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ANALYSIS OF GLYCOSAMINOGLYCANS INDUCED IN NEWLY FORMED CALCIUM OXALATE CRYSTALS USING AN UNDILUTED URINE SYSTEM

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Abstract

The aim of this study was to examine the effect of sodium pentosan polysulfate (SPP) in an undiluted urine system and to study its relative affinity to calcium oxalate (CaOx) crystals in the presence or absence of heparan sulfate (HS). CaOx crystals were induced with an overload of oxalate above the metastable limit in spun and filtered urine (SF) and ultrafiltered urine (UF). Then, the crystals were dissolved with EDTA (ethylenediaminetetraacetic acid), electrodialysed and lyophilized. The polyanions, HS or SPP were added to the UF prior to the addition of oxalate. Polyanions in crystal matrices were examined by cellulose acetate electrophoresis. Crystal volume and size were suppressed according to the increase of the concentration of SPP when compared with those of the UF. Scanning electron microscopy (SEM) showed marked aggregation of the crystals in the UF and no aggregation in the presence of SPP. HS was the only polyanion found in CaOx crystals formed after overload of oxalate in SF. Crystals formed in UF did not contain any polyanions. When SPP was added to UF, SPP appeared in the crystal matrix in accordance with its concentration. Once HS in physiological concentration was added to the UF containing SPP, HS and SPP obtained from crystals were strongly stained with Alcian blue in electrophoretic study, where SPP is stained stronger than HS. These results suggest that SPP strongly binds to CaOx crystals as well as HS and that HS and SPP competitively bind to the crystal, then, as a result, they are incorporated into the crystals. The fact that SPP suppressed the aggregation of CaOx crystals in undiluted urine showed the possibility that SPP might be one of the useful drugs for preventing CaOx urolithiasis.

Key Words: Urinary glycosaminoglycans, sodium pentosan polysulfate, heparan sulfate, calcium oxalate crystals, crystal aggregation, undiluted urine system.

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Introduction

Though a number of studies have clarified that some macromolecules are inhibitors of calcium oxalate (CaOx) crystallization, the role of macromolecules in the formation of CaOx stones remains unclear [15]. It is well known that glycosaminoglycans (GAGs) as well as proteins are present in the matrices of urinary stones [2, 12, 20, 38]. In normal urine, chondroitin sulfate (ChS) is the most abundant GAG at 60% and HS accounts for 15% [9]. The pattern of GAGs in stones differs from that in urine. Nishio *et al.* [23] reported that hyaluronic acid (HA) and heparan sulfate (HS) were extracted from the matrices of CaOx stones. Roberts and Resnick [29] reported that ChS was usually absent from urinary stone matrix and the two of GAGs in stones were HS and HA. Iwata reported the presence of HS in the matrix of uric acid crystals [14].

At present there are few reports about GAGs in the matrix of CaOx crystals. Yamaguchi *et al.* [46] reported that HS was the major GAG in CaOx crystal matrix. We found that HS was the only GAG in the newly formed CaOx crystals in spun and filtered urine (SF) and showed strong inhibition against crystal aggregation in undiluted urine. Moreover, HS was shown to be selectively included into crystals due to its relative binding affinity [44]. Sodium pentosan polysulfate (SPP) is a semi-synthetic polyanion and reported to be a strong inhibitor of CaOx crystallization [4, 6, 17, 19, 24, 25, 26, 41]. The role of SPP in an undiluted urine system as well as in crystal matrices has not been reported in the literature. In this paper, we report on the crystal size distribution and the analysis of the polyanions in the matrix of newly formed CaOx crystals in the presence of SPP. We also examined the interaction of HS and SPP using an ultrafiltered urine system, and evaluated it by electrophoresis and scanning electron microscopy (SEM).

Materials and Methods

Materials

All reagents were of analytical grade. SPP, heparan sulfate and Alcian blue 8 GX were obtained from Sigma

Chemical Co. (Tokyo, Japan). Cellulose acetate strips (CA-25010, 25 x 160 mm) were obtained from Schleicher and Schell (Dassel, Germany).

Collection and preparation of urine samples

Twenty-four hour urine specimens were collected from 5 healthy men aged 30-43 years old, then pooled. None showed any sign of blood by chemical test. Urine specimens were centrifuged at 6,000 X *g* for 60 minutes (RS-206, TOMY, Tokyo, Japan) and filtered through 8 μm followed by 0.22 μm Millipore GV filters (Millipore Corp., Bedford, MA, USA), being used as SF (spun and filtered urine), then ultrafiltered using hollow fiber bundle (AIP-1010, Asahikasei KK, Tokyo, Japan) with a nominal molecular cut-off of 10 kDa (UF; ultrafiltered urine). Urine samples were refrigerated until needed and were warmed to 37°C and refiltered (0.22 μm) just prior to use. Each 100 ml urine was prepared for further experiment.

