Urol Res (1985) 13:199–205

Urological Research

Evaluation of the Relative Inhibitory Potential of Fractionated Urinary Macromolecules

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Accepted: January 11, 1985

Summary. Ultrafiltration membranes of 10,000 d, 1,000 d and 500 d were used to remove urinary macromolecules from the urine of normal subjects and from the urine of stone forming patients. The filtrated urines were examined for their residual inhibitory potential for calcium-oxalate precipitation, by the discrimination method of Sarig et al. (D.I. test). The results of testing the filtrate were complementary to the information gained by analyses of retentates obtained in successive ultrafiltration. The method has an inherent advantage because the manipulation of solids retained on membranes may inadvertantly modify their inhibitory potential. At least two distinct groups of inhibitors were found in 20 normal urines. The first group has MW above 10,000 d while the second group of inhibitors has MW in the range of 500–1,000 d. The mean of the D.I. values increased dramatically from the normal range (< 0.6) to the stone former range (> 1.1) ($p < 0.001$) after the 500 d filtration. Some of the normal urines, even after the 500 d filtration, still had a degree of inhibitory potential. This inhibitory potential may be related to the inorganic compounds which were found in the urines. The inhibitory activity of macromolecules with MW above 10,000 d and below 500 Δ was negligible in 7 stone formers (SF) urines. The relative contribution of 500–1,000 d macromolecules is the highest both in SF and normal urines. *Conclusions:* 1) inhibitors in human urine are of wide range in MW; 2) stone formers and normals differ in the level of inhibitor activity at all MW ranges; especially in above 10,000 d and below inhibitors.

Key words: Calcium stone disease, Urinary inhibitors, Ultrafiltration.

Introduction

Modern concepts of urinary calculous disease include the role of matrix and deficiency of urinary inhibitors which play a key role in stone formation. It is recognized that the most effective inhibitors of calcium (CaOx) crystallization are organic compounds of the types similar to those known to compose the matrix of urinary stones. One of the promising avenues of research was to separate the inhibitors and potential promotors from normal healthy urines [1–7]. These compounds have been found promote either to [1–3] or to inhibit [4–7] CaOx precipitation. The identification of these substances is difficult because of the minute concentrations involved and because of the chemical complexity of urine. In addition many conflicting definitions of inhibitory activity have been suggested.

Isolation, purification and proper identification of organic constituents present in urine, are not easy tasks. The variety of the separation techniques is great and the influence of urinary macromolecules on calculogenesis remains controversial [8]. Moreover some of the fractions isolated from urine may be damaged in preparation [9, 10]. Molecules with molecular weight (MW) in the range of 50,000–300,000 daltons (d) were reported to show an effect on CaOx crystallization [11] that significantly differs from the effects observed at lower MW of 10,000–25,000 d. Molecules with less than 35,000 d have been classified as having "low" MW [12], but those in the range of 10,000–25,000 d are called "high" MW molecules by others [13], adding to the difficulty of interpreting the results. Several organic molecules have been found to be inhibitors of CaOx crystallization. Among these substances there are acidic polypeptides [6], macromolecules [7, 14] and glycosaminoglycans (GAGS) [14], which are recognized as the main urinary inhibitors [15]. Other authors reported the effect of small organic macromolecules, such as amino acids, on the crystallization of CaOx [16, 17].

In most of the above studies the accepted approach was to isolate the fractions from healthy normal urine and to compare their inhibitory potential to that of similar fractions separated from stone formers (SF) urine. However, it is not known which of the various inhibitors, are produced in decreased amounts in SF urines [18].

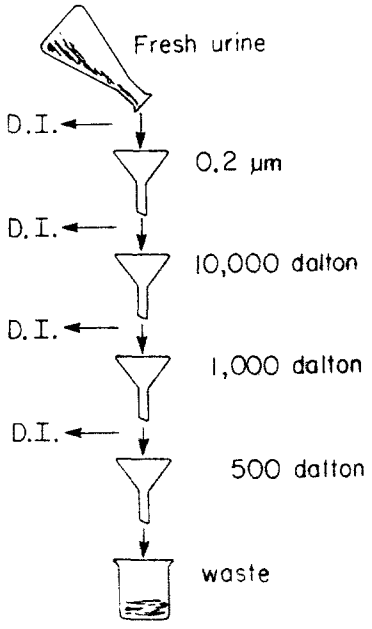

Fig. 1. A scheme of successive ultrafiltrations of fresh human urine

The present study used a different approach. We examined the residual inhibitory power in the urine after it was passed through different pore sizes of ultrafiltration membranes. Applying the method of Sarig et al. [19] we could measure the overall retarding potential of the residual urine toward CaOx precipitation and could define qualitatively the effect of inhibitory fractions filtered out from the urines.

