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Effect of urinary macromolecules on aggregation of calcium oxalate in recurrent calcium stone formers and healthy

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Summary. The inhibitory activity of urinary macromolecules on the aggregation of calcium oxalate crystals was studied using an aggregometer originally devised to measure thrombocyte aggregation capacity by means of the optical turbidity at 660 nm. The macromolecular fraction of the urine (molecular weight above 5000) of recurrent calcium stone formers showed much less inhibitory activity than that of healthy controls ($P < 0.05$). It was speculated on the basis of the results of gel filtration that there were some proteins (molecular weight about 10000–30000) which had inhibitory activities for the aggregation of calcium oxalate. This gives support to the assumption that macromolecules are important during the phase of aggregation of calcium oxalate crystals.

Key words: Aggregometer – Calcium oxalate – Crystal aggregation inhibitor – Normal subjects – Stone formers – Urinary macromolecules

It has been generally acknowledged that kidney stones result from the aggregation of individual crystals and/or their growth to large particles in the urine after nucleation. Some investigators have been studying the phenomenon of crystal aggregation for a long time [3, 7, 8]. However, the interpretation of the results concerning aggregation has been complicated by the simultaneous occurrence of crystal growth. Recently much more attention has been focused on crystal aggregation because of the importance of inhibitors of the process. Some studies have shown that normal urine contains some powerful inhibitors of crystal aggregation [4, 6, 10, 11].

In order to confirm that stone formers are less capable of inhibiting the aggregation of calcium oxalate crystals in urine, we measured inhibitory effects on aggregation in vitro using a devised technique. We also attempted to isolate some macromolecules (proteins) that inhibit aggregation by gel filtration of urine.

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Materials and methods

Preparation of urine

Early morning urine samples were collected without preservative from 31 recurrent calcium stone formers (23 males and 8 females, mean age 52 years) and from 29 healthy controls (23 males and 6 females, mean age 46 years). The healthy subjects were our hospital staff who had no previous history of kidney or urinary stone disease. No dietary restrictions were imposed on any of the subjects.

Urine specimens were prepared as follows, as soon as they reached the laboratory. Original pH was measured with a Corning pH meter 125 and the following analyses performed on the individual specimens of untreated urine: calcium by atomic absorption spectrometry, citrate by a modification of Nielson's method [13], oxalate by an ion chromatography method [12]. After filtration of the urine through a 0.45-μm filter (HVLP 02500, Millipore), 2.5 ml were desalted using a PD 10 column (Sephadex G-25M, Pharmacia, L.K.B. Biotechnology, Uppsala, Sweden) with a cut-off of 5 kDa and re-equilibrated with 3 ml 0.05 M TRIS-HCl buffer (pH 7.4). Subsequently the fraction was concentrated about threefold using a Centriprep-10 Concentrator (Amicon Division, W. R. Grace, Beverly, Mass.) and made up to a final volume of 1 ml. The concentration of protein in the final treated urine for assay of inhibitory activity was measured by Bradford's method [11].

Determination of the inhibition of calcium oxalate crystal aggregation using an aggregometer

An aggregometer (Hema Tracer 1, Model pat-4M, Niko Biosience, Osaka, Japan), devised to measure thrombocyte aggregation capacity by means of the optical turbidity at 660 nm, was used to estimate the inhibition of aggregation of calcium oxalate crystals in vitro. In our system (Fig. 1) 100 μl of 7.5 mM calcium chloride in 0.05 M TRIS-HCl buffer (pH 7.4) containing 0.15 M NaCl was stirred constantly with 50 μl of prepared urinary macromolecules (size of stirring tip 3 mm × 0.5 mm in diameter, 1000 rpm) at 37°C. Then, 150 μl of 5.0 mM sodium oxalate in the same buffer was added to the mixture. After reaching maximum turbidity, the aggregation

