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Inhibition of calcium oxalate crystallization by urinary macromolecules

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Summary. The crystallization of calcium oxalate (CaOx) was determined in dialyzed urine samples collected between 0600 and 1000 hours from 18 normal men, 10 normal women and 13 men and 10 women with CaOx stone disease. Each urine samples was supersaturated by the addition of calcium chloride and sodium oxalate, and CaOx crystallization was followed by quantification of the [^{14}C]-oxalate remaining in solution for 30 min after supersaturation of the sample. The rate of crystallization was compared with that in physiological saline. The surface area delimited by the urine and saline curves was used to express the inhibition of CaOx crystallization by urinary macromolecules (I_{UMM}). The I_{UMM} was significantly higher in urine from normal women than in that from stone-forming women ($P<0.05$), normal men ($P<0.005$), and stone-forming men ($P<0.02$). However, there were no significant differences between stone-forming men and stone-forming women, nor was I_{UMM} higher in normal men than in stone-forming men. A high concentration of inhibitors might protect women from CaOx stone formation and be one factor explaining the lower stone-formation rate in women. Although low values were more predominate in normal men than in normal women, there were no significant differences between the groups when the inhibition was corrected for differences in urinary volumes.

Key words: AP(CaOx) index – Calcium oxalate – Dialyzed urine – Urinary macromolecules

Several factors are considered to be important for the development of calcium oxalate (CaOx)-containing renal stones. One prerequisite is a urine sample that is saturated to a level at which crystals of CaOx are formed [9, 11]. This process is most likely the result of heterogeneous crystallization [4] induced by some nucleator or promoter [2]. The whole process is modified by inhibitors that might affect nucleation, crystal growth and crystal aggregation [3, 6, 7, 9, 11]. The exact nature of inhibitors and promoters is not known, but a number of substances have been reported to have such properties. Inhibition might thus be accomplished by macromolecules as well as small molecules in urine [7, 20].

Macromolecules have attracted the greatest interest because of their possible roles as both promoters and inhibitors [9, 12, 14]. In a previous study [20], we analyzed the crystallization of CaOx in dialyzed urine that had been diluted to a creatinine concentration of 7.5 mmol/l and recorded a slightly higher crystallization rate in urine from stone-formers. However, when direct assessment of the risk of CaOx crystallization was carried out in whole urine that had also been diluted to a standardized creatinine concentration, there was no evidence of lower inhibitory activity in urine from stone-formers [17].

The results have thus far been inconclusive; therefore, to obtain more information on this important matter, we compared the rate of crystallization in the macromolecular urinary fraction obtained by dialysis of 4-h urine samples collected from normal subjects and patients with CaOx stone disease.

Subjects and methods

Urine was collected between 0600 and 1000 hours from 18 normal men and 10 normal women and from 13 men and 10 women with CaOx stone disease. All samples were collected on an out-patient basis that included ordinary diet and drinking habits, and none of the subjects were on any medication known to affect urinary composition.

The samples were prepared as soon as possible and kept frozen until analysis, at which time urine was thawed, heated to 37°C, and carefully mixed. An aliquot was acidified with hydrochloric acid for analysis of calcium, oxalate, citrate, and magnesium according to methods described elsewhere [8, 19]. These variables were subsequently used for calculation of the AP(CaOx) index [16] as an estimate of the ion-activity product of CaOx.

The remainder of the urine sample was centrifuged for 10 min, and a 100-ml aliquot was transferred to a Spectrapor number 3 dialysis tube. The urine was first dialyzed for 5 h in 1,000 ml of deionized water, with exchange of water occurring every hour. Subsequently dialysis was carried out overnight in another 1,000 ml

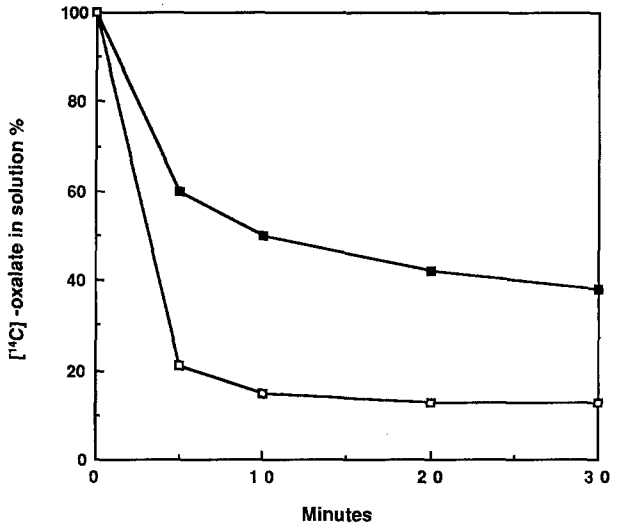

Fig. 1. Percentage of [^{14}C]-oxalate in solution at different times during the first 30 min following supersaturation of dialyzed urine (■) and saline (□)

deionized water and then on the next day for 3 h in glass-distilled water and for 3 h in 0.15 mol sodium chloride/l, whereby dialysis medium was exchanged every hour. Before further experiments were performed, 0.15 mol sodium chloride/l was added to give a final sample volume of 100 ml.

