodium oxalate and 10 ml of 0.15 *M* sodium chloride saturated with calcium oxalate. The corresponding additives of calcium chloride and sodium oxalate were 12 and 8 ml respectively, to sample B and 18 and 12 ml, respectively, to sample C. There was no addition of sodium chloride to samples A and B.

The number of crystals in the size intervals 2.8–4.5, 4.6–5.4 and 5.5–14.0 µm was determined in a Coulter counter (Model Z_B) with Channelyzer 30 min after establishment of the supersaturation.

Determination of crystal size distribution in whole urine

Urine samples collected over 4 h between 06.00 and 10.00 h from 33 normal subjects (19 men and 14 women) and 11 patients with CaOx stone disease (8 men and 3 women) were supersaturated by the standardized addition of 0.04 *M* sodium oxalate to obtain just above 100 crystals in the size range 3.5–5.0 µm according to principles described previously [27]. These samples were subsequently stirred during the following 120 min after which the crystal size distribution was determined in a Coulter counter. The number of crystals in the range 3.5–27.0 µm was recorded at intervals of 1.5 µm and the mean crystal volume (MCV) calculated by dividing the total crystal volume by the total number of crystals.

Statistical analysis was performed with Wilcoxon's rank sum test.

Results

The crystallization of CaOx as determined from the consumption of calcium in a 0.15 *M* solution of sodium chloride and in samples of dialysed urine at the original

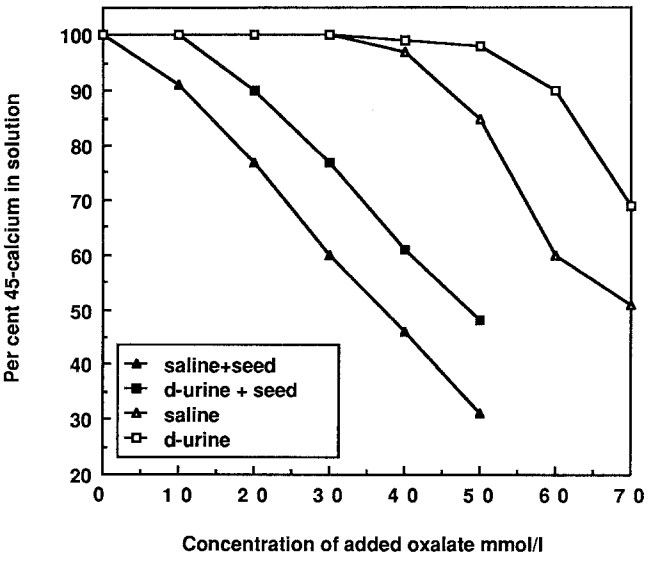

Fig. 4. Percentage of ^{45}Ca (*Percent 45-calcium*) remaining in solution in 0.15 *M* sodium chloride and dialysed (*d*-) urine, with and without seed crystals, 30 min after supersaturation by addition of increasing amounts of sodium oxalate

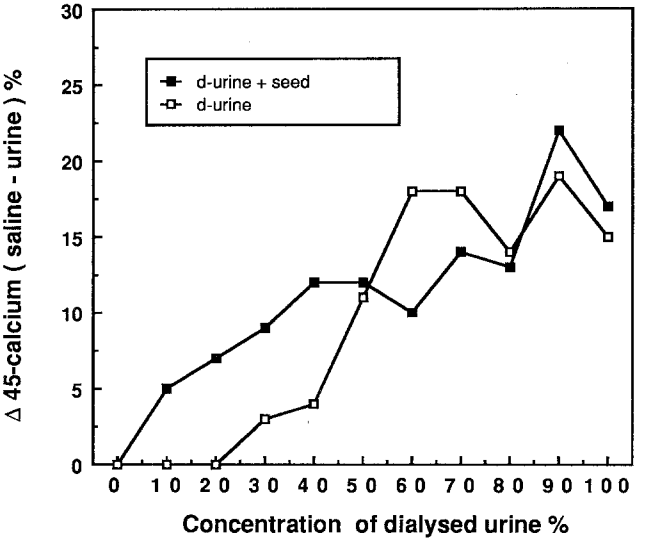

Fig. 5. Difference between ^{45}Ca crystallization in 0.15 *M* sodium chloride and dialysed urine of various relative concentrations, with and without seed crystals, 30 min after supersaturation by addition of a 50 m*M* sodium oxalate

concentration and diluted to 10% is shown in Fig. 2. In order to achieve a similar reduction of isotope concentration in solution, higher concentrations of oxalate were required in urine than in sodium chloride. This certainly reflects an inhibition on crystal growth by urinary macromolecules, because in the same samples without seed crystals, higher oxalate concentrations were necessary for corresponding levels of crystallization.