Crystal preparation

The method of inducing CaOx crystallization in undiluted urine has been described elsewhere [5, 34, 35]. Briefly, the minimum amount of oxalate required to produce crystals in 200 μl of urine by adding 2 μl of graded concentration series of sodium oxalate was used to determine the metastable limit by microplate and inverted microscopy [42]. Once the metastable limit had been measured, an amount of oxalate, 0.3 mmol/l (final concentration) in excess of this limit, was added to urine specimens drop-wisely. A Coulter counter (Model TA II, fitted with a NEC personal computer, aperture size; 100 μm; Coulter Co., Tokyo, Japan) was used to monitor the crystal particle size every ten minutes during a 90 minute incubation period in a 37°C shaking water bath. After 90 minute incubation, samples for SEM were prepared. The same amount of oxalate was added after 90 minutes and 120 minutes to increase the yield of CaOx crystals.

Into UF specimens, 0, 1, 2 and 10 μg/ml of SPP and/or HS were added just prior to use.

Isolation of crystal matrix

The crystals were washed on a 0.22 μm Millipore filter, 6 times with 0.1 M sodium hydroxide, and then with deionized and double distilled (d.d.) water to wash out surface substances completely, lyophilized, weighed and demineralized by the addition of 100 ml of 0.25 M EDTA (ethylenediaminetetraacetic acid; pH 8.0) to every 1 gram of crystals at 4°C.

The solution of demineralized crystal extracts was electrodialysed in the dialysis tube (Sigma, 10 kDa cut off) against 25 mM Tris, 192 mM glycine buffer (pH 8.3) with 4 changes of the buffer at 80 V initially, with a gradual increase to 120 V, then against d.d. water overnight at 4°C (3 times), and lyophilized. Crystal matrix extract was dissolved in d.d. water and desalted with an Econo-Pac 10DG column (6 kDa cut off; BIO-RAD Co., Tokyo, Japan) and lyophilized. All the steps were repeated 6 times. Only mean values of results are depicted.

Electrophoresis

Two μl of dissolved samples (20 μg/20 μl of distilled water) were applied to cellulose acetate strips and electrophoresed at 80 V for 45 minutes. 0.2 M calcium acetate (pH 7.2) was used as a buffer solution [16, 45]. As a reference, HS and SPP (each in 1 μg/μl) were run simultaneously in wide mini sub cell (BIO-RAD). Cellulose acetate strips were stained in 0.2 per cent Alcian blue 8 GX solution containing 0.05 M magnesium chloride, 0.025 M sodium acetate, 50% ethanol and 50% deionized water for 15 minutes and destained in same solution without Alcian blue for 120 minutes with 6 changes of destaining solution. A laser densitometer (Ultroscan XL, Pharmacia Biotech, Tokyo, Japan) was used to determine the relative content of each GAG. This experiment was repeated 6 times. In the case of protein rich urine, papain digestion was performed. Heparitinase was used to digest HS.

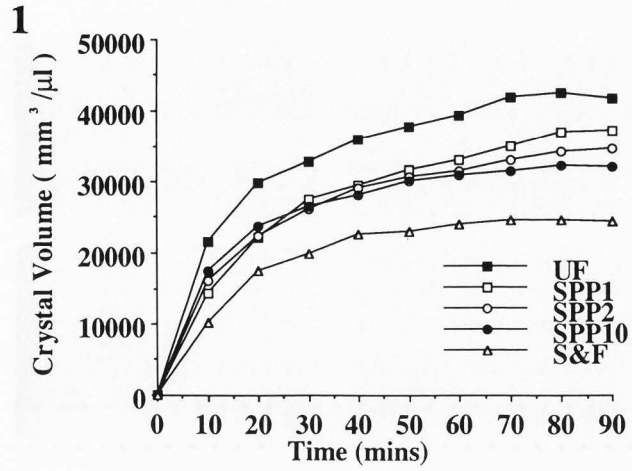
Scanning electron microscopic (SEM) study

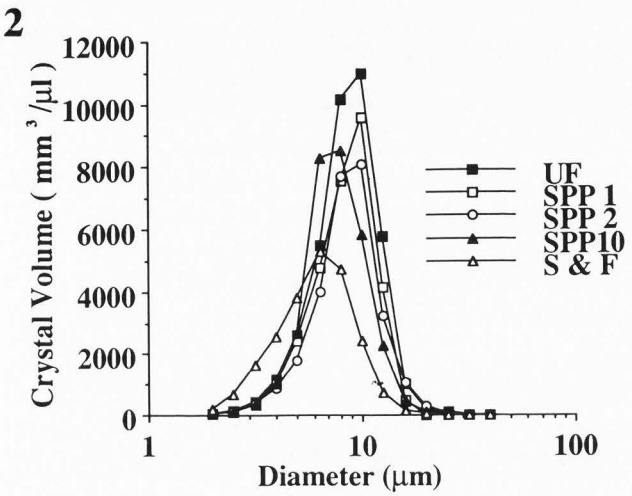
Urine samples for SEM were prepared at 90 minutes incubation. Each 500 μl urine was filtered on the 0.22 μm (10 mm) Millipore filter. Crystals on the filter were dried in a desiccator at room temperature, then mounted on stub and coated for SEM. The stubs were examined with a JEOL-JSM 840 (JEOL, Tokyo, Japan) SEM operated at an accelerating voltage of 15 kV, a probe current of 6 x 10^{-9} A, and a working distance of 15 mm.

