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ZETA POTENTIAL MEASUREMENT AND PARTICLE SIZE ANALYSIS FOR A BETTER UNDERSTANDING OF URINARY INHIBITORS OF CALCIUM OXALATE CRYSTALLIZATION

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Abstract

To better understand urinary inhibitors of calcium oxalate crystallization, both zeta potential measurement and particle size analysis were chosen to illustrate: (1) the potential therapeutic efficacy of G872, a semi-synthetic sulfated polysaccharide, in stone prevention; and (2) the relative contribution of various urinary fractions {e.g., ultrafiltered urine (UFU), Tamm-Horsfall protein (THP), urinary polyanions precipitated with cetylpyridinium chloride (CPC), urinary macromolecular substances with different concentration ratios (UMS10,50,90 and UMS'10,50,90) and THP-free urine (THPFU)} to total urinary inhibitory activity. The results showed: (1) addition of G872 significantly enhances urinary inhibitory activity and negative zeta potential values; (2) re-addition of the CPC to UFU completely restores urinary inhibitory activity; and (3) artificial urines prepared by mixing UMS'10,50,90 from THPFU with UFU differed in inhibitory activity from that prepared by mixing UMS10,50,90 from a pooled normal urine with UFU. Based on these experimental results, the following speculations can be made: (1) normal human urines are considered to be a protective colloidal system; (2) urinary inhibitory activity originates mainly from CPC and/or UMS; (3) normal THP is a protective material to maintain urinary inhibitory activity; and (4) mutual interaction between urinary inhibitors may change the total urinary inhibitory activity.

Key Words: Zeta potential, calcium oxalate, crystallization, nephrolithiasis, inhibitors, glycosaminoglycans, polysaccharides.

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Introduction

From a physico-chemical viewpoint the main factors dealing with renal stone formation in the overall urinary tract can be summarized in eq. (1) [11],

$$\Phi = \Phi\ (X, Y, Z) \qquad (1)$$

where Φ is the function describing a risk tendency for suffering from stone disease. Three variables are involved in this function which may determine the stone formation risk of an individual: X, the supersaturation of stone salts in urine (a thermodynamic parameter); Y, the urinary inhibitory activity against stone salts crystallization (a kinetic parameter); and Z, the crystal retention to urinary-tract epithelium cells (a colloid and interfacial chemical parameter).

In the past decade, the early events of stone formation as well as the roles of renal tubule cells in nephrolithiasis have been emphasized [24]. For the situation inside a nephron, a equation, similar to eq. (1), can be formulated {eq. (2)},

$$\Phi = \Phi\ (x, y, z) \qquad (2)$$

where Φ has the same meaning as in eq. (1). Three variables (x, y, z) associate to early events of stone formation occurring within the kidney. They are the renal handling of stone salt ions (x), synthesis/secretion of modifiers of stone salts crystallization (y) and cell-crystal interaction (z). Comparing eq. (2) to eq. (1), one may find the corresponding relationship between the two theories of stone formation (X, Y, Z versus x, y, z). Current opinion seems more and more to emphasize the importance of cellular and molecular levels [6].

It has been known that different behaviors of stone salt crystallization inside a nephron are associated with the properties of the interfaces between crystals and their surrounding liquids and between renal epithelial cells and their surroundings. Since the additivity of urinary inhibitors exists only in the mass, but not in their functions {eqs. (3) and (4)}, the following two factors should be considered: (1) the urinary environment such as urinary components, pH, ionic strength and supersaturation

Table 1. Artificial urine composition (A:B = 1:1).

A: solution	
Salt	g/l
$CaCl_2 \cdot 2H_2O$	1.765
Na_2SO_4	4.862
$MgSO_4 \cdot 7H_2O$	1.460
NH_4CL	4.643
KCl	12.133
B: solution	
Salt	g/l
$NaH_2PO_4 \cdot 2H_2O$	6.800
Na_2HPO_4	0.869
$Na_3Citrate \cdot 2H_2O$	1.168
NaCl	13.51
$Na_2C_2O_4$*	0.166

pH = 6.10; ionic strength = 0.33;
relative supersaturation of calcium oxalate = 1.29;
*Sodium oxalate was excluded from actual applied solution in this study.

may have an influence on the inhibitory activity of a given urinary inhibitor; and (2) the interaction between two given inhibitors may have an influence on the total urinary inhibitory activity.

$$M_{Total} = \sum_{i}^{k} M(LM)_i + \sum_{i}^{m} M(SM)_j \quad (3)$$

$$I_{Total} \neq \sum_{i}^{k} I(LM)_i + \sum_{i}^{m} I(SM)_j \quad (4)$$

in which M_{Total} is the total mass of inhibitors in a given urine; $\Sigma\ M(LM)_i$ is the total mass of various macromolecular inhibitors in the urine (i = 1...k); $\Sigma\ M(SM)_j$ is the total mass of various small molecular inhibitors in the urine (j = 1...m). Whereas I_{Total} is the total urinary inhibitory activity, $\Sigma\ I(LM)_i$ is the total contribution of i kind of macromolecular substances (LM) to the inhibitory activity (i = 1...k), and $\Sigma\ I(SM)_j$ is the total contribution of j kind of small molecular substances (SM) to the inhibitory activity (j = 1...m).

Supportive findings have been reported: (1) A given macromolecular substance such as Tamm-Horsfall protein (THP) shows a dual effect (inhibition and promotion) on stone salt crystallization under different experimental conditions depending on pH, ionic strength, calcium and citrate [17, 18, 20, 25]; (2) the same kind of material, but with a different molecular weight or existing state in solution, shows a different behavior to inhibition or promotion in calcium phosphate crystallization under identical experimental conditions [23]; and (3) a mixture of two different kinds of macromolecular inhibitor does not demonstrate a total inhibitory activity equal to the sum of their individual capacities (our unpublished data). These phenomena suggest that binding of urinary small or macromolecular inhibitors on stone salts crystals could determine their functions in stone salts crystallization.

In the present study, a modification of the zeta potential measurement is introduced. Three urinary parameters: inhibitory activity against calcium oxalate monohydrate (COM) crystal growth (IG) and agglomeration (IA), and zeta potential (ZP) have been chosen as markers to evaluate urinary inhibitor properties. Urine samples were collected from 10 stone-formers (SF) and 10 non-stone-formers (NSF) to define their inhibitory activity and ZP values in the presence and absence of G872. After the measurements, all NSF urines were pooled (pooled normal urine, PNU) for preparation of various urinary fractions including ultrafiltered urine (UFU), THP, THP-free urine (THPFU), urinary polyanions (CPC), as well as urinary macromolecular substances obtained form the whole urine (UMS) and from THPFU (UMS'). UMS and UMS' were tested at several concentrations: 10, 50 and 90%.

To better understand urinary inhibitors of calcium oxalate crystallization, the inhibitory activity of the pooled urines as well as their fractions has been studied. Special attentions will be paid to answer: (1) whether a exogenous semi-synthetic sulphated polysaccharide such as G872 may significantly enhance urinary inhibitory activity? (2) which urinary components contribute most to total urinary inhibitory activity? (3) what are characteristics of urinary inhibitors in human? and (4) what are the factors that may influence urinary inhibitory activity?