Next 3 ml calcium chloride (0.1 mol/l) and 2 ml sodium oxalate (0.01 mol/l) in 0.15 mol sodium chloride/l was added to 40 ml dialyzed urine. Isotope was added in the form of [^{14}C]-oxalate and the final volume was adjusted to 50 ml with 0.15 mol sodium chloride/l. The addition of sodium oxalate resulted in the crystallization of CaOx, which was followed by isotope determination in a scintillation spectrometer after 5, 10, 20, and 30 min. Urine that contained saline instead of sodium oxalate was used as a blank. Correction for quenching was performed automatically.

Figure 1 demonstrates the typical crystallization of CaOx in dialyzed urine and physiological saline. Kinetic analysis disclosed that the curves approximately fitted biphasic exponential equations. The inhibitory activity (I_{UMM}) in this system was expressed as the surface area delimited by the curves of isotope decay in the urine sample and in the sodium chloride solution during the first 30 min. The ion-activity product of CaOx was calculated by means of the EQUIL 2 programme [21]. Statistical analysis was performed using Wilcoxon's rank-sum test.

Results

It is noteworthy that the dialyzed urine had to be highly supersaturated before crystallization of CaOx could be obtained without a long lag phase. The ion-activity product of CaOx in our system was 5.9×10^{-8} (mol/l)^2, whereby the possible effects of macromolecules on the ion activities of Ca and Ox were not considered. These results indicated the presence of potent inhibitors of the crystallization process, which probably also affected the nucleation.

The results of the crystallization experiments are summarized in Table 1. The I_{UMM} value recorded for urine from normal women was significantly higher than that obtained for samples from stone-forming women ($P < 0.05$), normal men ($P < 0.005$) and stone-forming men ($P < 0.02$). On the other hand I_{UMM} was not significantly different between stone-forming men and women ($P < 0.05$). It was somewhat surprising that the value obtained for stone-forming men was significantly higher than that recorded for normal men ($P < 0.05$). The relationship between 4-h urinary volume and I_{UMM} is shown in Fig. 2 and 3. Although high urinary volume was usually associated with low inhibitory activity, there were no significant differences in urinary volumes between the different groups. As is evident from the results illustrated in Fig. 2, 10% of the normal women in our study (1/10) showed an I_{UMM} value of $< 1{,}000$ as compared with 70% of the stone-forming women; a value of $< 1{,}000$ was recorded in 17 of 18 normal men (95%) and in 9 of 13 stone-forming men (70%).

An estimate of the total inhibitory activity in 4-h urine samples was calculated as the product of I_{UMM} and the urinary volume expressed in litres. In this type of comparison the differences disappeared, indicating that the total excretion of inhibitory macromolecules during the 4-h period was similar in all groups. However, Fig. 4 shows the great variability observed between the different groups; whereas only 20% of the normal women showed values of < 200, corresponding values were found for 72% of the normal men and for 23% and 50% of the stone-forming men and women, respectively.

The risk of crystal formation in the different samples, expressed as the quotient of the AP(CaOx) index and

Table 1. Urinary findings in normal men (NM), stone-forming men (SFM), normal women (NW) and stone-forming women (SFW)

Urinary variable	NM	SFM	NW	SFW
Number of patients	18	10	13	10
AP(CaOx) index	1.40 ± 0.67	1.73 ± 1.56	1.41 ± 0.83	1.13 ± 0.77
AP(CaOx) index (s)	1.94 ± 1.6	2.16 ± 1.52	1.22 ± 0.8	1.17 ± 0.66
U volume (ml)	327 ± 281	363 ± 225	199 ± 107	281 ± 141
I_{UMM}	678 ± 197	887 ± 362	1,480 ± 212	847 ± 433
AP(CaOx) index/$I_{UMM} \times 10^{-3}$	2.28 ± 1.11	1.73 ± 0.91	1.03 ± 0.58	1.45 ± 0.78
$I_{UMM} \times$ U vol	207 ± 281	313 ± 169	254 ± 81	234 ± 151

