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Inhibition of Calcium Oxalate Crystallization in Urine

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Summary. Chromatographic separation of urine showed inhibition of calcium oxalate (CaOx)-crystallization among substances with both large and small molecular weights. Ultrafiltration showed that approximately 80 per cent of the inhibiting activity, as determined in 2 per cent urine, originated from substances with a molecular weight above 1,000. Dialysed urine was diluted to 7.5 mmol of creatinine per 1 and supersaturated with respect to CaOx. The rate of crystallization in these samples was slower in normal subjects than in stone formers ($p < 0.05$). The inhibiting activity in diluted urine from the two groups did not differ and neither did the concentration of alcian blue precipitable polyanions. From measurements in diluted urine it was apparent that inhibition was demonstrable with a urine concentration as low as 0.3 per cent.

Key words: Calcium oxalate, Chromatography, Dialysis, Inhibition, Urine.

Introduction

Most urinary concrements are composed of calcium oxalate (CaOx) or mixtures of CaOx and calcium phosphate. The most important step in the formation of these concrements is thought to be a heterogeneous nucleation of CaOx in a urine which is supersaturated with respect to this salt [3]. If the crystals are retained in the collecting system for a sufficient time, they will grow and aggregate to large crystals [8, 10], which frequently are observed in urine from recurrent CaOx stone formers [9, 14].

The process of CaOx crystallization is apparently to a large extent modified by inhibitors [4, 9], and a lower inhibitory activity has been recorded in patients with CaOx stone disease [2, 9, 12]. Several substances in urine have been ascribed inhibiting properties, e.g. glycosaminoglycans, glycoproteins, pyrophosphate, citrate, magnesium, ribonucleotides, and trace metals.

There are however, considerable technical difficulties in measuring the inhibiting activity, and the role of inhibitors in the process of CaOx stone formation is far from clear. In the present paper we report some results from studies on inhibition of CaOx-crystallization in urine.

Methods

Inhibiting Activity

An inhibition index (I) was obtained from measurements of the crystallization rate in a ^{14}C-labeled, metastably supersaturated solution. This CaOx-seeded method has been previously described in detail [12]. One ml of urine was added to 50 ml of this solution.

In the chromatographic separations 100 µl from each fraction was added to 5 ml of the metastable solution [12]. The inhibition was expressed as per cent of isotope remaining in solution 2 h after the addition of seed crystals.

To study the effects of dilution, 1 ml of urine, undiluted and diluted to 50, 30, and 15 per cent was added to 50 ml of the crystallization system. The inhibition resulting from addition of one ml of undiluted urine was set to 100 per cent.

Crystallization in Urine and Salt Solution

The crystallization of CaOx was studied in highly supersaturated unseeded and Millipore filtered solutions of physiological saline, urine-like salt solutions, and dialysed as well as pooled urines. The urine-like salt solution (Solution U) was a slight modification of the system proposed by Burns and Finlayson [9] and had the following composition: sodium chloride 105.5 mmol/l, sodium phosphate 32.3 mmol/l, sodium citrate 3.21 mmol/l, magnesium sulfate 3.85 mmol/l, sodium sulfate 16.95 mmol/l, potassium chloride 63.7 mmol/l, calcium chloride 4.5 mmol/l, sodium oxalate 0.32 mmol/l, ammonium hydroxide 17.9 mmol/l, and ammonium chloride 0.0028 mmol/l. pH was adjusted to 6.0.

All solutions were passed through Millipore filters with pore sizes of 0.45 and 0.22 µm before use in the crystallization experiments.

To 40 ml of solutions without calcium was added 6 ml of physiological saline, 2 ml of 0.1 mmol/l calcium chloride and 2 ml of 0.01 mol/l sodium-[^{14}C]-oxalate.