Materials and Methods

Zeta potential measurement

The principle and method of ZP measurement has been described elsewhere [2, 5]. In this study, a methodological modification was made. COM crystals were first incubated with whole urine, and the crystals coated by urinary inhibitors were washed and re-suspended to an artificial urine. The procedure can be described as follows: 1 ml urine sample was mixed with 50 μl of the COM seed crystal suspension (24 mg/ml). After incubation of the mixtures at 37°C with shaking for 2 hours, the crystals were collected by centrifugation and washed

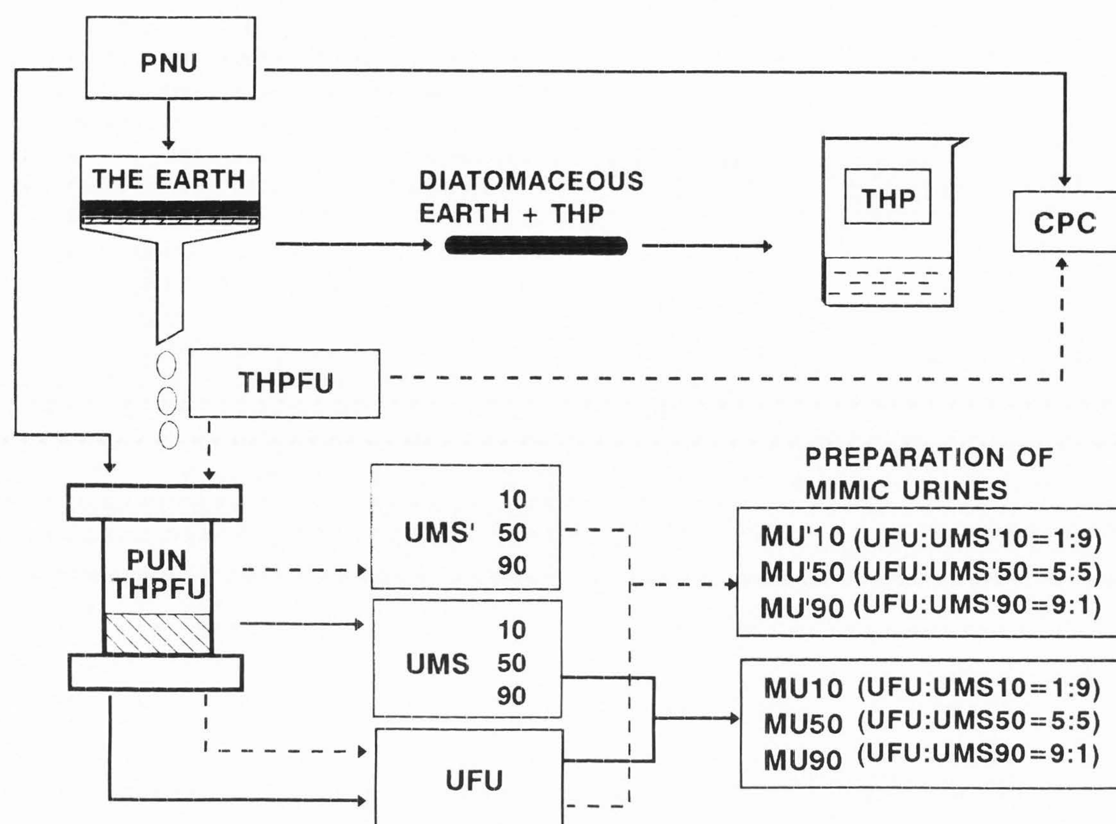

Figure 1. A schematic diagram of the preparations of urinary fractions from a pooled normal urine (PNU), the meaning of various symbols is: CPC: urinary polyanions precipitated with cetylpyridinium chloride; THP: Tamm-Horsfall protein isolated by adsorption with diatomaceous earth; THPFU: THP-free urine prepared by using the filter procedure with the earth; UMS or UMS': Urinary macromolecular substances prepared from original PNU or THPFU by using the ultrafiltration procedure; UFU: ultrafiltered urine; and MU/MU'10,50,90: mimic urines. These mimic urines were prepared by mixing of urinary macromolecular substances (UMS/UMS'10,50,90) with UFU, according to a given UFU to UMS/UMS' ratio's in volume. It should be noted that the composition of all mimic urines should be the same as corresponding to their original urines (PNU or THPFU).

two times with a solution saturated with calcium oxalate. The collected and washed COM crystals were re-suspended into 3 ml of 5 times diluted artificial urine (composition listed in Table 1). The mean ZP and ZP distribution of the COM crystals coated by urinary inhibitors were measured using the Coulter DELSA 440 (Coulter Electronics Ltd., Luton, U.K.).

To illustrate the effect of an addition of G872 to urine on the COM zeta potential, the same procedure was applied with addition of G872 (5 mg/l) to each urine tested. The method was also used in mimic urines prepared by mixing the UFU with urinary fractions such as THP, UMS, CPC and G872.

Inhibitory activity measurement

A COM seeded crystal growth system based on particle size determination (Coulter Multisizer II) together with a mathematical model as described by Ryall [26, 27] was chosen to address the inhibitory activity of crystal growth and agglomeration for a given urine and urinary fractions as well as other additives.

Urinary fraction preparation

The fresh morning urines (8:00-12:00 a.m.) were collected without preservative from 10 patients with calcium oxalate nephrolithiasis and 10 normal subjects. For determination of urinary inhibitory activity and ZP, 5 ml urines from each samples were centrifuged at 1500 g for 10 minutes and stored at -20°C until used. Remaining large volume urines of each sample were store at 4°C. The individual 10 normal urines were pooled and divided into three portions which were subjected to several procedures as described in Figure 1. Urinary GAGs concentration of the PNU was determined with dimethylmethylene blue-based spectrophotometry [10].

(a) The first portion of the PNU (100 ml) was ultra-

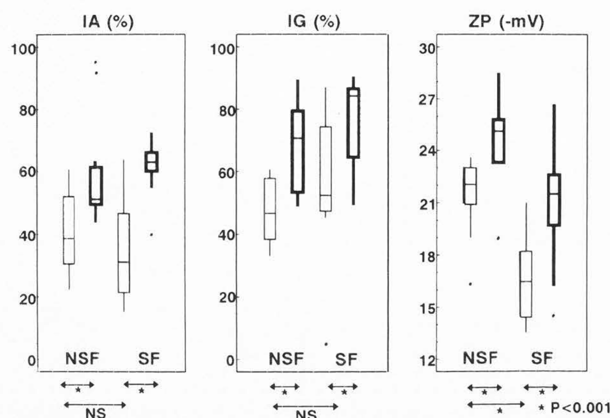

Figure 2. Scatter diagrams of data: (**a**) inhibitory activity of COM crystal agglomeration (IA%) in stone formers (SF) and non-stone formers (NSF) as well as the alterations of the inhibitory activity after addition of G872 (5 mg/l) to corresponding urines (bold line and box); (**b**) inhibitory activity of COM crystal growth (IG%); and (**c**) negative zeta potential values (ZP). The central box covers the middle 50% of the data values, between the upper and lower quartiles. The "whiskers" extend to the minimum and maximum values, and the central line is at the median. Extreme values are plotted as separate points useful for detecting outlier and asymmetric behaviour. NS: no statistical significance; *: statistically significant, $p < 0.001$).