Methods and Materials

a. First voided urine specimens were collected from a group of 20 healthy volunteers (7 females), all of them were on unrestricted diets. Their age ranged between 24 and 52 years and they had no history of stone episodes. All the urine specimens used were checked to be free of urinary infection and only clear urine samples were chosen for this experiment. The individual samples were filtered through 0.2 μ filter to remove cells fragments, debris or other solid materials. Afterwards, each sample was ultrafiltrated through Diaflo membranes (Amicon Co.) with MW cut-offs at 10,000 d, 1,000 d and 500 d. The filtration was performed at 5 °C and under 25 PSI nitrogen pressure.

At the termination of each filtration the overall inhibitory potential in the filtrates was determined using the Discrimination Index (DI) according to Sarig et al. [19]. Tested urine was equilibrated at 37 °C and 20 ml sample was mixed with approximately 75 ml of D.D. water. At this stage the calcium ion concentration was adjusted to 4×10^{-3} M (in 100 ml volume). This solution was mixed with 100 ml of 6×10^{-3} M solution of sodium oxalate. The calcium ion concentration was continuously measured with a specific calcium electrode. The first reading was taken at 30 s after mixing to allow for the specified 20 s response time of the electrode. The next reading was taken after 10 min. The logarithm of the ratio of calcium ion concentration after 30 s and 10 min was termed the Discrimination Index (DI). The DI expressed quantitatively the potential of the urine to inhibit calcium oxalate precipitation. The lower the DI value, the greater is the potential. The scheme of the experimental procedure is shown in Fig. 1.

b. The residues left on the membrane's surface were analyzed qualitatively for phosphates, sulphates, sugars and amino acids.

c. Statistical analysis. The means, variances and covariances of the DI values measured consecutively after each filtration, between SF and normals' urines were estimated by the maximum likelihood estimates for a multivariant Gaussian distribution [20]. The parameters of the normal group (where the DI value after the 500 d filtration were not measured for 3 subjects) were estimated through the missing information algorithm [21].

The changes of DI are functions of pore sizes expressed either in the usual dalton cut-offs or in a continuous mμ scale. The scales were correlated using molecular models in a non-stretched, close to spherical configurations.

The differences in the effect of consecutive removal of each macromolecular fraction among the studied populations as measured by the corresponding change in the DI value, were examined by Tukey-Kramer simultaneous conservative test procedure for pairwise comparison in one way analysis of variance [22]. Simultaneous tests were applied.

The rate at which the DI value changes, in each of the studied population, when analyzed as a function of the decrease in filtering membranes' log pore sizes, was estimated by the slope of the five-point curve of the DI mean values. The variances of the constructed estimates were derived from the estimated variance-covariance matrices of the DI values. The differences, in each of the studied populations, with respect to the rates of the change in the DI value after consecutive removal of inhibitory substances, were examined graphically and analytically. The analysis was performed through a t-test whose α-level critical values were derived from the distribution of Hotelling T^2 statistic [20] for testing the hypothesis of no difference in the five means of DI values. Such a conservative test was necessary since no a priori hypothesis with respect to possible difference in the rate was examined. The differences in the rates in which the DI values changes, between the studied populations, for each filtering membranes' pore size, were examined by the Welch approximate F-test [23]. Differences between any two populations were examined by Tamhane-Welch approximate t-test for pairwise multiple comparison in the unequal variance case [24].

Results

In Fig. 2 the initial DI values of 17 non-stone forming urines and the upward shifts effected by consecutive filtration are shown as functions of log mμ. The general trend reflected in Fig. 2 is that of an increase of the DI values i.e. a decrease in the inhibitory power of the urines, as the pore size decreases. The sharpest inflection is evident between the 1,000 and 500 d filtrations.