Urine and solution U were supersaturated by addition of 30 µmol of sodium-[^{14}C]-oxalate. With a final volume of 50 ml the

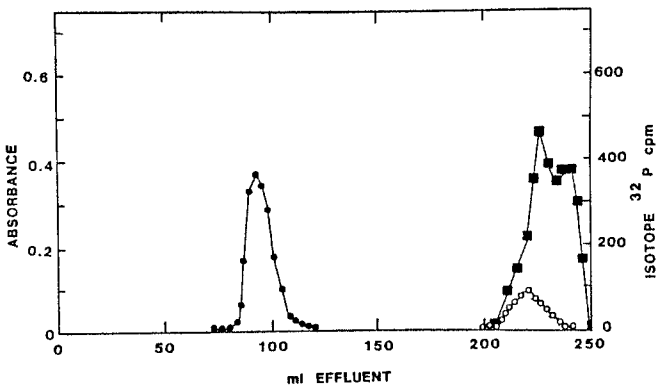

Fig. 1. Chromatographic separation of chondrotin sulphate (•), citrate (○), and phosphate (▪) on a column of Sephadex G-75 (height 900 mm, diameter 20 mm)

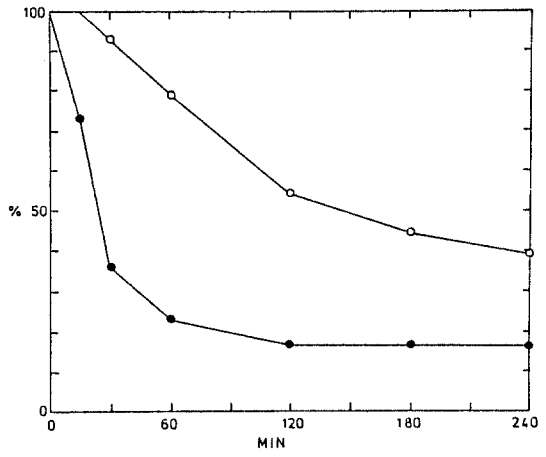

Fig. 2. Per cent of ^{14}C-oxalate remaining in solution in a urine sample (○) and solution U (•) during the first 240 min following increment of oxalate concentration with 0.6 mmol/l

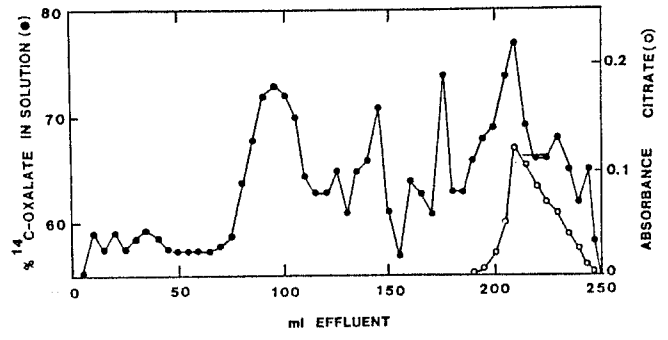

Fig. 3. Measurement of the inhibiting activty, expressed as ^{14}C-oxalate remaining in solution, in the different fractions from a chromatographic separation of urine on a column of Sephadex G-75. Citrate was analysed to demonstrate its occurrence in the chromatogram

urine concentration was 80 per cent and the increment in oxalate concentration 0.6 mmol/l.

At different times during the first 180 min after supersaturation, an aliquot of the sample was passed through a Millipore filter (pore size 0.22 μm) and the isotope determined in a scintillation counter.

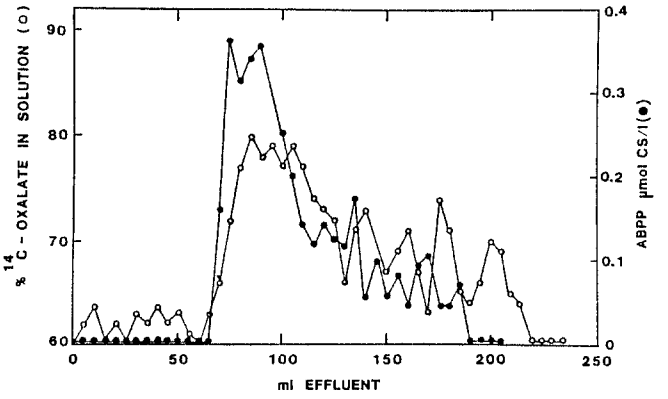

Fig. 4. The inhibiting activity and concentration of ABPP in the different fractions from a chromatographic separation of dialysed urine on a column of Sephadex G-75

Chromatographic Procedure

A sample of 100 ml of freshly voided or dialysed urine was evaporated to a final volume of 20 ml, filtered and applied onto a column of Sephadex G-75 (height 900 mm, diameter 20 mm). The column was eluted with distilled water and the effluent collected in 5 ml fractions.