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filtered through a membrane with a 5 KDa M.W. cutoff (catalogue no. 887856, Spectrum Medical Industries, Inc., Los Angeles, CA) to give an UFU and urinary macromolecular substance. During the ultrafiltration, the PNU was concentrated. Two milliliters of the concentrated urines were respectively collected at the following concentration ratio: 10%, 50% and 90%, which were respectively named as urinary macromolecular substances (UMS10, UMS50 and UMS90). The remaining UFU was kept as medium and stored at 4°C.

(**b**) The second portion (500 ml) was used to isolate THP and to prepare THPFU according to Serafini-Cessi and associates [30]. Briefly, 5 g of diatomaceous earth (Sigma, St. Louis, MO) suspended in deionized water was poured into Buchner funnels of 70 mm in diameter (Sigma-Aldrich Techware, Bornem, Belgium), lined with No. 1 Whatman filter paper. The layer of diatomaceous earth is washed first with deionized water and then with 0.025 M PBS (phosphate buffered saline) of pH 7.5, containing 0.14 M NaCl. The urine was filtered through the prepared diatomaceous earth layer, and THPFU as a filtrate was obtained. To obtained the urinary THP, the diatomaceous earth with THP adsorbed was washed with 100 ml PBS, scraped off and resuspended in water with stirring occasionally for 30 minutes. The earth suspension was centrifuged at 20,000 g for 20 minutes and the supernatant containing THP was dialyzed against water and lyophilized. It has been shown that the majority of THP present in urine can be removed following this procedure.

The same procedure as described in (a) was applied to 100 ml of the THPFU to acquire UMS without THP. A second (THP-free) series of urinary macromolecular substances (UMS'10, UMS'50 and UMS'90) is obtained according to the ultrafiltration procedure.

(**c**) The third portion of PNU (50 ml) and 50 ml THPFU obtained from (b) were used to prepare urinary polyanions (CPC). Briefly, 1 ml 5% cetylpyridinium chloride (Sigma) was mixed with 50 ml urine (PNU or THPFU) and incubated overnight at 4°C. After centrifugation at 10,000 g for 10 minutes, the obtained pellet, CPC, was washed twice with absolute ethanol, dried and dissolved in 2 M LiCl solution. After other centrifugation, the supernatant was mixed with ethanol to final concentration of 90%. Following storage of the mixture at 4°C for 48 hours, the final CPC was dissolved in deionized water. The concentration of the CPC was defined according to de Jong *et al.* [10].

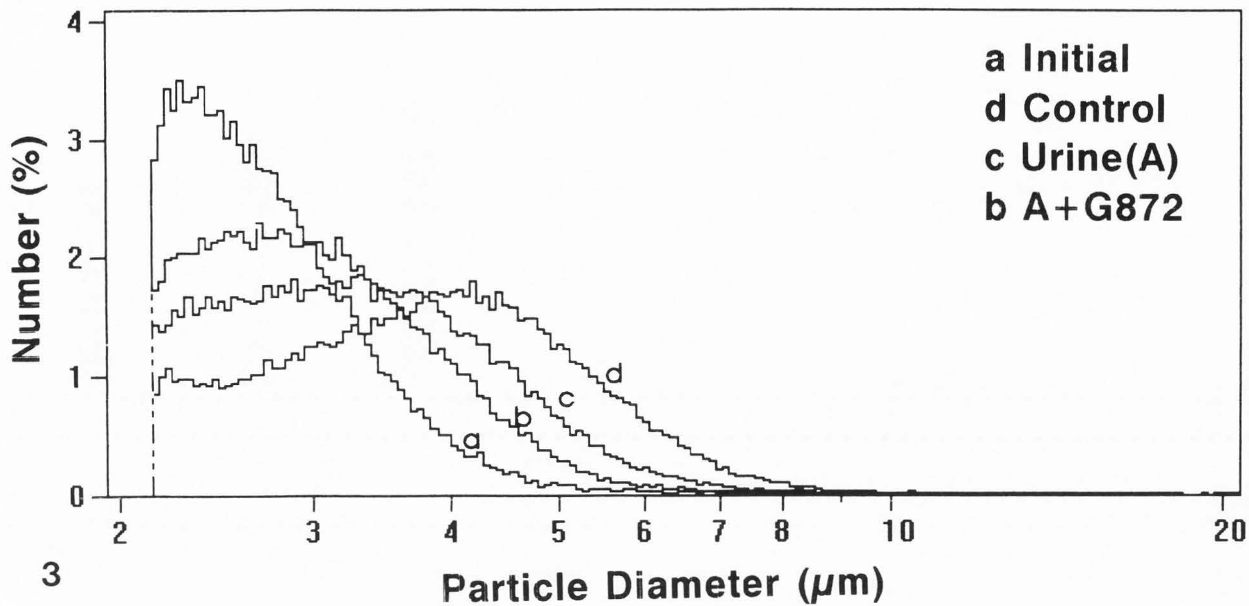
Thus, the original PNU and THPFU as well as four isolated fractions including UFU, THP, CPC and urinary macromolecular substances (UMS10,50,90 and UMS'10,50,90) were obtained. From these fractions, a series of mimic urines (MU'10,50,90 and MU10,50,90) were reconstructed by adding UMS to UFU in the mentioned ratio in volume. The preparation procedures are summarized in Figure 1.

Preparation of COM seeded crystals

COM crystals were prepared as described before [5]: one liter of calcium chloride solution (0.25 M) was added drop-wise to one liter of sodium oxalate solution (0.25 M) over a period of 1 hour at room temperature. The COM seed crystals produced were washed several times with deionized water until no chloride ions were detected. The COM seeded crystal suspension (24 mg/l) was prepared and aged at 70°C for 2 hours with stirring. The prepared COM seeded crystals were kept at room temperature at least for three weeks before use. The COM seeded crystal has been characterized by Philips APD X-ray diffraction (Philips NV, Eindhoven, Netherlands), and their spectrum completely agreed with the spectra of commercial available COM.

Statistical methods

Determinations were performed in duplicate. A statistical software package (STATGRAPHICS V6.0, STSC, Inc., Rockville, MD) was chosen to perform the Wilcoxon matched-pair signed-rank test (a non-parameter statistical test) and one-way analysis of variance to compare the median values of each two groups.