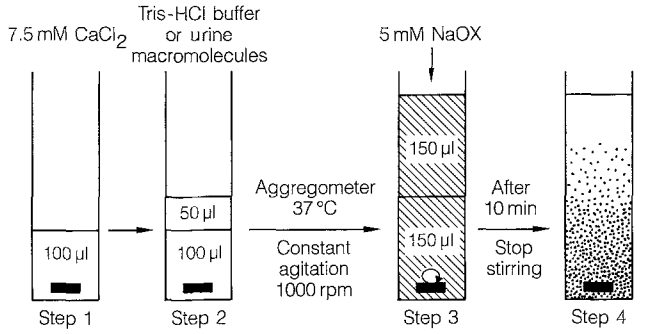


Fig. 1. System for measuring the effect of urinary macromolecules on calcium oxalate crystal aggregation using an aggregometer. One hundred microlitres of 7.5 mM calcium chloride in 0.05 M TRIS-HCl buffer (pH 7.4) containing 0.15 M NaCl is stirred constantly (step 1) with 50 µl of prepared urinary macromolecules (size of stirring tip 3 nm × 0.5 mm in diameter, 1000 rpm) at 37°C and turbidity set at point 0 (step 2). The turbidity is measured at the time. Then, 150 µl of 5.0 mM sodium oxalate in the same buffer is added to the mixture (step 3). After reaching maximum turbidity, the aggregation of calcium oxalate crystals developed at 37°C. Ten minutes later stirring was stopped and the spontaneous sedimentation of the aggregated crystals in the suspension monitored for 10 min (step 4). The optical density at 660 nm decreased in proportion to the degree of crystal aggregation

of calcium oxalate crystals developed at 37°C. After 10 min stirring was stopped and spontaneous sedimentation of the aggregated crystals in the suspension was monitored for 10 min. The optical density (600 nm) decreased in proportion to the degree of crystal aggregation. This phenomenon is explained by the principle that a constant terminal particle velocity equals the rate of sedimentation [5], and was applied to the estimation of the antiaggregation activity of a urinary protein by Hess et al. [14].

Calculation of the inhibition of calcium oxalate crystal aggregation

The decrease in turbidity at 5 and 10 min after stopping the stirring was measured on a turbidity curve. Inhibitory activity (%) was expressed as:

$$\text{Inhibitory activity (\%)} = \left(1 - \frac{\text{Change in turbidity in test solution}}{\text{Change in turbidity in standard solution}}\right) \times 100$$

where the standard solution is 50 µl of TRIS-HCl buffer added to the cuvette instead of the urinary macromolecule sample.

Gel filtration

Twenty-four hour urine samples from two healthy men were collected without preservative. After centrifugation (2000 rpm, 10 min) the urine was concentrated approximately 100-fold using a high-volume Pellican cassette system (Millipore) with filtration membrane (PTGC OLCH2, nominal molecular weight limit 10000, Millipore). The concentrated urine was desalted using a PD 10 column (Sephadex G-25M, Pharmacia) with a cut-off at 5 kDa, and then re-equilibrated with 0.05 M TRIS-HCl buffer (pH 7.4). The resultant fraction (10 ml, pH 7.4) was applied to a Superrose 12HR (2 × 50 cm, Pharmacia) gel filtration system equilibrated with 0.05 M TRIS-HCl buffer (pH 7.4) containing 0.15 NaCl. Each 10 ml of fraction was collected at a flow rate of 1 ml/min.

Results

As shown in Table 1 there are no significant differences in urinary biochemical parameters, including the concentration of protein (estimated in the final treated urine), between the stone formers and healthy subjects.

Figure 2 shows the two turbidity curves obtained using the aggregometer method. After stirring was stopped, the turbidity diminished rapidly in urine sample A (from a recurrent stone former) while it decreased gradually in urine sample B (from a normal subject). Scanning electron micrographs taken during the initial phase and at 10 min from both samples are shown below the turbidity curves. A mixture of calcium oxalate dihydrate and monohydrate is induced by the present system. The micrographs reveal the massive aggregation of calcium oxalate crystals in sample A compared with the small degree of aggregation in sample B. However, there are no differences between the initial distributions of the crystals.

Figure 3 shows the anti-aggregation activities of urinary macromolecules from recurrent calcium stone former and healthy subjects. Inhibitory activity (%) in stone formers is significantly less than that in the healthy controls ($P < 0.05$).

The relationship between inhibitory activity and urinary proteins in the final treated urine is shown in Fig. 4, to test the possibility that the increased calcium oxalate aggregation in stone formers reflects a higher concentration of macromolecules in the urine. However, there is no correlation between the degree of inhibitory activity and the concentration or urinary protein.