Results

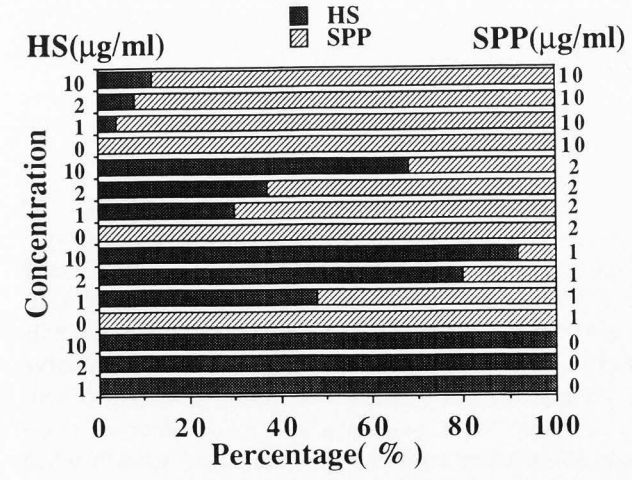
SF and UF as well as UF that contained HS(UF+HS) or UF that contained SPP(UF+SPP) showed the same metastable limit. The volume of additional oxalate required to form crystals was same in each solution. Crystal volume of UF was significantly increased in accordance with time (Fig. 1). UF+SPP showed a slightly lower volume after 10 minutes compared with UF. SF urine showed a significantly lower volume compared with UF or UF+SPP. Figure 2 shows crystal size-volume distribution at 90 minutes incubation. In UF, crystal size and volume were larger than in UF+SPP and SF.

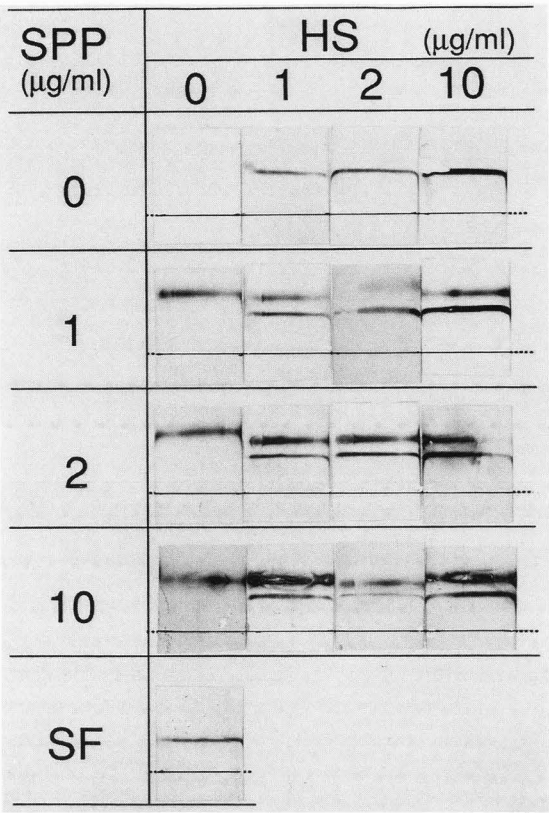
The HS (1 μg/μl) standard clearly identified on cellulose acetate strip was completely digested and disappeared from the strip by the use of heparitinase. Crystals derived from each 100 ml of SF contained HS only. This band showed the same migration distance





Figures 1 and 2. Crystal volume according to time course measured by Coulter counter (**Fig. 1**). Crystal size distribution at 90 minutes after load of oxalate measured by Coulter counter (**Fig. 2**). UF: ultrafiltered urine; SPP1, 2, 10: UF containing SPP at final concentrations of 1, 2, and 10 μg/ml, respectively; S&F: spun and filtered urine.





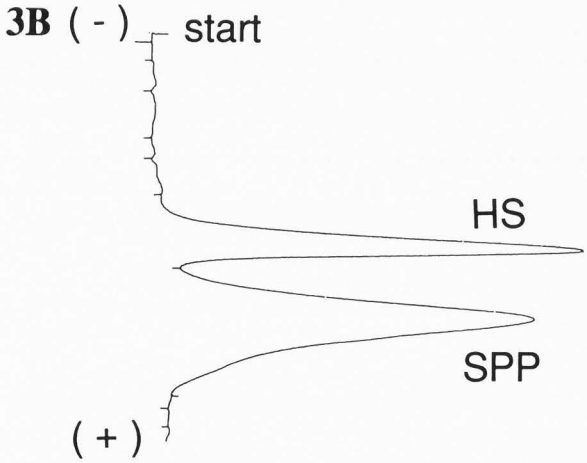


Figure 3. Alcian Blue stained cellulose acetate strips of SPP and HS included in crystal matrix at each concentration (μg/ml) of applied HA and/or SPP (**Fig. 3A**). HS: heparan sulfate; SPP: sodium pentosan polysulfate. Densitometric analysis of Alcian-blue stained electrophoretic profile of HS and SPP at the concentration of 1 μg/μl as standards (**Fig. 3B**).