Decreased concentrations of dialysed urine evidently resulted in a reduced inhibitory activity and accordingly an increased rate of crystallization (Fig. 3). Whereas an ion-activity product for CaOx of 3.7×10^{-8} $(M)^2$ was

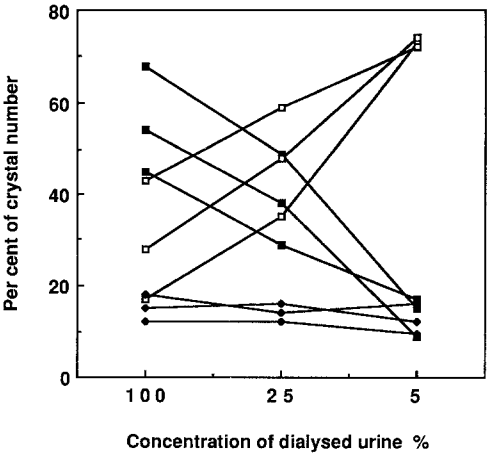

Fig. 6. Fraction of small (■), medium-sized (♦) and large (□) crystals 30 min after supersaturation of three different samples of undiluted, 25% and 5% dialysed urine without seed crystals

Table 1. Mean crystal volume (MCV) 120 min after supersaturation of 4-h whole urine samples to a level at which just above 100 crystals in the size range 3.5–5 μm were detected

Urine samples from	Number	Mean MCV (±SD) (μm^3)	Median MCV (range) (μm^3)
Normal men	19	286 ± 136	257 (82–690)
Normal women	14	254 ± 122	224 (117–591)
Stone-forming men	8	167 ± 139	132 (66–496)
Stone-forming women	3	92 ± 37	83 (60–133)

necessary to reduce the ^{45}Ca concentration ot 50% in the undiluted dialysed urine, an ion-activity product of 3.2×10^{-8} $(M)^2$ was sufficient for a similar crystallization in 10% dialysed urine. This corresponded to DG values of 3.6 and 3.4, respectively. In sodium chloride solution the same crystallization was recorded at an ion-activity product of approximately 2.4×10^{-8} $(M)^2$.

Figure 4 summarizes the extent of calcium crystallization in seeded and unseeded dialysed urine and sodium chloride following the addition of increasing concentrations of sodium oxalate. As expected, a higher supersaturation was necessary to obtain crystallization in the unseeded urine sample, but crystallization otherwise appeared to occur in a similar way in the two urine systems. Moreover, it is shown in Fig. 5 that even in the absence of seed crystals increased concentrations of dialysed urine were associated with a reduction of calcium precipitation, reflected by greater differences between the amount of calcium precipitated in sodium chloride and that precipitated in dialysed urine.

Crystal size distribution 30 min after supersaturation of urine samples with different relative concentrations of urinary macromolecules (Fig. 6) shows that dilution of dialysed urine from 100% to 5% was associated with a shift from a predominance of small crystals (2.8–4.5 μm) to one of large crystals (5.6–14.0 μm).

Table 1 gives the MCV values in whole urine from stone-formers as well as from normal subjects recorded 120 min after supersaturation of the samples to a level at which a similar number of crystals was recorded. The surprinsing finding in this experiment was the significantly higher MCV ($P < 0.01$) in urine from normal subjects as compared with urine from stone-formers. This difference was demonstrated both between normal men and stone-forming men ($P < 0.02$) and between normal women and stone-forming women ($P < 0.05$). The explanation for this is not evident but might be an effect of differences during the initial phase of crystal formation, possibly reflecting a higher concentration of macromolecules with nucleating properties in normal urine.

Discussion

The influence of the macromolecular fraction of urine on the crystallization of CaOx was assessed in different experimental systems. One major aim with these measurements was to define a system in which macromolecules could be used in concentrations similar to those in untreated urine. In as much as there might be differences in crystallization properties between diluted and undiluted urine, such measurements should be advantageous [26]. The drawback with dialysed urine is that the effect of small molecular inhibitors will not be accounted for, but on the other hand all experiments were carried out in solutions with a known supersaturation level.

A significant reduction of ^{45}Ca in solution after 30 min was usually not observed until the ion-activity products of CaOx in 100% dialysed urine exceeded approximately 1×10^{-8} and 2×10^{-8} $(M)^2$ for seeded and unseeded samples, respectively.