Each fraction obtained from the gel filtration column was measured for its inhibitory activity on calcium oxalate

Table 1. Values of urinary parameters

	n	Protein (µg/ml)	Calcium (mg/l)	Oxalate (mg/l)	Citrate (mg/dl)	pH
Healthy controls	29	100.0 ± 41.3	78.9 ± 68.2	31.34 ± 16.34	31.5 ± 22.7	6.0 ± 0.5
Stone formers	31	116.0 ± 45.2	93.7 ± 47.3	32.29 ± 18.28	22.6 ± 13.5	6.1 ± 0.5

There are no significant differences between the two groups.
Protein is measured in the final treated urine; the other four parameters are measured in the original urine

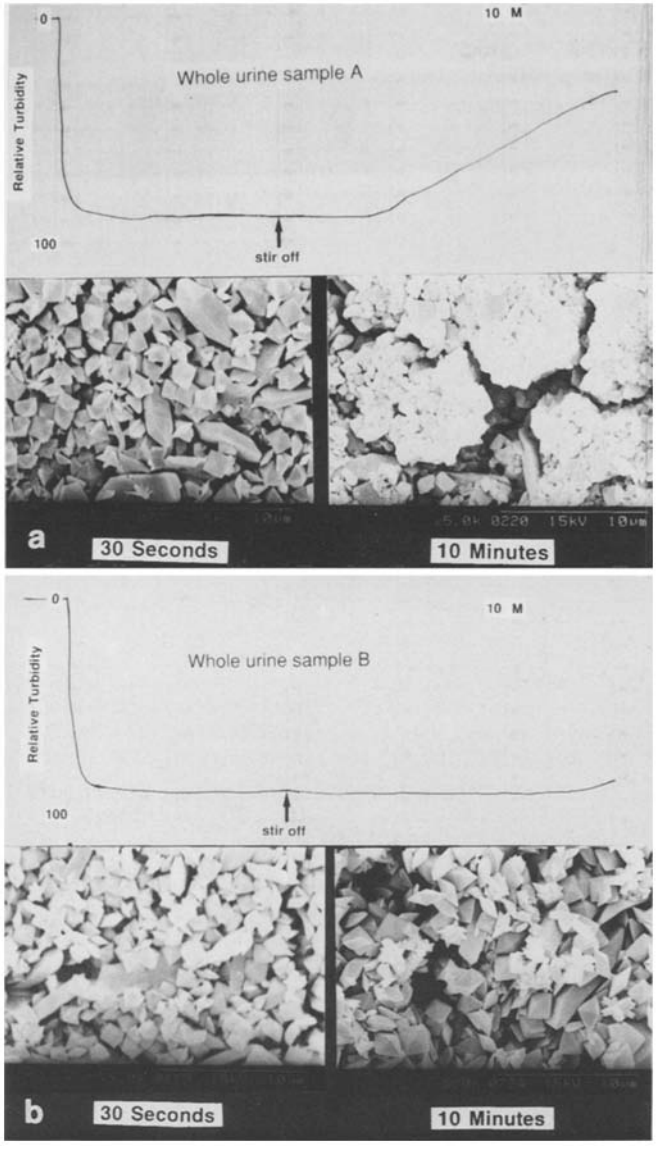


Fig. 2a, b. Turbidity curves from two urine samples and scanning electron micrographs. The turbidity of urine sample A from a recurrent stone former diminished rapidly and shows massive aggregation of the calcium oxalate crystals on scanning electron microscopy at 10 min (**a**). On contrast the turbidity of urine sample B from a healthy control decreased very gradually and shows little aggregation (**b**). However, there are no differences between the initial distribution of the crystals, as shown in the micrographs taken at 30 s after the addition of sodium oxalate. The crystals are mixtures of calcium oxalate dihydrate and monohydrate

crystal aggregation; the results are shown in Fig. 5. The peak of inhibitory activity roughly corresponds to the peak of optical absorbance at 280 nm. The approximate molecular weight of the substances with in the inhibitory peak is between 10000 and 30000.

Fig. 5. The inhibitory activity (%) of each gel filtration fraction (Superrose 12HR, 2×50 cm, Pharmacia) from normal concentrated urine. The peak of inhibitory activity roughly corresponds to the peak of 280 nm optical absorbance. The approximate molecular weight of substances with in the inhibitory peak is between 10000 and 30000. Carbonic anhydrase (29000) and cytochrome *c* (12400) are used as markers of molecular weight

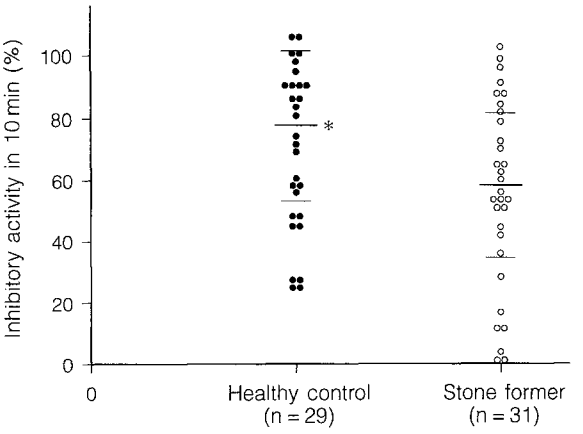


Fig. 3. Inhibitory activity (%) of urinary macromolecules in healthy controls (*filled circles*) and recurrent calcium oxalate stone formers (*open circles*). The urinary macromolecules in the stone formers inhibit calcium oxalate crystal aggregation much less than those of healthy controls ($P < 0.05$)