Figure 4 (at left). Densitometric analysis of Alcian-blue stained electrophoretic profile of SPP and HS after destained cellulose acetate strip. Relative percentage of HS (left) and SPP (right) included in crystal matrix at each concentration (μg/ml) of applied HS and/or SPP.

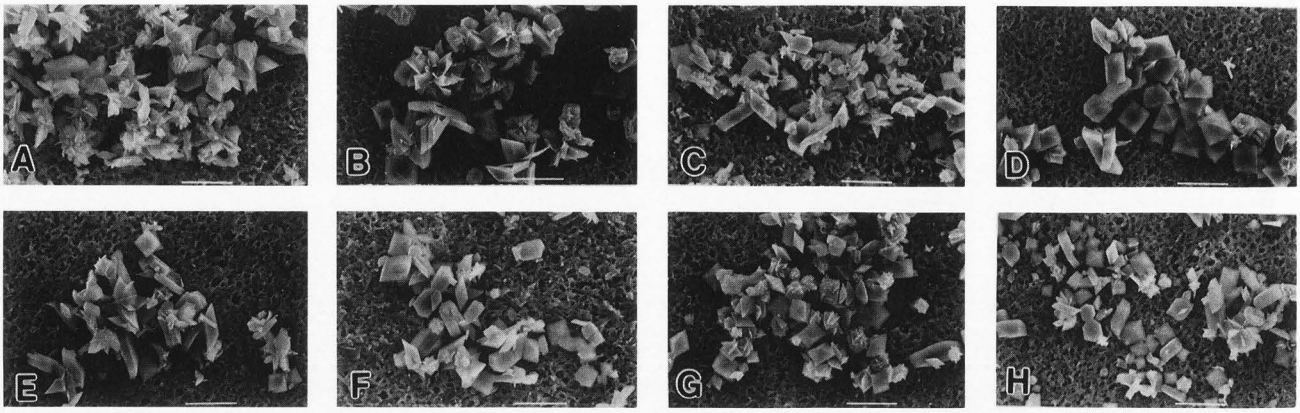


Figure 5. Scanning electron micrographs of calcium oxalate crystals formed in various concentrations of SPP (0, 1, 2, and 10 μg/ml) and HS (0, 1 and 2 μg/ml) added to ultrafiltered urine (UF). SF: spun and filtered urine. (**A**) SPP 0 / HS 0 (UF); (**B**) SPP 1 / HS 0; (**C**) SPP 2 / HS 0; (**D**) SPP 10 / HS 0; (**E**) SPP 1 / HS 2; (**F**) SPP 2 / HS 2; (**G**) SPP 10 / HS 2; and (**H**) SF.

when compared with the migration of standard and disappeared after heparitinase digestion and, though not shown in Figure 2, still remained after protein digestion using papain. In crystals derived from UF, no visible GAG bands were seen. These findings were same as previous report [44]. When SPP was added into UF in final concentration of 1 μg/ml, a single GAG band was observed. This band was considered to be the same as SPP from the migration distance of standard.

In order to determine whether there might be a relationship between SPP and HS in forming the crystals, another experiment was performed. Various concentrations (0, 1, 2, 10 μg/ml) of SPP and HS were added to UF, then crystals were prepared in the same manner. Table 1 shows each concentration of HS and SPP added to UF. Figure 3A shows the results of electrophoresis of GAGs from crystals. HS was found in crystals using UF+HS in concentration of 1, 2, 10 μg/ml, as well as SF. SPP from the samples of each concentration of 1, 2, 10 μg/ml was clearly stained (Fig. 3A). GAG bands obtained from crystal matrix were examined by the use of densitometer. The densitometric result of electrophoresis when the mixture of HS and SPP at 1 μg/ml were added to UF is demonstrated in Figure 3B. Densitometry of 1 μg/ml of HS and SPP showed that the average ratio of HS to SPP was 48% to 52%. The results of relative percentage of HS and SPP contained in crystals are shown in Figure 4. At similar concentrations of SPP, the relative content of HS increased in accordance with its concentration. When the concentration of HS and SPP was the same at 2 and 10 μg/ml, it is apparent that the amount of HS incorporated into the crystals decreased in relation to increasing concentrations of SPP. This effect is most noticeable at the concentration of 10 μg/ml.

Table 1. Concentration of HS and SPP added to ultrafiltered urine.