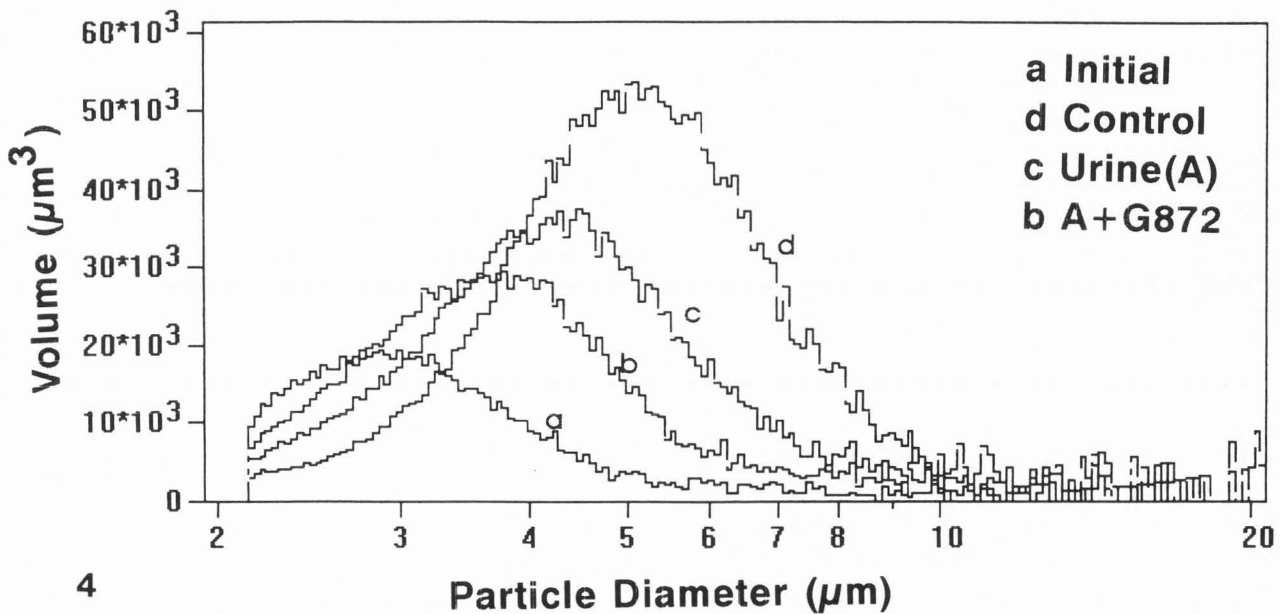


Figure 3. Changes in crystal number distributions in the presence and absence of an addition of G872 to an given normal urine (No. 5): (**a**) initial distribution, (**b**) after 4 hours incubation without any additive, (**c**) with 2% urine and (**d**) with 2% urine plus G872 (5 mg/l). The data for (b), (c) and (d) were collected from Coulter Multisizer II following 4 hours of COM seeded-crystal growth in a metastable solution.

Figure 4. Changes in crystal volume distributions in artificial urine: (**a**) initial distribution, (**b**) after 4 hours incubation without any additive, (**c**) with 2% urine and (**d**) with 2% urine plus G872 (5 mg/l).

Results

G872 enhances urinary inhibitory activity and negative zeta potential values

Figure 2 shows a comparison of inhibitory activity of crystal growth (IG) and agglomeration (IA), and zeta potential (ZP) in the urines from stone-formers (SF) and non-stone formers (NSF). No significant difference of urinary inhibitory activity was found between SF and NSF. Only the median of ZP value is significantly higher in normal urine than in stone-forming urine (NS = -21.4 ± 2.31 vs. NSF = -16.6 ± 2.53 mV; $p < 0.05$).

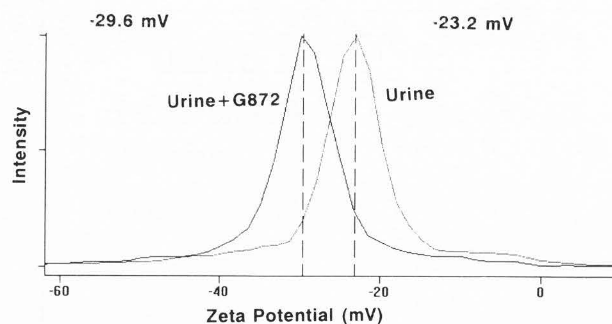


Figure 5. The zeta potential distribution of COM seeded crystals in an artificial urine medium following incubation with normal urine and urine plus G872 (5 mg/l). The mean zeta potential values are -23.2 and -29.6 mV respectively.

It was found that addition of 5 mg/l G872 to SF and NSF urines significantly enhances the urinary inhibitory activity against COM crystal growth and agglomeration and the negative zeta potential values, suggesting a potential therapeutic efficacy of G872 in stone prevention. In other words, exogenous sulphated polysaccharides, e.g., G872, could be useful for stone prevention if such compounds can appear unchanged in urine with a sufficient concentration following their (oral) administration.

The same amount of G872 added to the same volume of UFU (see Fig. 8, shown later) or to artificial urine [7] shows a much higher inhibitory activity and negative ZP, emphasizing the importance of the concept of net-inhibitory activity again [29].

As an example, Figures 3, 4 and 5 demonstrate the effect of addition of G872 to a normal urine (No. 5) on COM particle size distributions in number (Fig. 3) and in volume (Fig. 4), and on zeta-potential distribution (Fig. 5) of COM seed crystals. As observed in Figure 3, after 4 hours incubation the total crystal number and portion of small crystal moiety are much higher in the presence of G872 than in its absence. Moreover, the total volume and mean diameter of COM seeded crystals are much less in the presence of G872 than in its absence (Fig. 4). The addition of G872 to this urine (No. 5) shifts the ZP distribution to more negative direction, suggesting these COM seeded crystals can get a high negative charge due to the adsorption of G872 to the crystal surface (Fig. 5).

Comparison of inhibitory activity and zeta potential value of PNU to its derivatives (THPFU and UFU)

Figure 6 shows the parameters IG, IA and ZP from pooled normal urine (PNU), THP-free urine (THPFU) and ultrafiltered urine (UFU). It can be seen that the inhibitory activity against crystal agglomeration and growth and the negative ZP values are reduced in the

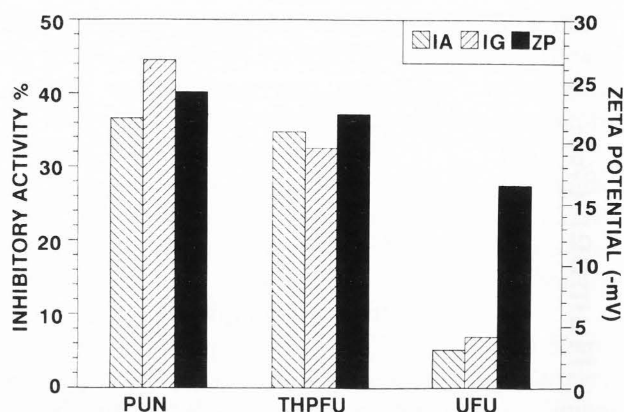


Figure 6. Comparison of inhibitory activity against crystal growth (IG) and agglomeration (IA), and the zeta potential values (ZP) of a pooled normal urine (PNU) with its derivatives such as THP-free urine (THPFU) and ultrafiltered urine (UFU).

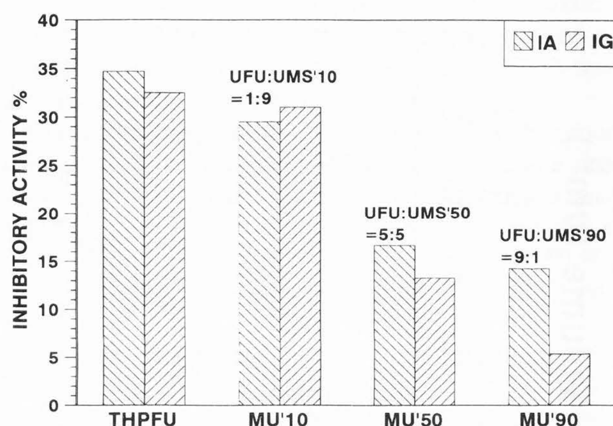


Figure 7. Comparison of inhibitory activity (IA, IG) of THP-free urine (THPFU) with its corresponding mimic urines MU'10, MU'50 and MU'90. It should be noted that THPFU and all mimic urines prepared have a similar composition (see text for details).

UFU, suggesting that UMS (MW > 5000 Da) mainly account for urinary inhibitory activity. Although no differences of these parameters are found between PNU and its corresponding THPFU, the role of THP on COM crystallization cannot be ignored.

