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URINARY GLYCOSAMINOGLYCANS ARE SELECTIVELY INCLUDED INTO CALCIUM OXALATE CRYSTALS PRECIPITATED FROM WHOLE HUMAN URINE

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Abstract

Urinary glycosaminoglycans are selectively included into calcium oxalate (CaOx) crystals precipitated from whole human urine: The presence of glycosaminoglycans (GAGs) in the organic matrix of urinary stones, and their known effects on CaOx crystallization have prompted speculation regarding their role in CaOx urolithiasis. The aim of this study was to examine the involvement of GAGs in the early stages of CaOx crystallization in human urine. Urine samples were collected from healthy men and CaOx crystallization was induced by the addition of a sodium oxalate load. The crystals were harvested and demineralized, and the GAG content of the resulting extract analysed by cellulose acetate electrophoresis. Only one GAG, heparan sulphate (HS) was detected in the organic matrix of the crystals; chondroitin sulphate (ChS), the most abundant urinary GAG, was conspicuously absent. Further experiments, in which varying amounts of HS and ChS were added to ultrafiltered (10,000 Da) urine prior to induction of calcium oxalate crystallization, showed that ChS was included into the crystals only when HS was absent from the urine. It was concluded that the selective inclusion of GAGs into crystals and stones is a function related more to relative binding affinity than to ambient GAG concentration and that HS and ChS compete for specific binding sites on the crystal surface.

Key Words: Urine, glycosaminoglycans, urolithiasis, calcium oxalate, stone matrix, crystal matrix, chondroitin sulphate, heparan sulphate, inhibitory activity.

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Introduction

All urinary stones, regardless of their mineral content, contain a network of organic matrix that is distributed throughout their crystalline structure and accounts for 2-6% of their total weight [5, 27, 31]. Because its presence would suggest that it may fulfil some directive function in the stone's formation, many years of research have been devoted to its study. However, its role remains enigmatic, largely because early workers were unable to dissolve 75% of the lyophilized stone matrix, and this seriously hampered attempts to characterize its component macromolecules [6]. Nonetheless, study of the 25% of matrix which they **could** dissolve yielded some information which at least provided a clue as to which macromolecules may be involved in a stone's formation. The major portion of matrix consists of protein [4], with the remainder comprising an ill-defined mixture of various materials ranging from "inorganic ash" to bacteria and cellular membranes [12]. More recently, a second class of macromolecules, the glycosaminoglycans (GAGs), have also been detected in matrix [16, 19] and these are worthy of particular scrutiny because a number of studies have demonstrated that some of them are inhibitors of calcium oxalate (CaOx) crystallization in aqueous inorganic media [3, 7, 10, 17, 20, 21, 22, 25].

Although a recent study [34] found that the only GAG in CaOx stone matrix was heparan sulphate (HS), others have previously reported [16] that CaOx dihydrate stones contain two GAGs, namely, HS and hyaluronic acid (HA), a finding confirmed by Roberts and Resnick [19], who also demonstrated the presence of these GAGs both in CaOx monohydrate stones and in those containing calcium phosphate. Perhaps, the most intriguing aspect of these studies is that though both HS and HA occur in normal human urine, they are minor constituents: HA is present in only trace amounts, while HS accounts for some 15-20% of the total GAG complement [13, 30, 32]. Chondroitin sulphate (CS), on the other hand, comprises approximately 60% of urinary GAGs [13, 30, 32], but though present in small amounts in