The properties of this chromatographic system are shown in Fig. 1, where a mixture of citrate, [^{32}P]-phosphate, and chondroitin sulfate was passed through the column.

Dialysis of Urine

Samples of more than 100 ml of freshly voided urine from 9 normal subjects and 9 CaOx stone formers collected between 6.00 and 10.00 h were filtered and dialysed against tap water, deionized water, and distilled water. Finally the solution was equillibrated with physiological saline.

Analytical Procedures

Alcian blue precipitable polyanions (ABPP) were analyzed as described by Whiteman [15] and citrate according to Grunbaum and Pace [6].

Results

The rate of CaOx crystallization was measured in a urine sample (AP_{CaOx} = 2.6 x 10^{-8} M^2) and solution U (AP_{CaOx} = 2.6 x 10^{-8} M^2) following increment of the oxalate concentration with 0.6 mmol/l. It is apparent from Fig. 2 that despite a considerable crystallization driving force in both samples, the crystallization rate was much slower in urine. This difference can probably best be explained by the absence of crystallization inhibitors in solution U.

Chromatographic separation of urine on a column of Sephadex (Fig. 3), disclosed inhibiting activity in fractions with large as well as small molecular substance. When urine was dialysed prior to the chromatographic procedure, the inhibiting activity occurred together with ABPP, which indicated the presence of glycosaminoglycans and/or ribonucleotides in these fractions (Fig. 4).

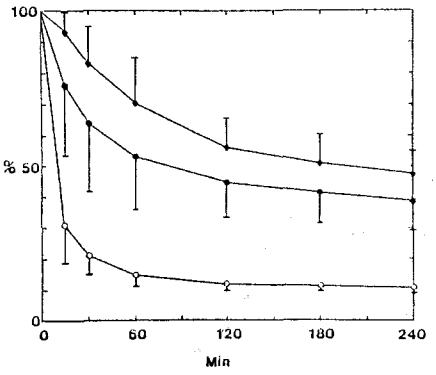

Fig. 5. Per cent of ^{14}C-oxalate remaining in solution after supersaturation of dialysed urine from recurrent CaOx stone formers (•), and normal subjects (♦), and physiological saline (○). *Bars* indicate standard deviation

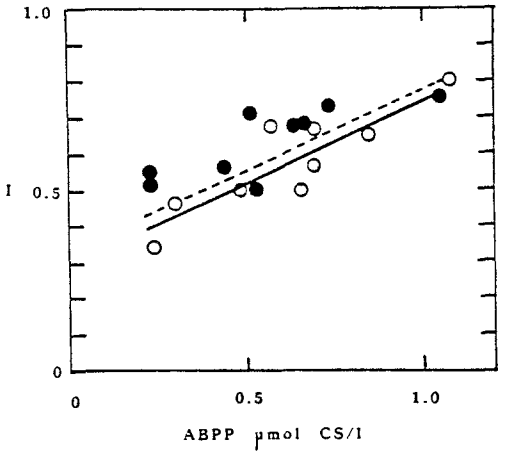

Fig. 6. Relationship between inhibition index (I) and ABPP expressed as μmol of chondroitin sulfate (CS) per litre in dialysed urine from stone formers (•) and normal subjects (○)

There was no significant difference in crystal growth rate in diluted urine before and after dialysis of urine. Passage of urine through an ultra filter with an exclusion limit at 10,000 daltons also showed remaining activity in the macromolecular range indicating inhibiting activities of macromolecules with molecular weights below 10,000. However, when passed through a filter excluding molecules above 1,000 daltons, the inhibition index was reduced by approximately 80%.

The CaOx crystallization rate was measured in dialysed urine from 9 normal subjects and 9 patients with recurrent CaOx stone disease and compared with the crystallization rate in physiological saline after supersaturation with calcium chloride and sodium oxalate. Figure 5 shows that crystallization occurred at a significantly slower rate in urine from normal subjects ($p < 0.05$).