Data represent mean values ± SD. U vol, urinary volume

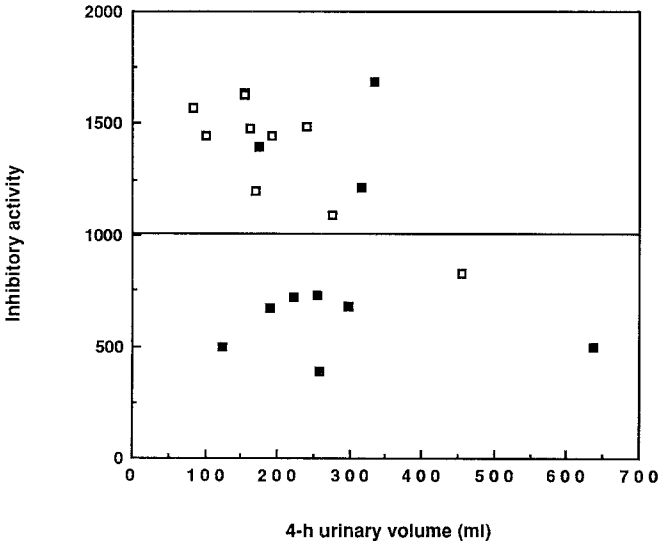

Fig. 2. Relationship between 4-h urinary volume and I_{UMM} in normal (□) and stone-forming (■) women

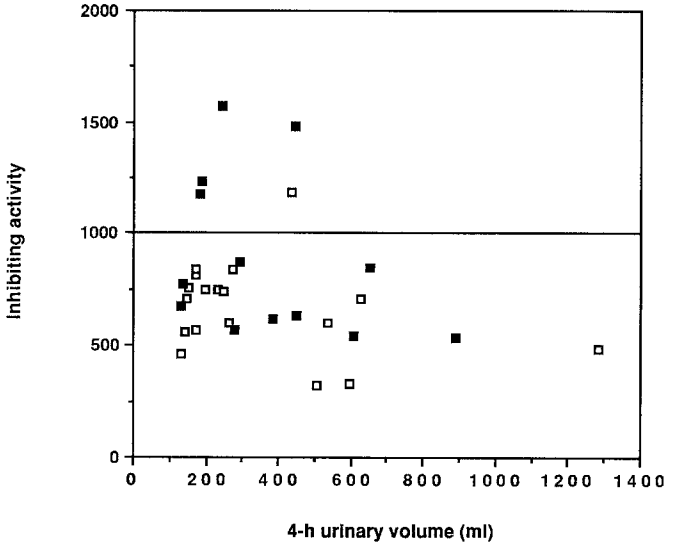

Fig. 3. Relationship between 4-h urinary volume and I_{UMM} in normal (□) and stone-forming (■) men

I_{UMM} is presented in Fig. 5. All normal women showed values of <2. Of the normal men and the stone-forming men and women, 63%, 36%, and 22%, respectively, exhibited values of >2. Significantly higher values were recorded in normal men ($P<0.02$) and stone-forming men ($P<0.01$) as compared with normal women.

Discussion

It should be emphasized that the rate of crystallization measured in dialyzed urine in the present study might reflect the sum of the promotive and inhibitory properties of the macromolecules. The dialysis excluded substances with a molecular weight of $<3{,}000$ Da and there might

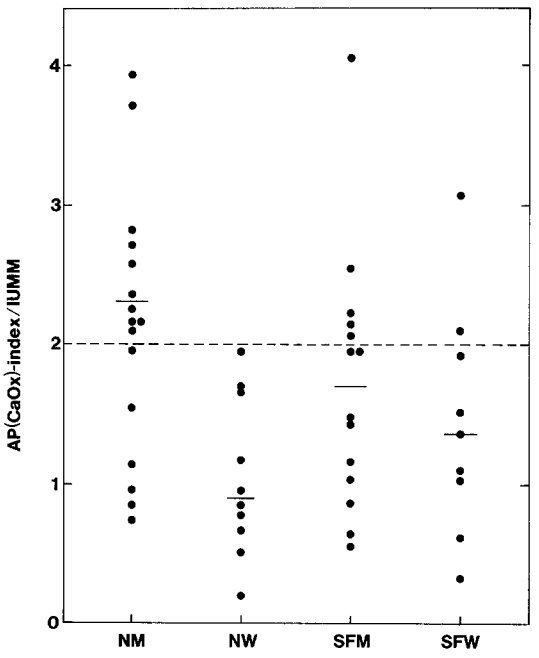

Fig. 4. Total inhibition expressed as $I_{UMM} \times$ volume: product in normal men (*NM*), normal women (*NW*), stone-forming men (*SFM*) and stone-forming women (*SFW*)