Different property of urinary macromolecular substance derived from between PNU and THPFU

As compared with the original PNU, no significant differences of inhibitory activity (IA and IG) were found in the first series mimic urines (MU10,50,90) prepared by re-addition of UMS10, UMS50 or UMS90 to the UFU (not shown), whereas a much lower inhibitory activity was found in the second series mimic urines

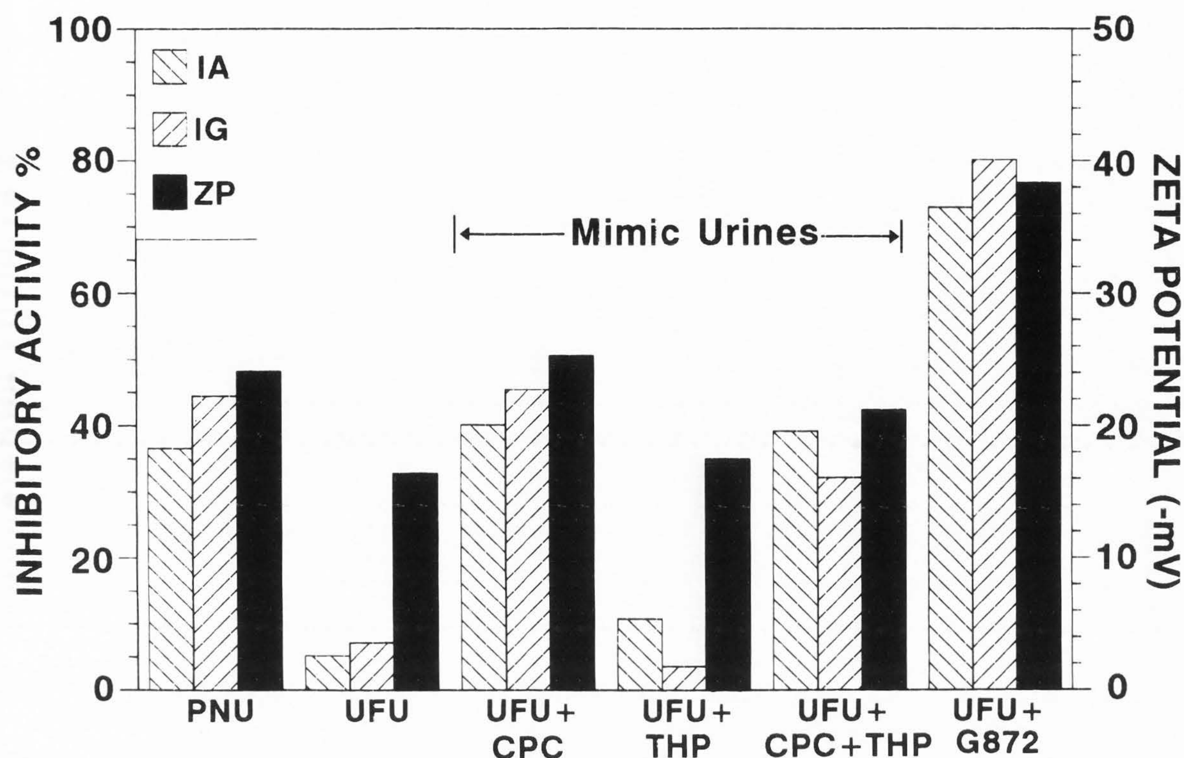


Figure 8. Comparison of inhibitory activity (IA, IG) and zeta potential values of the pooled normal urine (PNU) with its corresponding mimic urine including UFU, UFU plus G872 5 mg/l (UFU + G), UFU plus CPC (UFU + CPC), UFU plus THP (UFU + THP) and UFU plus CPC and THP (UFU + CPC + THP).

(MU'10,50,90) prepared by addition of the THP-free UMS'10, UMS'50 and UMS'90 fractions to the UFU (Fig. 7).

Interestingly, the inhibitory activity of these mimic urines (MU'50 and MU'90) is much lower than those of their original one: THPFU (Fig. 7) and PNU (Fig. 6). When we assume that fractionation and recombination compose a reversible reaction {eq. (5)}, the prepared mimic urine obtained by mixing UFU and UMS's at a given UFU and UMS's ratio (1:9 for UMS10, 5:5 for UMS'50 and 9:1 for UMS'90) should act similar as the THPFU tested at the same concentrations.

$$\text{THPFU} \rightharpoonup \text{UFU} + \text{UMS'10(50,90)} \qquad (5)$$

However, in the present study, the reconstituted urines appear to inhibit less than the original urine. This suggests that the reaction described in eq. (5) is an irreversible process. It is conceivable that the reduction of inhibitory activity in the mimic urines (MU'50 and MU'90) is due to denaturalization of the urinary macromolecular substances (UMS'50 and UMS'90) during their preparation.

Relative contributions of various urinary fractions to total urinary inhibitory activity and negative zeta potential

Figure 8 shows the inhibitory activities and negative ZP values in ultrafiltered urine (UFU) and their reconstructed mimic urines, e.g., mixtures of UFU with CPC, THP, THP + CPC and G872. As compared with PNU, it is obvious that: (1) addition of G872 (5 mg/l) to UFU showed the highest potency to increase IA, IG and ZP; (2) re-addition of CPC is able to restore the parameters to normal (PNU); and (3) re-addition of THP alone does not restore inhibitory activity.

Discussion

In the past two decades, some important urinary macromolecular inhibitors {e.g., nephrocalcin [21], THP [17], uropontin [31], GAGs [1], crystal matrix protein (prothrombin activation peptide fragment 1) [33] and inter-α-trypsin protein [32]} have been isolated, purified and identified. Some functional differences of these inhibitors such as THP and GAGs between SF and NSF have also been reported [14, 17]. In spite of these advances, however, we still do not completely understand the role of these inhibitors in stone formation. For example, some urinary components have demonstrated a strong inhibitory effect on both calcium oxalate and calcium phosphate crystallization in various model systems *in vitro* [13, 22], to date, however, no systematic and randomized clinical trails are found in literature to show