The inhibition index in these samples was positively correlated to the concentration of ABPP, expressed as μmol of chondroitin sulfate per litre (Fig. 6), but there was

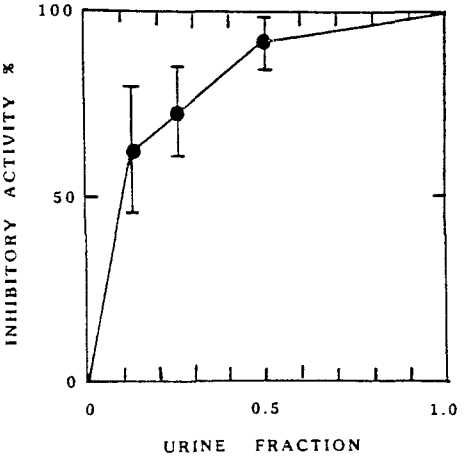

Fig. 7. The dilution effect on urinary inhibiting activity. By definition 100% was the inhibition brought about by a 2% urine concentration in a metastably supersaturated, seeded crystallization system

no significant difference in ABPP concentration between stone formers and normal subjects.

The effect of decreased urine concentration on the inhibiting activity is shown in Fig. 7. The inhibiting activity of 1 ml of urine added to 50 ml of our metastably supersaturated crystallization system was set to 100%, which thus is the inhibition brought about by a 2% urine concentration. The activity was only slightly affected by decreasing urine concentrations to 1%, and more than half of the inhibition remained after reducing urine concentration to 0.3%. These results clearly demonstrate the high crystal growth inhibiting potential of urine.

Discussion

The inhibiting properties of urine are probably of great importance for prevention of crystallization during periods with a high CaOx supersaturation. A deficiency of inhibitors might thus increase the risk of CaOx stone formation and from measurements in diluted urine a lower inhibiting activity was observed in stone formers [2, 9, 12]. However, it has been proposed that results obtained with diluted urine not necessarily are valid for whole urine [3]. The results with dialysed urine in this study support this assumption.

A most important observation is the very high inhibiting potential of urine, with a demonstrable inhibition of crystal growth in metastably supersaturated crystallization systems, despite a concentration as low as 0.3 per cent of the original. It is therefore unlikely that small differences in inhibiting activity would have any major influence on stone formation, unless inhibitors in concentrated urine behave differently from that found in diluted urine. From a clinical point of view, dilution of urine by increased drinking, will therefore certainly not have a negative effect on the inhibiting properties.

In the chromatographic separations of substances with inhibiting activities it is evident that a large number of urine constituents contributes to the total inhibitory activity. The net effect is certainly not simply additive [16] and deficient excretion of one or several inhibitors might therefore well be compensated for by other inhibitors. As previously demonstrated by many authors [2, 5, 7, 11, 13] macromolecules were found to be important contributors to the total inhibition. When stone formers were compared with normal subjects in terms of crystallization properties in dialysed urine there was a slower rate of crystallization in urine from normal subjects. In as much as the ABPP-concentrations were at the same level in both groups, the observed difference in crystallization rates might be attributable to qualitative differences of the macromolecular pattern in urine.

It is difficult to measure the inhibiting properties in whole urine. The reason for this is clearly demonstrated in Fig. 2 where the rate of CaOx crystallization in whole urine is much slower than the crystallization in a salt solution despite a high level of supersaturation. Another possibility to induce crystallization would be to add a sufficient amount of seed crystals. Although seeded systems are better standardized and have a higher degree of reproducibility [8], the amount of seed crystals required for whole urine is too large to be physiological. The highly supersaturated unseeded crystallization systems used by us are not physiological, but despite the absence of seed crystals the reproducibility in the experiments reported here were quite acceptable, and the isotope exchange during the period was apparently low. Following addition of oxalate only, or oxalate and calcium the crystallization probably starts by a heterogeneous nucleation on impurities in the solution rather than by a homogeneous nucleation. The formed crystals will subsequently grow and aggregate in the supersaturated solution. The measured decrease in soluble ^{14}C-oxalate is therefore certainly a net result of nucleation and crystal growth.

These results support previous findings of the importance of macromolecular substances in the inhibition of CaOx-crystallization. Analysis of crystallization properties in undiluted or only slightly diluted dialysed urine might give important information in this respect. Further studies in this area are in progress in our laboratory.

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