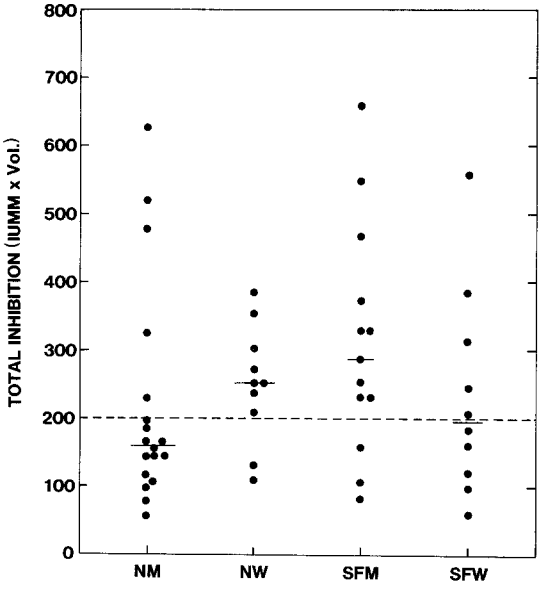

Fig. 5. AP(CaOx) index/($I_{UMM} \times 10^{-3}$): quotients in normal men (*NM*), normal women (*NW*), stone-forming men (*SFM*) and stone-forming women (*SFW*)

have been differences in inhibition in the presence of smaller molecules. No seed crystals were added; thus, the effects recorded most probably resulted from nucleation and subsequent crystal growth. As the size distribution of crystals was not determined, inhibition of aggregation was not considered. Although the macromolecular inhibitors might be particularly efficient in preventing aggregation of crystals, it is evident that much slower crystallization occurred in urine as compared with a urine-free salt

solution. Unseeded systems are considered to be slightly less reproducible than seeded systems, but the former might more appropriately resemble the physiological situation in urine and take into account a possible macromolecular promotive activity [12].

It is well known that the measurement of inhibitors is associated with a number of problems. In whole urine the effects of supersaturation, promotion, and inhibition cannot be easily distinguished. Therefore, inhibitors have traditionally been determined in systems of diluted urine [10, 18]. When we compared the inhibitory activity between stone-formers and normal subjects in systems in which urine had been diluted to a standardized creatinine concentration and subsequently dialyzed, we observed slightly higher inhibition in urine from normal subjects [20], similar to the results we had previously obtained using diluted urine [18]. The difference between stone-formers and normal subjects, however, was small and its clinical significance was doubtful. Similar results have been reported by other authors [13, 15], although some have obtained more pronounced differences in inhibitory properties [1, 3, 5–7].

One important observation in the present series of experiments involved differences between normal men and women in I_{UMM} values as well as quotients of the AP(CaOx) index and I_{UMM}. Furthermore, low values for total inhibitory activity were more predominant in normal men than in normal women; the higher concentration of macromolecular inhibitors probably counteracts CaOx crystal formation in women. A lower risk of CaOx crystal aggregation in women has previously been reported by Ryall and co-workers [13].

A previously described saturation inhibition index [10] was used to discriminate stone-formers from normal subjects, but inhibition was determined in low concentrations of urine in the present study. Assessment of the inhibitory properties of macromolecules and calculation of AP(CaOx) index/I_{UMM} quotients in our patients failed to confirm such a difference. In fact, the similar quotients obtained for the different groups were compatible with our previous results of direct determination of the risk of CaOx crystallization in urine [17]. It could be argued that the supersaturation level was unusually high in our normal subjects. The reason for this is not known but might partly be explained by the lower urinary volumes measured in this group.

In conclusion, the results we obtained from a kinetic analysis of CaOx crystallization is dialyzed urine showed that the I_{UMM} value for normal women was higher than that for either normal men or stone-forming men and women. Moreover, the inhibitory activity in this macromolecular fraction was high, and urine had to be considerably supersaturated for the crystallization to occur. Concentrations recorded in stone-forming men were higher than those observed in normal men, possibly due to a greater production of macromolecules in some patients with stone disease as a response either to periods of high CaOx supersaturation or to existing stones in the urinary tract.

Although it is reasonable to assume that abnormal crystallization in some CaOx stone-formers can be explained by deficiences in macromolecular inhibition, our results show that macromolecular inhibition of nucleation and crystal growth as determined in bladder urine cannot be used to discriminate stone-formers from normal subjects. However, an imbalance resulting from a high supersaturation and low I_{UMM} is likely to result in a pronounced risk of CaOx crystallization.

The crystal volume resulting from crystallization in a seed-free solution is probably smaller than that in seeded systems and the larger crystal surface area might affect the result; nevertheless, crystallization in an unseeded system appears to resemble that in urine. Although the macromolecules might have influenced crystal aggregation differently in four groups [13, 14], this process was not assessed in the present experiments.

It should be emphasized that different methods yield different results, and comparative studies of the value of different analytical systems, including the assessment of crystal aggregation are highly desirable. Furthermore, the determination of inhibitory activity in urine samples collected during well-defined periods and diluted to a standardized urinary volume might provide additional information.

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