	HS (μg/ml)			
	0	1	2	10
SPP	1	1/1	1/2	1/10
(μg/ml)	2	2/1	2/2	2/10
	10	10/1	10/2	10/10

Scanning electron micrographs were obtained at each experiment (Fig. 5). In all samples, CaOx dihydrate crystals were precipitated. Interesting results were obtained from SEM study. In UF, CaOx dihydrate crystals were formed and most of them were aggregated, which was confirmed in crystal size analysis shown in Figure 2. With increasing concentration of SPP, the crystals were shown not to be aggregated. HS showed almost the same results as those of SPP. Addition of HS at the concentration of 2 μg/ml to UF+SPP showed minimal aggregation which is thought to be almost same as SF. In SF, most of the crystals were single and somewhat aggregated.

Discussion

An undiluted urine seems to be the best system to observe the affinity of macromolecules for CaOx crystals that might have inhibitory activity. Though many macromolecular substances have been reported to efficiently inhibit growth or aggregation of CaOx crystals [35], undiluted urine may help to determine their relative inhibitory power. Though chondroitin sulfate (ChS), which is the most abundant GAG in normal urine, is reported to inhibit crystal aggregation and growth when

used in seeded crystal system, it showed little or no inhibition against CaOx crystals in an undiluted urine system [36]. A previous study [44] clearly demonstrated that HS was selectively incorporated into CaOx crystals when using the undiluted urine system. ChS was included into crystals only when HS was added at 0 or 1 μg/ml.

In the normal concentration of urinary glycosaminoglycans, HS is a stronger inhibitor than ChS. From the present study, HS and SPP bind the growing crystals competitively. SPP is stronger than HS when these substances are used at the same concentration. Fellström *et al.* [7] reported almost the same results. They measured the relative binding affinities of GAGs to CaOx crystals in organic media and reported that the inhibitory activity of GAGs is largely a function of their charge density, SPP > heparin > ChS. On the other hand, Angel and Resnick [1] reported the binding affinity of CaOx crystals with various GAGs and obtained different results; heparin > hyaluronic acid > ChS > SPP, though the analytical method was different.

How GAGs are involved or incorporated into CaOx crystals is unknown. Scurr and Robertson [39] reported that heparin, ChS and RNA became negatively charged in accordance with increasing concentration and this resulted in the increase of negative zeta potential, causing a repulsion between crystals and inhibition of aggregation. More precise characterization of GAGs, such as, charge or sulfation is needed to clarify the genesis of crystal formation.

The studies of Robertson *et al.* [30, 31] suggest that a reduction in inhibitory activity could be related to a decrease of GAGs in about half of the cases of stone formation. The difference of GAG excretion in urine among the normal subjects and stone patients, has been confirmed by subsequent investigations [3, 10, 13, 18, 21, 22, 32, 33, 37], though there was one report that no difference existed between healthy persons and CaOx stone patients [11]. Recently, Shum *et al.* [40] reported that urinary HS of normal control subjects showed higher inhibitory activity than that of stone formers.

SPP is reportedly a powerful inhibitor of CaOx crystal aggregation and growth in seed crystal system [4, 6, 17, 19, 24, 25, 26, 41]. SPP is an anticoagulant now used for treating interstitial cystitis [27]. Moreover, SPP has the possibility of preventing stone formation. In this report, we showed that SPP exists in crystals and that SPP has an inhibitory role on growth and aggregation of CaOx crystals in undiluted urine. It is thus reasonable to assume that SPP might inhibit or prevent the CaOx crystal formation, growth and aggregation *in vivo*. HS and other macromolecules in urine could not be expected to increase from an oral load because of their molecular weights. SPP is reportedly excreted in the urine at about 3% of the oral dose [28]. Since the usual dose is 300 to 400 mg/day, the calculated excretion rate rises above 10 mg/l, that is enough to inhibit crystal aggregation.

About clinical use, Fellström *et al.* [8] reported SPP had a role in the treatment of recurrent stone disease from the results that 48% of the patients became stone free and half of the patients who continued to form stones had smaller ones which were easily passed. Though complete prevention was not obtained, their trial was thought to be valuable and the use of SPP might be beneficial.

There are macromolecules other than HS in crystal matrix [43]. Doyle *et al.* [5] have isolated a 31 kDa crystal matrix protein (CMP) as a powerful inhibitor of crystal aggregation. Further studies are required to clarify the relationship between HS and CMP.

In summary, this study indicates that SPP is a potent inhibitor of CaOx crystal aggregation and growth and that SPP as well as HS are incorporated into crystals in undiluted urine. The resultant relative content of SPP in the crystal matrix was higher than that of HS.

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References

[1] Angel AH, Resnick MI (1989). Surface interaction between glycosaminoglycans and calcium oxalate. J Urol **141**: 1255-1258.

[2] Boevé ER, Cao LC, Verkoelen CF, Romijn JC, de Bruijn WC, Schröder FH (1994). Glycosaminoglycans and other sulfated polysaccharides in calculogenesis of urinary stones. World J Urol **12**: 43-48.