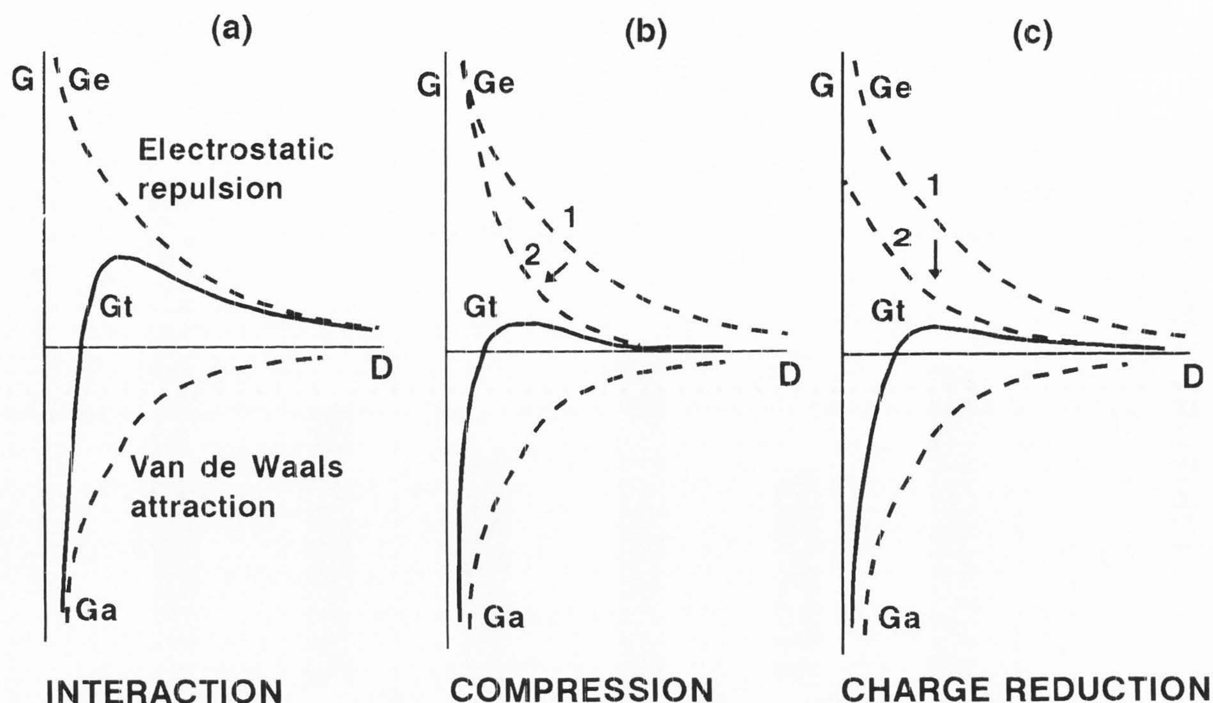


Figure 9. A schematic diagram of the connection between DLVO interaction and COM crystal agglomeration. D: the distance between two crystal particles; G: Gibbs free energy. When two particles approach each other, they undergo (1) electrostatic repulsion (G_e) and (2) van der Waals attraction (G_a) resulting in an energy barrier at the total energy curve (G_t). The crystals agglomerate together before they must overcome this barrier (**a**). The double electron layer may be compressed by an increase in ionic strength (1→2) (**b**), or by a reduction of charge (1→2) (**c**) resulting in decreasing the energy barrier. Both situation (b) and (c) will lead to particle agglomeration.

the significant differences of urinary inhibitory activity between SF and NSF. In addition, although the dual effect of urinary inhibitors has been recognized [4, 14, 23, 29], little is known about the causes leading to low urinary inhibitory activity. The following open questions are listed and are waiting to be answered in further investigation:

(1) What is the molecular defect of urinary inhibitors in stone formers?

(2) How do the urinary inhibitors play a role in pathogenesis of urolithiasis?

(3) Can the advances of inhibitor theory be used in clinical practice for stone prevention?

Of course, these questions are very wide, and at this moment we do not have sufficient knowledge to answer all of them. An attempt was made in the present study to illustrate the properties of urinary inhibitors and their relative contributions to total urinary inhibitory activity.

The concept that human urine can be a protective colloidal system was first put forward by Butt in 1952 [3]. For many reasons, considering the human urine as a colloid system would be better than considering it a simple solution. Maintaining the stability of urinary colloidal system could be an efficient approach to stone prevention. Zeta potential, as a very sensitive marker, has been chosen to study the stability of the urinary colloidal system and the mechanisms of crystal agglomeration [2, 5, 8, 16, 17, 28]. In the present study, the method of ZP measurement has been modified and used successfully. As compared with previous ZP measurement [28], the new method described in this paper enables us to eliminate the interference of urinary pH and ionic strength with ZP measurement. Moreover, the new ZP measurement could be further used to study the interactions between two crystals coated by the same and/or different macromolecules in a given medium which could be useful for a better understanding of the mechanisms of inhibition by urinary macromolecules inhibitors in COM crystallization, especially concerning COM crystal agglomeration.

In this study, a significant difference of negative ZP values was found between stone-formers and non-stone-formers (Fig. 2). Moreover, it was observed that addition of G872 to SF and NSF urines significantly increases in the negative ZP values (Figs. 2 and 5). Re-addition of CPC to UFU completely restored the urinary inhibitory activity (Fig. 8). These findings indicate that there is a highly negative charge density on the COM

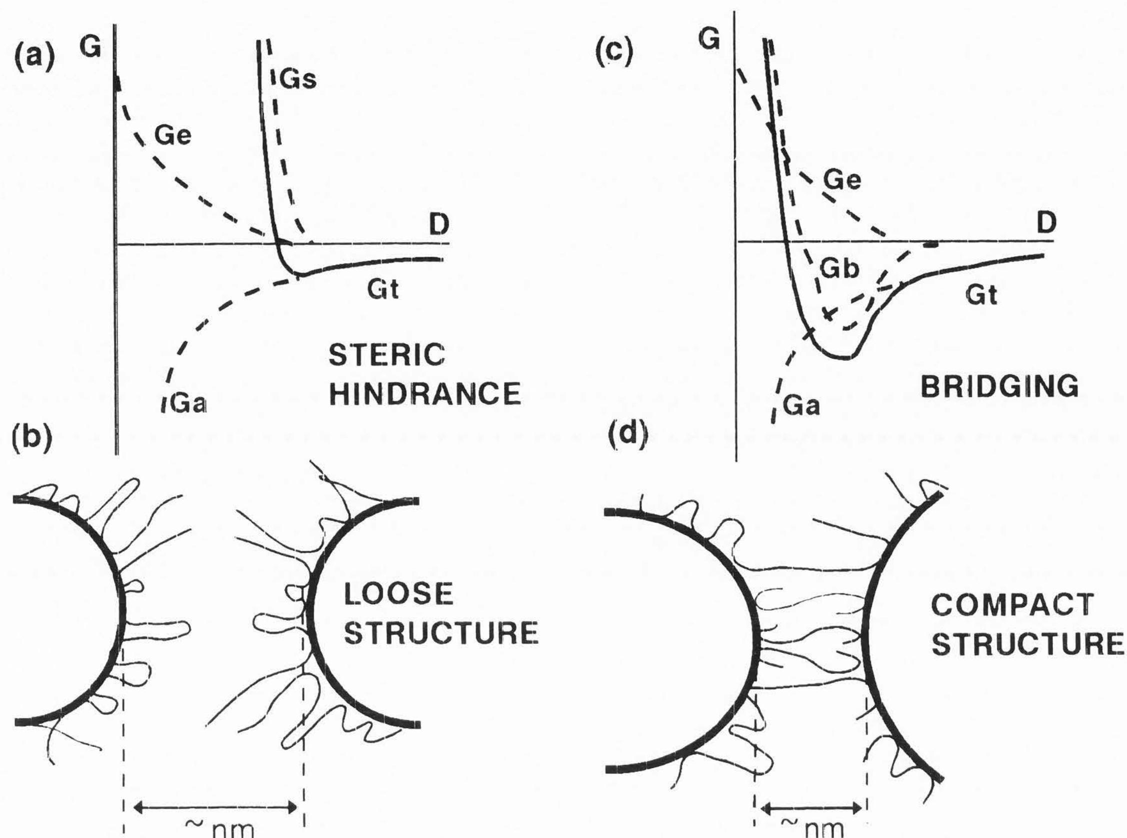


Figure 10. A schematic diagram of binding modes of urinary macromolecular substances (UMS) on COM crystal surface and corresponding changes of Gibbs free energy (G): a loosely structural layer formed by adsorption of UMS, which leads to a significant increase of total energy (steric hindrance) (**a** and **b**); a compact structural layer formed by adsorption of UMS, which leads to a significant decrease of total free energy (steric bridging) (**c** and **d**). G: surface free energy; G_a: van der Waals attraction; G_e: electrostatic repulsion; G_b: steric hindrance or steric bridging energy; and G_t: total energy.

crystals following the adsorption of CPC. The same could be observed after G872 addition to UFU. The more negative the mean ZP value at the interface between COM crystals and surrounding solution, the higher the dispersion of the COM crystals, which was indeed shown by the particle size analysis (Figs. 3 and 4).