both magnesium ammonium phosphate [19] and apatite stones [16], it has not been detected in CaOx stone matrix [16, 19, 34]. The findings of Nishio *et al.* [16], Roberts and Resnick [19] and Yamaguchi *et al.* [34] indicate that the inclusion of GAGs into kidney stone matrix does not simply reflect their proportional representation in human urine. Though this discrepancy may be attributable to the relative binding affinities of each GAG for the CaOx crystal surface [1, 11], equally, it may be the consequence of a number of other factors, including difficulties with dissolving matrix, as discussed above. Furthermore, identification of matrix GAGs, like proteins, has been frustrated by the fact that their molecular structure and properties can be altered by the aggressive procedures required for their isolation from the stone, ageing, polymerization and their very involvement in the crystallization process [9, 14, 18]. To these can be added a further complicating factor: macromolecules in matrix can derive both from the normal urinary complement and from endothelial trauma caused by the inchoate or developed stone during its passage through the renal collecting system and urinary tract [9]. It is possible therefore, that GAGs may be present in matrix as a result of the participation of normal urinary GAGs in the crystallization of CaOx, the binding to crystal surfaces of GAGs released by tissue damage, that are not normally present in urine, or both.

Collectively, these problems have conspired to obscure the role of urinary GAGs in stone pathogenesis. However, to a large extent the problems associated with the analysis of GAGs in renal stones can be overcome by studying those included in CaOx crystals freshly precipitated from human urine, an approach which has been successfully adopted for studying the involvement of urinary proteins in CaOx crystallization [9, 19]. The aim of this study was therefore to determine which urinary GAGs are involved in the formation of CaOx crystals newly deposited from fresh human urine.

Materials and Methods

Materials

All reagents were of analytical purity. Unless stated otherwise, the biochemicals were purchased from the suppliers listed in a previous paper [9]. Chondroitin sulphate, heparan sulphate, chondroitinase ABC (EC 4.2.2.4), heparitinase (EC 4.2.2.8) and D-glucuronic acid were obtained from Sigma Chemical Co., St. Louis, MO). All solutions were prepared with the highest quality water from a "Hi Pure" water purification system fitted with a 0.2 μm pore-size filter (Permutit Australia, Brookvale, NSW, Australia). Cellulose acetate strips (Sepraphore III, 2.5 x 15.2 cm) were obtained from Gelman Sciences (Ann Arbor, MI, USA) and stained with Alcian blue (Sigma).

Collection and preparation of urine samples

Twenty four hour urine specimens, refrigerated throughout the collection period, were obtained without preservative from 5 healthy men aged between 30 and 43 years. Each specimen was tested to exclude the presence of blood using Multistix test strips (Miles Laboratories, Mulgrave, Victoria, Australia). The samples were then pooled and subjected to one of the following treatments. Whole urine (**WU**) was obtained by passing the urine through a 20-μm pore-size nylon mesh (Swiss Screens; Sydney, Australia; Nytal HD20) to remove cellular debris. Spun and filtered urines (**SF**) were prepared by centrifugation at 10,000 x g for 30 minutes in a J2-21M/E centrifuge (Beckman Instruments, Palo Alto, CA), followed by filtration through 0.22-μm pore-size filters (GSWP04700; Millipore Corp; Bedford, MA), while ultrafiltered urines (**UF**) were similarly treated prior to ultrafiltration using a hollow fibre bundle (H1P10-20; Amicon Corp, Danvers, MA) with a nominal molecular mass cut-off of 10 kDa. Urine samples were refrigerated until required and warmed to 37°C immediately prior to use.

Induction of CaOx crystallization

Preparation of CaOx crystals from urine and isolation of their organic matrix were performed as described by Doyle *et al.* [9], with the urinary metastable limit being determined using a modification [28] of the original method of Ryall *et al.* [24]. Thirty mmol/l of sodium oxalate, in excess of the measured metastable limit, was added drop-wise to the urine and the specimen was incubated in a shaking water bath at 37°C for 1 hour. To increase the yield of CaOx crystals, the same amount of oxalate was added after 1 hour, and again after 2 hours. The reaction was carried out for a total of 3 hours.

In the initial experiments, HS and ChS were added to ultrafiltered urine at final concentrations of 1, 2 and 10 μg/ml respectively.