[3] Caudarella R, Stefani F, Rizzoli E, Malavolta N, D'antuona G (1983). Preliminary results of glycosaminoglycan excretion in normal and stone-forming subjects: Relationship with uric acid excretion. J Urol **129**: 665-667.

[4] Danielson BG, Fellström B, Wirkström B (1989). Glycosaminoglycans as inhibitors of renal stone formation. In: Urolithiasis. Walker VR, Sutton RAL, Cameron ECB, Pak CYC, Robertson WG (eds.). Plenum Press, New York. pp. 101-104.

[5] Doyle IR, Ryall RL, Marshall VR (1991). Inclusion of proteins into calcium oxalate crystals precipitated from human urine: A highly selective phenomenon. Clin Chem **37**: 1589-1594.

[6] Fellström B, Danielson BG, Ljinghall S, Wikström B (1985). The inhibition of calcium oxalate

crystal growth by chondroitin sulfates, heparin, pentosan polysulfate and Tamm-Horsfall glycoprotein. In: Urolithiasis and related clinical research. Schwille PO, Smith LH, Robertson WG, Vahlensieck W (eds.) Plenum Press, New York, pp. 887-890.

[7] Fellström B, Lindsjo M, Danielson BG, Karlsson FA, Ljunghall S (1989). Binding of glycosaminoglycan inhibitors to calcium oxalate crystals in relation to ionic strength. Clin Chim Acta **180**: 213-220.

[8] Fellstöm B, Backman U, Danielson BG, Wikström B (1994). Treatment of calcium stone disease with the synthetic glycosaminoglycan pentosan polysulfate. World J Urol **12**: 52-54.

[9] Goldberg JM, Cotlier E (1972). Specific isolation and analysis of mucopolysaccharides (glycosaminoglycans) from human urine. Clin Chim Acta **41**: 19-27.

[10] Grases F, Llompart I, Conte A, Coll R, March JG (1994). Glycosaminoglycans and oxalocalcic urolithiasis. Nephron **68**: 449-453.

[11] Hesse A, Wuzel H, Vahlensieck W (1986). The excretion of glycosaminoglycans in the urine of calcium-oxalate-stone patients and healthy persons. Urol Int **41**: 81-87.

[12] Hesse A, Wuzel H, Vahlensieck W (1991). Significance of glycosaminoglycans for the formation of calcium oxalate stones. Am J Kid Dis **XVII**: 414-419.

[13] Hwang TIS, Preminger GM, Poindexter J, Pak CYC (1988). Urinary glycosaminoglycans in normal subjects and patients with stones. J Urol **139**: 995-997.

[14] Iwata H, Kamei O, Abe Y, Nishio S, Wakatsuki A, Ochi K, Takeuchi M (1988). The organic matrix of urinary uric acid crystals. J Urol **139**: 607-610.

[15] Khan S, Hackett RL (1993). Role of organic matrix in urinary stone formation: An ultrastructural study of crystal matrix interface of calcium oxalate monohydrate stones. J Urol **150**: 239-245.

[16] Kodama C, Kodama T, Yoshizawa Z (1988). Method for analysis of urinary glycosamonoglycans. J Chromatography **429**: 293-313.

[17] Martin X, Werness PG, Bergert JH, Smith LH (1984). Pentosan polysulfate as an inhibitor of calcium oxalate crystal growth. J Urol **132**: 786-788.

[18] Michelacci YM, Glashan RQ, Schor N (1989). Urinary excretion of glycosaminoglycans in normal and stone forming subjects. Kidney Intl **36**: 1022-1028.

[19] Miyazawa K, Suzuki K, Tsugawa R (1989). The quantitative study of inhibitory effect of pentosan polysulfate and chlorophyllin on the experimental calcium oxalate stone. Jpn J Urol **80**: 861-869.

[20] Morse RM, Resnick MI (1988). Urinary stone matrix. J Urol **139**: 602-606.

[21] Nesse A, Garbossa G, Romero MC, Bogado CE, Zanchetta JR (1992). Glycosaminoglycans in urolithiasis. Nephron **62**: 36-39.

[22] Nikkilä MT (1989). Urinary glycosaminoglycan excretion in normal and stone-forming subjects: Significant disturbance in recurrent stone formers. Urol Int **44**: 157-159.

[23] Nishio S, Abe Y, Wakatsuki A, Iwata H, Ochi K, Takeuchi M, Matsumoto A (1985). Matrix glycosaminoglycan in urinary stones. J Urol **134**: 503-505.

[24] Norman RW, Scurr DS, Robertson WG, Peacock M (1984). Inhibition of calcium oxalate crystallization by pentosan polysulfate in control subjects and stone formers. Brit J Urol **56**: 594-598.