However, we did not find any significant difference in the ZP values between PNU and THPFU and its mimic urines such as MU'10,50,90 (not shown), irrespective of the big differences in inhibitory activity between them (Fig. 7). This finding suggested that there could be a different binding property between the urinary macromolecular substances in the THPFU and in its mimic urines. It also shows that inhibitory potential and ZP are two different entities which act and are influenced independently of each other.

To explain the phenomena, it is necessary to mention the interaction between two separated particles which has been described by Finlayson [11] and Robertson [29] {see eq. (6)}:

$$V_T = V_A + V_R + V_V \tag{6}$$

where V_T: the total interaction potential between two particles; V_A: van der Waals attraction; V_R: electrostatic repulsion; and V_V: viscous binding. In general, both DLVO (Derjaguin, Landau, Verwey and Overbeek) interaction (Fig. 9) [19] and steric interaction (Fig. 10) [12] can be taken to understand the stability of urinary colloidal system as well as the mechanism of crystal agglomeration. From Figure 9, it can be imagined that increase of ionic strength or reduction of surface charge compress the double layer resulting in decreasing of the energy barrier which may benefit for crystal agglomeration. In our case, G872 and CPC with high negative charge bound to COM crystal surfaces may enhance electrostatic repulsion leading to crystal agglomeration prevention. Moreover, it is also necessary to emphasize that the viscous binding may contribute to the total interaction potential by the steric effect, resulting in promotion of either crystal dispersion by steric hindrance

(Figs. 10a and 10b) or of crystal flocculation by bridging (Figs. 10c and 10d). The steric effect may be mainly determined by molecular structure of the substances bound on COM crystals. When a macromolecular substance is transferred from solution to crystal surface, this will induce not only a DLVO repulsion force, but also a strong steric hindrance. The data shown in Figures 2, 3, 4, 5 and 8 support the concept of DLVO repulsion and steric interaction. Therefore, one may not be surprise to see the dual effect of a given inhibitor on COM crystallization. To clarify the potential therapeutical efficacy of a give exogenous polysaccharide such as G872, it could be necessary to further investigate the urinary inhibitors in their existing state and their secondary structure changes in urine.

From our experimental data about IA and IG shown in Figures 6, 7 and 8, the results can be symbolized to eq. (4) as follows: Due to $I_{PNU} \approx I_{THPFU} >> I_{UFU}$ (Fig. 6), $I_{UFU} \approx I_{UFU+THP}$ and $I_{PNU} \approx I_{UFU+CPC}$ (Fig. 8), but $I_{THPFU} >> I_{MU'50} > I_{MU'90}$ (Fig. 7). It can be speculated that: (1) urinary inhibitory activity mainly derives from urinary macromolecular substances and (2) normal THP could play a protective effect on urinary macromolecular substances because of $I_{PNU} \approx I_{THPFU}$, but $I_{THPFU} >> I_{MU'50} > I_{MU'90}$ (Fig. 7). Although no effort was made in this study to identify molecular defect of UMS, the different properties between UMS10,50,90 and UMS'10,50,90 indicated that the existing state and molecular structure of UMS'50 and UMS'90 could be changed during the removal of THP or ultrafiltration procedures. The secondary-structure alteration of UMS'50 and UMS'90 could lead to the difference of inhibitory activity between THPFU and its mimic urines (Fig. 7). In other words, the property of THP-free urine cannot be restored in the mimic urines prepared by mixing ultrafiltered urine (UFU) with UMS'50 and UMS'90. Our finding may support observation from Grover *et al.*, who recently reported a protective effect of THP on calcium oxalate crystal aggregation induced by urate in human urine *in vitro* [15].

It has been believed that an abnormal function of urinary inhibitors is directly connected to its structural change. Any functional and structural changes of urinary inhibitors could originate from: (1) abnormal urinary environment; or (2) from abnormal cellular metabolism. Renal tubular cell injuries induced by various causes may be a necessary condition for urolithiasis especially for its early state which has been shown in recent morphological investigations [9]. Therefore, the study on structure-function relation of urinary inhibitors could be extended towards crystal-cell binding interrelations in further investigations.

Based on the results obtained, the following conclusions and/or speculation can be drawn: (1) Normal human urine can be thought of as a protective colloidal system with a temporary stability in thermodynamics due to urinary macromolecular protective substances. (2) Urinary inhibitory activity originates from urinary macromolecular substances (UMS) or urinary polyanions (CPC). (3) Normal THP may be a protective substance for the urinary macromolecular inhibitors. (4) Abnormality of urinary inhibitors may be induced by an abnormal urinary environment caused by changes in pH, ionic strength and supersaturation and/or by an abnormal metabolism of renal tubular cells. (5) Interaction between urinary inhibitors may change the total urinary inhibitory activity.

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Discussion with Reviewers

R.L. Ryall: The methodology used to assess inhibitory activity is of great concern. This technique was published about 14 years ago, and we have since published work demonstrating its inability to quantify inhibitory activity accurately. One concern is that crystal growth and aggregation are not fully independent processes; because the amount of CaOx deposited during the reaction is dependent upon the available surface area, crystal growth expressed as increasing volume will depend upon the degree of aggregation of the crystals. A second worry is that the lower limit of detection of the Coulter Counter excludes many crystals from being recorded by the instrument and this can introduce large errors in the calculation of inhibitory activity. These problems can only be addressed by use of a computer model to obtain accurate estimates of inhibitory activity [39]. Our work has been extended by Dr. Michael Hounslow of the Department of Chemical Engineering, University of Cambridge. A non-iterative computer model has been available for analyzing Coulter Counter data obtained from reaction involving simultaneous CaOx crystal growth and aggregation [38]. For this reason, the data related to inhibitory activity measurements are invalid and therefore, should not be used.

G.H. Nancollas: The finding that the addition of G872 produces more small crystallites (lower crystal volume and diameter) with greater zeta potential, may suggest secondary nucleation processes at surfaces on which the additive molecules have been immobilized.

H.G. Tiselius: The effects on the inhibition of crystal growth and crystal aggregation was measured by its reflection in the zeta potential as well as by data derived from size distribution in a Coulter Counter. Of these two methods, the zeta potential is probably most significant because the accuracy of this technique is probably greater than that of crystal size analysis.