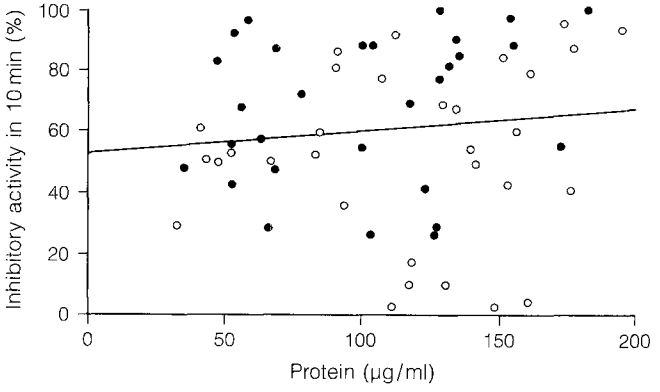
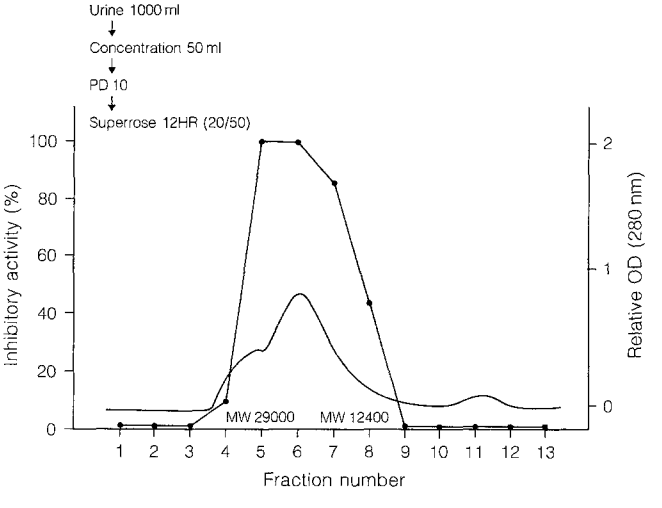


Fig. 4. There is no correlation between inhibitory activity (%) on crystal aggregation in 10 min and the concentration of protein (μg/ml) in ($y = 52.698 + 7.0471e - 2x$; $r^2 = 0.012$). *Filled circles*, healthy controls; *open circles*, the final treated urine recurrent calcium oxalate stone formers



Discussion

Urine had very powerful inhibitory action on crystal aggregation, and 1–10% urine distinctly inhibited the aggregation [3, 9]. However, low-molecular-weight constituents, such as magnesium, orthophosphate and citrate, have little or no effect on crystal aggregation [3], and some studies have indicated that the major part of the inhibitory activity resides in the urinary macromolecules [2, 3, 6, 11].

In our investigation, the urinary macromolecles of recurrent stone formers had markedly less inhibitory activity than those of healthy controls, as has been described previously [6, 7, 11]. The aim of the present study was to elucidate the difference between the urinary macromolecules of recurrent stone former and of healthy controls with respect to their inhibitory effect on crystal aggregation. However, the uncritical application of inhibitory activity as a method for evaluating the risk of stone formation seems questionable, because there is considerable overlap between the two groups.

It is well known that much slow crystallization occurs in human urine as compared with an experimental calcium oxalate solution. In the present, unseeded system, there is much rapid crystal nucleation and aggregation, and some crystal growth occured simultaneously. However, on the basis of the findings of scanning electron microscopy the predominant phenomenon in the system is crystal aggregation and we consider that simultaneous crystal growth may be negligible. Although urine at a concentration as low as 1% has the ability to inhibit aggregation in a small-scale experimental system, considerable and powerful inhibitory capacity would be needed to inhibit the rapid and massive aggregation in the present system. Thus, our aggregometer system may be suitable for estimating the inhibition of crystal aggregation by urinary substances or therapeutic agents.

We did not find any correlation between inhibition of aggregation and the concentration of urinary protein. There are numerous proteins in human urine, some of which might have a role in preventing aggregation while others might promote stone formation. We are currently looking for proteins with a molecular weight between 10000 and 30000 that have a significant inhibitory effect, and will go on to isolate these from concentrated urine in the near future.

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