Isolation of crystal matrix

Crystals precipitated from the UF urines were isolated by 0.22 μm Millipore filtration. However, those deposited from the WU specimens were removed by centrifugation at 10,000 x g for 20 minutes in a Beckman J2-21M/E centrifuge, because whole human urine cannot be filtered [8, 33] owing to the presence of polymerized Tamm-Horsfall glycoprotein (THG). The crystals were then washed with 0.1 mol/l NaOH and distilled water to remove any glycosaminoglycans present in the small amounts of residual urine associated with the crystals. This step was repeated 6 times prior to their demineralization in 0.25 mol/l ethylenediaminetetraacetic

acid (EDTA) solution (pH 8.0) for three days at 4°C as described in detail by Doyle *et al.* [9]. Following exhaustive electrodialysis to remove the EDTA, the demineralized crystal extracts were lyophilized to yield a colourless, flocculant material that was processed as described below.

Electrophoresis

After dissolution of the lyophilized extract (20 μg/20 μl of distilled water), 2-5 μl was applied to cellulose acetate strips and electrophoresed for 90 minutes in 0.2 mol/l of pH 7.2 calcium acetate buffer [29] at a constant voltage of 80 V). HS and ChS were run simultaneously as standards at a concentration of 1 μg/μl. After electrophoresis, the cellulose acetate strips were stained for 30 minutes in 0.2% Alcian blue in 25 mmol/l sodium acetate buffer (pH 5.8), containing 50 mmol/l magnesium chloride and 50% ethanol. The membrane was then destained in several changes of the same buffer.

GAGs were provisionally identified by comparing the bands on the electrophoretogram with the migration distances of the standards included in each run. Identification was confirmed by digesting the matrix solution, prior to electrophoresis, with chondroitinase and heparitinase. Samples of crystal matrix were incubated with chondroitinase ABC (0.5 unit of enzyme/100 μg uronic acid) in 0.1 mol/l Tris-HCl buffer (pH 8.0) at 37°C for 18 hours, or with heparitinase (2 units of enzyme/100 μg uronic acid) in acetate buffer (pH 6.7) at 43°C for 60 minutes. Total GAG was estimated by determining uronic acid concentration using the method of Blumenkrantz and Asboe-Hansen [2] using D-glucuronic acid as standard.

Results

In all cases, calcium oxalate monohydrate crystals were precipitated. Figure 1 shows electrophoretograms of HS and ChS standards, matrix extract of CaOx crystals precipitated from whole urine (**WU**), centrifuged and filtered urine (**S&F**) and ultrafiltered urine to which HS (**UF+HS**) and ChS (**UF+ChS**) had been added; bands are shown before and after heparitinase digestion. Only one band was visible in the matrix from the whole (**WU**) and spun and filtered (**SF**) urines. The band was identified as HS, since its migration distance was identical to that of standard HS, and it disappeared after digestion with heparitinase. It was unaffected by papain digestion, suggesting that the HS in the crystals was not present as a proteoglycan.

As expected, no GAG bands were detected in electrophoretograms of the matrix of CaOx crystals deposited from **UF** urines. However, when HS was added to **UF** urine at a final concentration of 2 μg/ml, and CaOx crystal formation induced, the electrophoretogram of the resulting crystal extract showed the presence of the same band as was observed with the extract derived from the **SF** urine.

Table 1. Concentrations of HS and ChS added to samples on the same ultrafiltered urine.

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

Because these results indicated that heparan sulphate is selectively included into CaOx crystals to the exclusion of other urinary GAGs, a further experiment was carried out where CaOx crystals were precipitated from **UF** urine which had been supplemented with ChS at a final concentration of 10 μg/ml. In this case, the electrophoretogram demonstrated the presence of a weakly staining band that had the same migration characteristics of ChS. This band disappeared after digestion with chondroitinase, but not with heparitinase, confirming that it was chondroitin sulphate.