Authors: Although the weakness or limitation existing in the Coulter Counter model has long been recognized by many investigators including ourselves, the model as a tool in stone research is still used in many laboratories. We are impressed by Dr. Ryall's special contribution to the model and believe that the principle of the model is correct and the existing problems in this model are common as compared with other crystallization model systems *in vitro*. In our own experience, the currently used Coulter Counter model looks good for evaluation of inhibitory activity of single and pure inhibitors such as GAGs and the results can be compared with Constant Composition model. But, for a complex sample such as human urine, the model may sometimes have deficiencies because urine composition cannot be controlled. Moreover, because they have to be diluted (usually to around 1%), different inhibitors are diluted to varying extents, and the resulting overall activity may bear no relation to the inhibitory effect that the urine may have on CaOx crystallization in the undiluted state. These difficulties, perhaps, concern study on methodological standardization. **In our opinion, the main problem of this model is how to record and evaluate the extra fine particles and extra large particles. The former has gone beyond the detection range of Coulter Counter single tube technique and the latter may related to additional foreign catalytic surface.** Both extra fine and large particles may associated with the possibility of second nucleation, de-agglomeration of poly-crystals and additional foreign catalytic surface. For instance, a potential secondary nucleation induced by adding G872 to human urine, as pointed out by Dr. Nancollas, could not be detected with the single tube technique of Coulter Counter.

R.L. Ryall: I firmly believe that the model you used, and which you point out, we have used ourselves, has much to offer stone research. However, there are two factors that need to be taken into account: (a) Coulter Counter data need to be corrected to take account of the deficiencies I have listed. (b) Data must not be used to draw conclusions about an inhibitor's possible action in urine *in vivo*. In addition, even you find that the model "looks good for evaluation of inhibitory activity of a single and pure inhibitors such as GAGs," I can only stress, once again, that the data are only useful to draw conclusions about an inhibitor's action in an inorganic solution. For example, chondroitin is a powerful inhibitor of aggregation in a seeded, inorganic metastable solution, but it has no material effect in urine and this is perfectly in keeping with its absence from both CaOx stone and crystals. Could you explain in detail the real difference between aggregation and agglomeration? The term "agglomeration" has crept into stone research in recent years with no author ever bothering to explain its meaning.

Authors: We agree with your principle mentioned in (a) and (b), and are glad to hear that Drs. Hounslow and Bramley of the University of Cambridge are willing to help us in improving our current model. However, we think that any results from any *in vitro* crystallization model systems used currently may not completely represent the situations in whole urine and *in vivo*. Your example about the absence of chondroitin in stone matrix and crystal matrix does not exclude the role of chondroitin in stone formation. More recent evidence [40] has demonstrated the difference of chondroitin between stone-formers and non-stone-formers. Considering a change of available surface area of seeded crystals induced by crystal flocculation or coagulation, which may

have an influence on crystal growth, it could be necessary to define the terms of aggregation and agglomeration. Nancollas [22] stated: "The terms flocculation and coagulation refer to the tendency for small suspended particles in a liquid to form aggregates or agglomerates. Aggregates may be regarded as groups of primary particles joined at their faces and having surface areas significantly less than the sum of the areas of their constituent particles. In agglomeration, the primary particles may be regarded as being joined at edges and corners so that the surface area of the resulting particles is not markedly different from the sum of the areas of the individual components." We suppose that agglomeration could be a main manner of crystal flocculation in the used Coulter Counter model.

S.R. Khan: Why is there so little difference in inhibition and agglomeration between the normals and the stone formers, and yet the ZP is much lower for the stone formers?

R.L. Ryall: For the data presented in Figure 2, all urines were centrifuged at 1500 x g for 10 minutes and were also frozen at -20°C. It is known that such treatment may cause the loss of substantial amounts of THP [35] and induce precipitation of urinary proteins and CaOx crystal [37]. Did the authors check the effects of their urine treatment procedures on THP and total protein and GAG concentrations? What steps were taken to ensure after thawing that precipitated macromolecules and crystals were completely redissolved? Were the effect of freezing and thawing on ZP and inhibitory activity measurements checked?

Authors: We do not know why no significant difference of urinary inhibitory activity was found in this study between stone patients and normals. Too small sample count (n = 10 for each) could be one of the reasons because we found a significant difference of these parameters in our previous investigation [34]. A second reason could be related to treatment and store of the urine samples which we shall seriously consider checking the influence of urinary pretreatment on the results of urinary inhibitory activity and ZP. It may be necessary to mention that only 5 ml urine for each individual samples underwent such treatment, because we were pressed for time. The pooled urine and various urinary fractions were prepared without centrifuging and freezing stone procedures.

G.H. Nancollas: The zeta potential is an extremely sensitive parameter for measuring changes in surface charge. In order to make proper comparisons, the isoelectric point is more useful. The authors should make ZP measurements as a function of pH on both the COM pure surfaces and those which have been coated with the macromolecules of interest. In the ZP measurements, the COM crystal were re-suspended in diluted artificial urine. This contains considerable concentrations of phosphate ion and it is quite possible that other phases (e.g., calcium phosphates) will precipitate on the crystal surface. Did the authors do energy-dispersive X-ray (EDX) measurements in order to verify that this did not occur?

R.L. Ryall: Why were the zeta potential measurements made in artificial urine and not measured directly in the urine samples, which would more accurately reflect physiological conditions?

Authors: Although it is not difficult to measure directly the ZP of COM crystals in whole urine, the results from such whole urine could be difficult to evaluate due to undefined pH, ionic strength and other reasons. The new approach of the ZP measurement presented in this paper may overcome the above difficulties. After incubation of COM crystals in whole urine, the COM crystals coated by urinary macromolecules have all original feature of these compounds and the results from re-suspending COM in artificial urine can fully reflect the characteristics of urinary macromolecules. The precipitation of calcium phosphate on COM could occur in re-suspension process as pointed out by Dr. Nancollas, therefore, the suggested EDX measurements should be done later.

H.G. Tiselius: From a therapeutic/prophylactic point of view, the effects of G872 appear promising and the effects on the ZP are obviously reflected in corresponding changes in the crystal size distribution. In these experiments, G872 was added to give a final concentration of 5 mg/l. Is it likely that this concentration can be attained in urine following an oral load of this preparation?

Authors: Little is known about metabolism of endogenous GAGs as well as exogenous sulfated polysaccharides, such as, G872. The final concentration of 5 mg/l was chosen according to the pharmacokinetic study of SP54 [36]. The results from *in vitro* models suggest potential use of G872 for stone prevention, however, the actual situations concerning the role of G872 in stone-forming rats and human urolithiasis are much complex. Up to now, we could not measure the precise amount of urinary G872 following an oral load. Moreover, we could not find a significant increase of total GAGs excretion as well as of urinary inhibitory activity in stone-forming rats induced with ethylene glycol and vitamin D_3 following oral G872 administration. However, a preliminary clinical study demonstrated a significant increase of urinary GAGs excretion following oral G872 administration (200 mg/day). This may suggest a different metabolic response to G872 between humans and rats.

S.R. Khan: Why are the normal urines pooled and not the stone former urines?

R.L. Ryall: I am afraid that I still cannot agree with your statement about the protective effect of THP on urinary macromolecular inhibitors, because you did not perform any experiments to examine molecular defects of urinary inhibitors.

Authors: Indeed, in the present study no special attempt was made to identify the difference of urinary inhibitors between SF and NSF. However, an irreversible process was found in THPFU {see eq. (5) and corresponding text for details}, but was not in PNU which prompted us to make the speculation about protective effect of THP.

R.L. Ryall: The procedure you use for removing THP is very good. However, I must ask you whether you are certain whether the diatomaceous earth procedure you use removes only THP. Are you certain that all the other macromolecules are not removed?

Authors: The result from ELISA [2] suggest that all urinary THP can be removed from original urine using the diatomaceous earth procedure. But, we are not sure whether other urinary macromolecules are also removed. This point needs to be investigated further.

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