The spread of the initial DI values measured by the coefficient of variation (standard deviation/mean) is considerable (1.16). Figure 2 indicates the existence ot two groups among the healthy controls. A subdivision of the non stone forming population into a group of healthy individuals and an "in-between" group was observed in a full scale statistical survey and was commented upon [19]. This population may contain either potential stone formers stones, or people who may form stones only in unfavorable conditions. In this study the mean DI value of 0.41 among the non stone

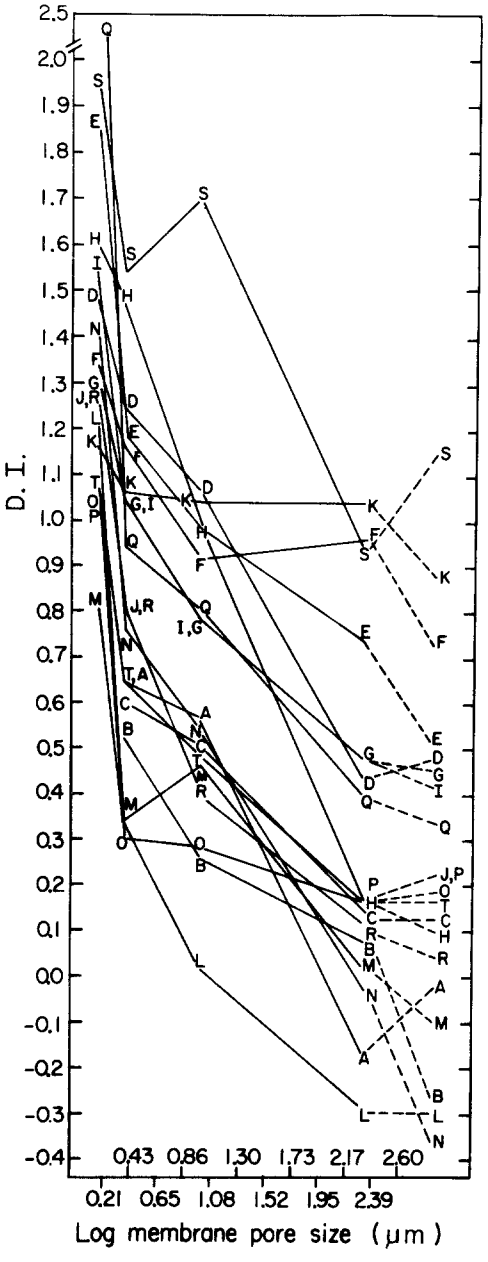

Fig. 2. The change of DI values in 17 healthy urines plotted against the log of pore size of the ultrafiltration membranes

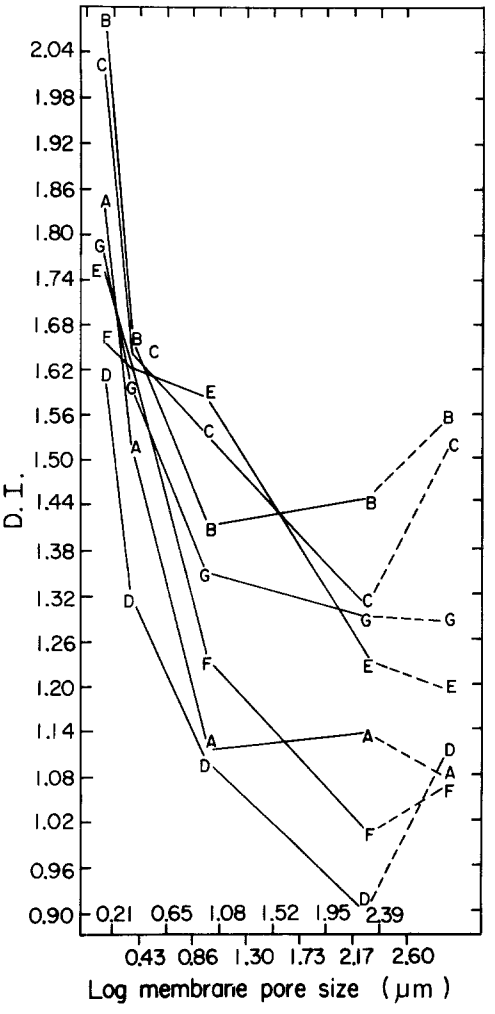

Fig. 3. The change of DI values in 7 stone formers plotted against the log pore size of the ultrafiltration membranes

formers [19] was taken as the cut-off point between the two sub-groups.