[25] Norman RW, Scurr DS, Robertson WG, Peacock M (1984). Sodium pentosan polysulfate as a polyanionic inhibitor of calcium oxalate crystallization *in vitro* and *in vivo*. Clin Sci **68**: 369-371.

[26] Osswald H, Weinheimer G, Schütt I-D, Ernst W (1989). Effective prevention of calcium-oxalate crystal formation in vitro and in vivo by pentosan polysulfate. In: Urolithiasis. Walker VR, Sutton RAL, Cameron ECB, Pak CYC, Robertson WG (eds.). Plenum Press, New York. pp. 141-144.

[27] Parsons CL (1982). Prevention of urinary tract infection by the exogeneous glycosaminoglycan sodium pentosan polysulfate. J Urol **172**: 167-169.

[28] Parsons CL, Schmitt JD, Pollen JJ (1983). Successful treatment of interstitial cystitis with sodium pentosanpolysulfate. J Urol **130**: 51-53.

[29] Roberts SD, Resnick MI (1986). Glycosaminoglycans content of stone matrix. J Urol **135**: 1078-1083.

[30] Robertson WG, Peacock M, Marshall RW, Marshall DH, Nordin BEC (1976). Saturation-inhibition index as a measure of the risk of calcium oxalate stone formation in the urinary tract. New Engl J Med **294**: 249-252.

[31] Robertson WG, Peacock M, Heyburn PJ, Marshall DH, Clark PB (1978). Risk factors in calcium stone disease of the urinary tract. Br J Urol **50**: 449-454.

[32] Ryall RL, Marshall VR (1983). The value of the 24-hour urine analysis in the assessment of stone formers attending general hospital out patient clinic. Br J Urol **55**: 1-5.

[33] Ryall RL, Marshall VR (1984). The relationship between urinary inhibitory activity and endogeneous concentrations of glycosaminoglycans and uric acid: comparison of urines from stone-formers and normal subjects. Clin Chim Acta **141**: 197-204.

[34] Ryall RL, Hibberd CM, Marshall VR (1985). A method for studying inhibitory activity in whole urine. Urol Res **13**: 285-289.

[35] Ryall RL, Harnett RM, Hibberd CM, Edyvane KA, Marshall VR (1989). The effect of macromolecules on the crystallization of calcium oxalate in human urine.

In: Urolithiasis. Walker VR, Sutton RAL, Cameron ECB, Pak CYC, Robertson WG (eds.). Plenum Press, New York. pp. 133-135.

[36] Ryall RL, Harnett RM, Hibberd CM, Edyvane KA, Marshall VR (1991). Effect of chondroitin sulfate, human serum albumin and Tamm-Horsfall mucoprotein on calcium oxalate crystallization in undiluted human urine. Urol Res **19**: 181-188.

[37] Sallis JD, Lumley MF (1979). On the possible role of glycosaminoglycans as natural inhibitors of calcium oxalate stones. Invest Urol **16**: 296-299.

[38] Sallis HD (1987). Glycosaminoglycans as inhibitors of stone formation. Mineral Electrolyte Metab **13**: 273-277.

[39] Scurr DS, Robertson WG (1986). Modifiers of calcium oxalate crystallization found in urine. II. Studies on their mode of action in an artificial urine. J Urol **136**: 128-131.

[40] Shum DKY, Gohel MDI (1993). Separate effects of urinary chondroitin sulfate and heparan sulfate on the crystallization of urinary calcium oxalate: differences between stone formers and normal control subjects. Clin Science **85**: 33-39.

[41] Suzuki K, Miyazawa K, Tsugawa R (1989). Inhibitory effect of sodium pentosan polysulfate on the formation, growth and aggregation of calcium oxalate in vitro. Jpn J Urol **80**: 526-531.

[42] Suzuki K, Miyazawa K, Tsugawa R (1989). A simple method for determining the metastable limit of calcium oxalate. In: Urolithiasis. Walker VR, Sutton RAL, Cameron ECB, Pak CYC, Robertson WG (eds.). Plenum Press, New York. pp. 65-66.

[43] Suzuki K, Moriyama M, Nakajima C, Kawamura K, Miyazawa K, Tsugawa R, Kikuchi N, Nagata K (1994). Isolation and partial characterization of crystal matrix protein as a potent inhibitor of calcium oxalate crystal aggregation: evidence of activation peptide of human prothrombin. Urol Res **22**: 45-50.

[44] Suzuki K, Mayne K, Doyle IR, Ryall RL (1994) Urinary glycosaminoglycans are selectively induced into calcium oxalate crystals precipitated from whole human urine. Scanning Microsc **8**: 523-532.

[45] Thorne ID, Resnick MI (1984) A methodology for the characterization of urinary glycosaminoglycans. J Urol **131**: 995-999.

[46] Yamaguchi S, Yoshioka T, Utsunomiya M, Koide T, Osafune M, Okuyama A, Sonoda T (1993). Heparan sulfate in the matrix and its inhibitory effect on calcium oxalate crystallization. Urol Res **21**: 187-192.