The amount of CaOx seed crystals in each sample was 0.34 μmol which, in volume of 5.15 ml, corresponds to a CaOx concentration of 70 μ*M*. This is certainly higher than the crystal concentration in the urine of most stone-formers. Hallson [11] reported a CaOx crystal concentration up to about 10 μ*M* in stone-formers with a slightly elevated concentration of oxalate, although greater amounts of crystals were found among hyperoxaluric patients. Whereas our analytical system might thus possibly have underestimated the inhibiting activity, there was apparently little risk of the opposite.

The presence of macromolecules reduced the rate of crystallization even at a relative concentration of dialysed urine as low as 10% of the original. This clearly shows that urine has a superfluous capacity to inhibit CaOx crystal growth (Figs. 2, 3). Nevertheless, with the amount of seed used in these experiments, there was evidently a clear relationship between the concentration of dialysed urine and the inhibition of crystal growth. That urine has a high capacity to inhibit CaOx crystallization is well recognized [14], and in view of the pronounced inhibition with very low concentrations of urine in metastably supersaturated solutions [23, 29] this is not surprising.

Although unseeded systems have a poorer reproducibility than seeded systems [19], the patterns of crystallization were similar in the two systems as shown in Fig. 4 and 5. For the isolated determination of crystal growth inhibition a seeded system therefore is superior, but studies of the initial steps of crystallization have to be carried out in seed-free solutions. Furthermore, CaOx dihydrate is the most common crystalline phase in urine at least during the initial part of crystallization [9, 25], whereas CaOx monohydrate is the only form of seed that can be used experimentally.

The changing rates of crystallization demonstrated with different relative concentrations of urinary macromolecules indicate that this analytical system might be useful for the detection of variations in inhibitory activity between stone-formers and normal subjects. Such work is in progress in our laboratory.

Another important observation was the relationship between the relative concentration of urinary macromolecules and crystal size distribution. The predominance of small crystals at high macromolecular concentrations is consistent with an aggregation inhibition exerted by the macromolecules [8, 13, 23, 26]. It needs to be emphasized, however, that another contributing explanation might be a macromolecule-mediated influence on the initial phase of CaOx crystallization. Thereby, macromolecules might bind microcrystals [12, 15, 22] or create zones with increased concentrations of calcium and oxalate [14]. For the same supersaturation level, a high concentration of macromolecules might provide many sites for crystallization, resulting in a large number of small crystals, whereas a low concentration accordingly would result in fewer but larger crystals [5].

The paradoxical finding of a higher MCV in whole urine from normal subjects 120 min after supersaturation to a level at which slightly more than 100 crystals in the size range 3.5–5.0 μm occurred appears to be opposite to the general opinion that urine from stone-formers contains larger crystals than urine from normal subjects. However, this is most likely an effect of the experimental conditions, and one possible explanation of this phenomenon are differences between the groups during the initial phase of crystallization. If we assume that there is a higher concentration of nucleating macromolecules in normal urine, this would imply a large number of nucleating sites. In contrast, fewer sites might be available in urine from stone-formers. In order to produce crystals of sufficient size to be detected in the Coulter counter, normal urine thereby had to be more supersaturated than stone-formers' urine. Thus, at the start of the 120-min crystallization period, normal urine was supersaturated to a higher level than urine from stone-formers resulting in the formation of crystals with a larger MCV in normal urines. Further studies are necessary to prove the validity of this assumption.

Determination of the role of macromolecules during the initial phase of crystallization is difficult because the subsequent steps are modified by inhibition of both crystal growth and aggregation. It is possible that the same macromolecules in addition to the inhibition of growth and aggregation might also contribute to nucleation [7, 22]. Theoretically, stone-formers might have a low concentration [2, 23, 28, 29] or structurally abnormal crystallization-modifying macromolecules [18],

whereby a high supersaturation with CaOx results in the formation of large crystals which subsequently are insufficiently proctected from further growth and aggregation [6].

The major conclusions from the results in this investigation are that low concentrations of urinary macromolecules are associated with both a higher rate of CaOx crystal growth and the formation of larger crystals or crystal aggregates than high concentrations. Whether differences exist between stone-formers and normal subjects in terms of crystallization properties in whole urine is still a question of debate which deserves further study in well-standardized crystallization systems.

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Hans-Göran Tiselius, MD
Department of Urology
University Hospital
S-58185 Linköping
Sweden