Figure 3 shows the DI values of 7 stone formers as a function of the log pore size. The ordinate scale of DI values is more expanded than in Fig. 2 to accomodate the closely packed curves. The overall trend is very similar to that found in the population of normals, though the actual modification values are smaller.

Higher standard deviations at the termination of each filtration were found among the non stone formers (Table 1) compared to those among the stone formers. Since the mean of the DI values at the termination of each filtration (Table 1) was higher among the stone formers, the differences in diversity were more pronounced when expressed in units of the coefficient of variation (Table 1).

The differences in the effects of the removal of macromolecules with respect to the three population groups and with respect to the molecular fractions inside each group, were statistically analyzed. Fig. 4 shows the bar diagram of the average change of DI effected by the four filtrations according to the normal pore size divided into the response of the stone-formers, the in-between group and the healthy normals.

The steepest change was caused by the removal of the 500–1,000 d fraction, while the 1,000–10,000 d causes a comparatively small change. Though the effect of the 0.2 mμ filtration was almost negligible, the negative changes in DI value in the stone formers group may point to the possible removal of colloidal promoters of nucleation.

The removal of macromolecules of a size smaller than 0.2 μ from the urines was found to cause a significant reduction in the mean inhibitory power (in DI values) among the "healthy" group and SF population (two tailed P value for each filtration < 0.01). In the urines of the "in-between"

Table 1. Mean standard deviation (SD) and coefficient variation (CV) of the DI value at the termination of each filtration among stone formers and non-stone formers

Group	Filtering Size														
	1.5 mμ			2.5 mμ			10 mμ			200 mμ			300 mμ		
	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
Stone formers	1.82	0.17	0.09	1.57	0.12	0.07	1.33	0.19	0.14	1.19	0.19	0.16	1.26	0.20	0.16
Non-stone formers	1.39	0.40	0.28	0.85	0.36	0.42	0.69	0.37	0.54	0.32	0.37	1.16	0.25	0.39	1.52

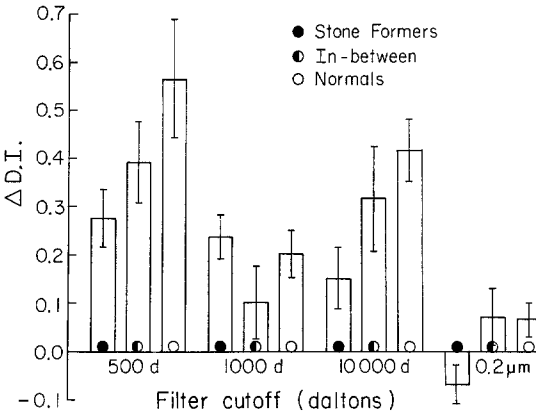

Fig. 4. The bar-histogram of changes in inhibitory potential (ΔDI) of stone formers' and normals' urines effected by ultrafiltration

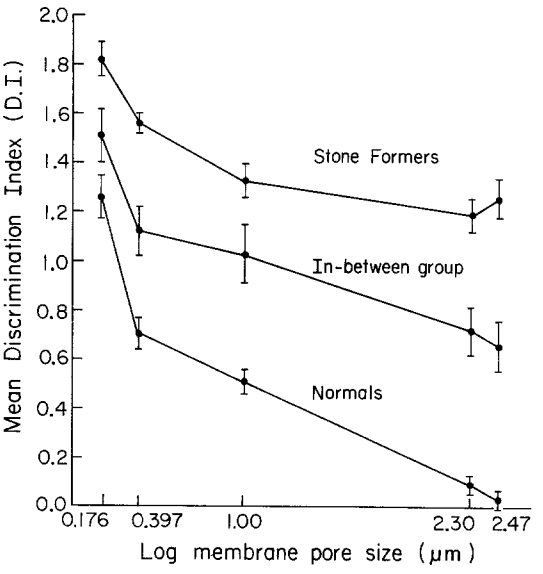

Fig. 5. The mean inhibitory potential in stone formers' and normals' urines, expressed in DI values, as a function of log membrane pore size, after successive ultrafiltrations

group the mean of the inhibitory power (in DI values) was reduced significantly only after the removal of macromolecules in the range sizes of 10,000 d up to 0.2 μ and 500 d up to 1,000 d (two tailed P value after each filtration < 0.01).