Discussion with Reviewers

D. Kok: Could you please define the term "crystal matrix"? Does the crystal matrix contain molecules which were not removed by the washing treatment, e.g., because they were trapped inside aggregates? If the substances were actually inside crystals or particles, can you tell whether they are there because they induced nucleation or because they once got adsorbed to the crystal surface and subsequently got overgrown by crystal material.

Authors: In this paper, crystal matrix means the macromolecular substances derived from the CaOx crystals. Washing solution did not show any detectable amount of GAGs or protein. Matrices are thought to bind to the crystal surface and incorporate into the crystals.

D. Kok: Did you perform quantitative comparisons, and if so how were these done? If you did not do these type of experiments, you can only state that SPP reduces the size of the particles formed.

Authors: Crystal volume and size distribution study between UF and SF, UF+SPP/HS showed statistically significant results. Although it was difficult to perform quantitative comparisons in SEM, many aggregated crystals were seen only in UF, not in SF or UF+SPP/HS.

S.R. Khan: What is the physiological concentration of heparan sulfate in normal and stone formers urine? What is the origin of GAGs excreted in the urine particularly the heparan sulfate? Are GAGs present as GAGs or as a part of mucoprotein or associated with membranes etc.?

Authors: In human urine, both of normal and stone former, the concentration of HS was calculated to be 1-2 μg/ml [9, 21, 22]. We did not examine the origin of HS or other GAGs in this experiment. Further study is needed to clarify the exact localization or characterization of HS in the kidney such as proteoglycans.

S.R. Khan: SPP 1, 2, 10 results are very close together in Figures 1 and 2, showing crystal volume and crystal number. What were the standard deviations? Was there any significant difference between the three different amounts of SPPs used?

Authors: Statistically significant difference was observed between UF and UF+SPP10 for crystal volume in Figure 1 and for mean crystal size in Figure 2.

L.C. Cao and E.R. Boevé: Could the binding behavior of urinary GAGs on CaOx crystals in this urine with such high ionic strength be different from original urine? Could the high oxalate concentration used to induce CaOx crystallization influence the binding behavior of urinary GAGs on the newly formed crystals?

Authors: Since the urine with high ionic strength and high oxalate concentration was used, there is a possi-

bility that binding behavior was slightly different from original urine. But, an undiluted urine seems to be the best system to observe the affinity of macromolecules for CaOx crystals that might have inhibitory activity.

L.C. Cao and E.R. Boevé: The crystal matrix protein is supposed to be selected from the urine and incorporated in newly forming CaOx crystals as reported by Ryall in 1991. In the present manuscript, the authors describe a similar phenomenon for HS. Can the authors comment on the reason why they have different results? Is the HS or crystal matrix protein located on the crystal surface only or both inside and outside the crystals?
Authors: We already reported CMP and HS coexisted in crystal matrix extract [43]. The results of references [43] and [44] suggests that HS and CMP adsorbed on different binding sites; and HS and CMP were thought to locate both inside and on the surface of the crystals. Recently, we showed the presence of CMP using CMP antibody and immunogold by backscattered electron microscopy (International J Urol (1995) **2**: 87-91).

M.D.I. Gohel and D.K.Y. Shum: During the isolation of crystal matrix, the surface was washed out. The reviewers believe that if GAGs were to act as growth and/or aggregation inhibitors, GAGs would be exerting its influence on the **surface** of the crystals and not in the crystal matrix. Those found in the crystal matrix are possibly promotors of nucleation. Hence, the surface washing should have been recovered for investigations of the nature and crystallization properties of macromolecules therein.
Authors: Washing solution, which was concentrated, electrophoresed and examined by SDS-PAGE and cellulose electrophoresis, did not show any detectable amount of GAGs or proteins. From the results of this experiment and references [43, 44], matrices were thought to be bound to the crystal surface and finally incorporated into the crystals.

P. Binette: Does SPP bind to other crystals?
Authors: We did not have any data of SPP about other crystals. There are some reports about other crystals as follows: Uric acid crystals {Grases *et al.* (1991) Urol Res **19**: 375-380}; and CaP crystals {Boeve *et al.* (1990): Urol Res **18**: 62}.

P. Binette: A small percentage of SPP is secreted in the urine. Is it bound to crystals excreted in spontaneous crystalluria?
Authors: We did not perform such an experiment yet.

P. Binette: It is stated that SPP is an anticoagulant. What protein(s) does it bind to?
Authors: We do not know the binding protein(s) of SPP.

R.W. Norman: Is there any evidence to suggest that SPP might function differently in the urine of women or male/female stone-formers as opposed to healthy controls?
Authors: To answer the question, further experiment is needed.

R.W. Norman: Do you have any evidence that oral administration of this drug will allow reproduction of your findings when the drug is directly added to the pooled urine collections?
Authors: We did not perform the experiment of oral administration. In near future, we want to try to clarify the usefulness of oral administration of SPP.