Removal of macromolecules in the range size of 10,000 d up to 0.2 μ from urine of a healthy group reduced the inhibitory power (in DI values) more significantly (the corresponding P value for pairwise multiple comparison test = 0.05) than the removal of the macromolecules with the same MW from the urines of SF population. The differences in the DI values after the removal of macromolecules in the range of 500–1,000 d between the "healthy" group and the SF population did not reach statistical significance (the corresponding P value for pairwise multiple comparison test = 0.13).

None of the other observed differences (Fig. 4) between the "healthy" group and SF population and between either of these two groups and the "in-between" groups was found to be significant (the corresponding P value for pairwise multiple comparison test $\geqslant 0.24$).

When the estimated mean values of DI computed on the basis of all subjects through the missing information algorithm [21] were plotted against the continuous log pore size (r), the three curves (Fig. 5) show the differences between the groups relevant to statistical analysis. The curves are close to linear for the stone formers and the in-between group, and linear for healthy normals in the range of $2.5\ m\mu \leqslant r \leqslant 300\ m\mu$. The equations of the lines can be expressed by

$$DI = a + b \log (r)$$

The rate of change of DI with respect to pore size (r) in group ϕ, is given by

$$\left[\frac{d(DI)}{d(r)}\right]_{\phi} = \frac{b_1^{\phi}}{r} \text{ for } 1.5\ m\mu \leqslant r \leqslant 2.5\ m\mu \quad \text{(I)}$$

$$\frac{b_2^{\phi}}{r} \text{ for } 2.5\ m\mu \leqslant r < 300\ m\mu \quad \text{(II)}$$

The values of b_1 and b_2 and the corresponding standard errors are listed in Table 2. The results indicate that the rate

Table 2. Estimates of the rate in which the DI value changes as a function of the log of the filtering membranes' pore sizes and their standard errors

Group	Filtering Size Range			
	1.5–2.5 mμ		2.5–300 mμ	
	Estimate (b_1)	Standard error	Estimate (b_2)	Standard error
Stone formers	1.145	0.233	0.196	0.023
In-betweens	1.351	0.386	0.208	0.055
Normals	2.697	0.382	0.320	0.032

of change in the log (1.5 μm) to (2.5 μm) interval in all the three populations are higher than the corresponding rate of change in the log (2.5 μm) to log (300 μm) interval. Due to the small sizes of the SF and the "in-between" groups, the observed difference reached statistical significance only among the healthy population (a two tailed P value for T^2 test = 0.008).

When the rate of change in the DI value with respect to the filtering membranes pore size were compared among the three populations (Table 1), significant (P value for Welch test $\leqslant 0.05$) higher rates were observed in the "healthy" group as compared to the stone formers and the "in-between" group. The difference was statistically significant (the corresponding P value for pairwise multiple test $\leqslant 0.001$) when the "healthy" group was compared to the SF group. No significant difference (comparison test $\leqslant 0.005$) was observed between stone formers and the "in-between" group.

The qualitative analysis of the compounds which have been filtered out from the urines showed that the compounds retained by the 10,000 d membrane contained sugars, sulphates and phosphates. The compounds retained by the 500 d membrane consisted mainly of amino groups.

Discussion

This study reinforces the evidence that urine contains a variety of organic macromolecular and inorganic compounds which are involved in the CaOx crystallization process.

Significant differences were reported with respect to the content of macromolecules in urine of SF as compared to that in urne of normal persons. The results indicate that these macromolecules vary with regard to their MW distribution, total quantity and composition [10, 12, 25, 26]. The effect of these urinary substances reported in the literature vary between inhibition to enhancement of CaOx crystallization [1–7]. In this study assessment of the effect on CaOx precipitation, that may be built up after the removal of urinary macromolecules from the urine, was attempted. This aim was attained using the DI test [19] to measure the relative contribution of the various urinary macromolecules to the inhibitory potential of the urine. This information should be supplementary to that obtained by the analysis of the solid fractions retained on the filters. As the identification of the inhibitors in the macromolecular fractions is not an easy task, and the amounts are extremely small, analyses are, as a rule, performed on pooled urine [9, 10]. The simplicity of the DI test allowed us to carry out individual evaluations to be combined in statistical analysis.

The diversity in the urinary macromolecules (presence and activity) among the members of SF population is less pronounced than in the non SF population (Figs. 2, 3 and Table 1). It could indicate that individuals differ with respect to the content, concentration, and probably the nature of their urinary inhibitors to CaOx precipitation.

Moreover, there is a certain degree of inhibition in the urine of CaOx SF as it can be seen in Fig. 3. Thus, the filtration process caused a reduction in the inhibitory potential existing in SF urine. The comparison between Fig. 2 and Fig. 3 and the supplementary information of Table 1, could indicate that SF urines contain inhibitors in insufficient quantity or activity. This finding is in agreement with the conclusions of others [27].

A non continuous gradation of MW with at least two distinct groups of macromolecular inhibitors exist in the urine of non SF population (Table 2 and Fig. 4). The first group of inhibitors has MW above 10,000 d while the second group has MW in the range of 500–1,000 d. Our results suggest that while the inhibitors of MW above 10,000 d are absent or inactivated in the urine of SF, inhibitors of 500–1,000 d are nevertheless present in their urine. Considerable individual differences in the types and species of urinary inhibitors were also reported by Fleich [18].

In the majority of the filtrated urines the DI values did not change markedly after the filtration through a 1,000 d membrane (Figs. 2 and 3). In a few urines, the first significant change in the DI value was observed, following filtration through the 1,000 d membrane. This suggests the presence of a third kind of organic macromolecular inhibitor, characterized by a MW of 1,000–10,000 d, as previously reported by Nicar et al. [28].

From the present results a reasonable explanation for the existence of the "in-between" population, as suggested elsewhere [19], can be deduced. The "in-between" population differ from the "purely healthy" in one kind of inhibitor. The 1,000 d–10,000 d fraction is absent or inactivated in the "in-between" group but the above 10,000 d and 1,000 to 500 d fractions are present and active in an amount sufficient to prevent stone formation.

The present study focused on the relative contribution of the macromolecules to the inhibitory power of the urine. It is generally accepted that evaluation of the relative contribution of the different known inhibitors is very difficult. It appeared to numerous authors that the known inhibitors represent only a small part of the total inhibitory activity [29]. In fact a large part of the activity of unknown nature was ascribed to macromolecules [7, 30]. The DI test enabled us to show the relative importance of the macromolecular

inhibitors of MW 500–1,000 d as compared to other macromolecular inhibitors present in urine. The removal of the 500–1,000 d inhibitory fraction from the urine caused the steepest decrease in the inhibitory power (Table 1 and Fig.s. 4, 5). The residual inhibitory power after the 500 d filtration in the healthy group was significantly higher (one tailed P value < 0.001) than the residual inhibitory power in SF urines. According to the DI scale [19] there is negligible inhibitory power left in SF urine after the 500 d filtration. It is striking that the mean of DI values after the 500 d filtration among the non SF population coincides with the mean of the DI values determined in 84 SF urine samples (1.26:1.25) [31]. This could suggest that the non stone former population have inhibitors of MW or molecular size less than 500 d in their urine. These inhibitors are most probably inorganic ions and they may be absent or inactivated in SF urine. It is known that several small size molecules have been found to be active in preventing CaOx precipitation. Pyrophosphate, citrate and magnesium [32] are reported to be quite effective. Various other metals may also act as inhibitors but it was uncertain whether the effects were strong enough to play an important role in urine [33]. It was argued by several investigators that there is almost no difference in the inhibitory content between SF and normal urines. The present results suggest that the difference could be ascribed to the activity of these inhibitors rather than to their presence. The activity of 500–1,000 d fractions is evident in SF and normal urine but SF have almost no activity of the above 10,000 d fraction of organic macromolecules and almost no inorganic inhibitory activity.

Our quantitative analysis shows that the above 10,000 d fractions contain sugars, sulphates and phosphates. These compounds could indicate the presence of GAGS in this fraction as also reported by others [9, 34]. GAGS are thought to be inhibitors of aggregation [35]. The dominance of amino groups in the 500–1,000 d fraction content may indicate the presence of small peptides [12, 36] or amino acids [16, 17]. The importance of these compounds in the nucleation and crystal growth processes was reported previously [37].

The present results are in agreement with the hypothesis which suggests several kinds of inhibitors in urine [18]. According to a recent report the combined effects diverse substances acting on different parts of stone forming process may be synergistic [38]. Inactivation of at least two inhibitory species in SF urine could weaken this effect. Though our results do not prove the existence of synergism, they may indicate the validity of this concept.

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