Since this result provided further evidence that the inclusion of GAGs into CaOx crystals is selective, and suggested that binding of urinary GAGs to the crystal surface is competitive, an additional experiment was undertaken in which increasing concentrations of HS and ChS were added to samples of the same ultrafiltered urine in a grid construction as shown in Table 1. Crystallization was induced by the addition of oxalate as before, and after isolation of the matrix extract from the CaOx crystals precipitated from each of the 16 urine samples, the lyophilized materials were dissolved and electrophoresed. The electrophoretograms are shown in Figure 2. In the absence of any added GAGs, and at ChS and HS concentrations of 0 and 1 μg/ml, respectively, no bands were visible. ChS was detected in the crystal extract only at a final urinary concentration of 10 μg/ml, and then, only in the complete absence of HS or in the presence of the lowest concentration of HS tested (1 μg/ml), where a faint band was visible. In contrast, HS was visible in the extract at a concentration of 1 μg/ml in the presence of ChS concentrations of 1 and 2 μg/ml, and was always observed at higher concentrations. Although the results were not quantified, it is apparent that the amount of HS incorporated into the crystals decreased in relation to increasing concentrations of ChS. This effect is most noticeable at an HS concentration of 2 μg/ml. The competition between ChS and

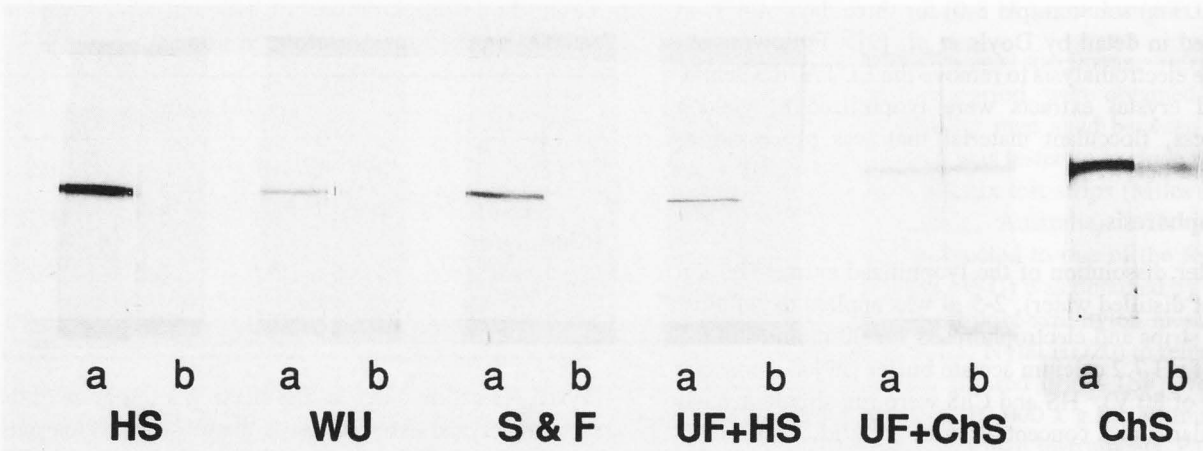

Figure 1. Cellulose acetate electrophoretograms of standards and GAGs extracted from CaOx crystals precipitated from urine, before (**a**) and after (**b**) digestion with heparitinase: **HS**, heparan sulphate standard (1 μg/μl); **WU**, extract of crystals deposited from whole (untreated) human urine; **S&F**, extract of crystals obtained from urine that had been centrifuged and filtered; **UF + HS**, crystal extract derived from ultrafiltered urine to which HS had been added at a final concentration of 2 μg/ml; **UF + ChS**, extract of crystals precipitated from ultrafiltered urine that had been supplemented with ChS at a final concentration of 10 μg/ml; **ChS**, chondroitin sulphate standard (2 μg/μl). No bands were visible in extracts of crystals generated in ultrafiltered urine.

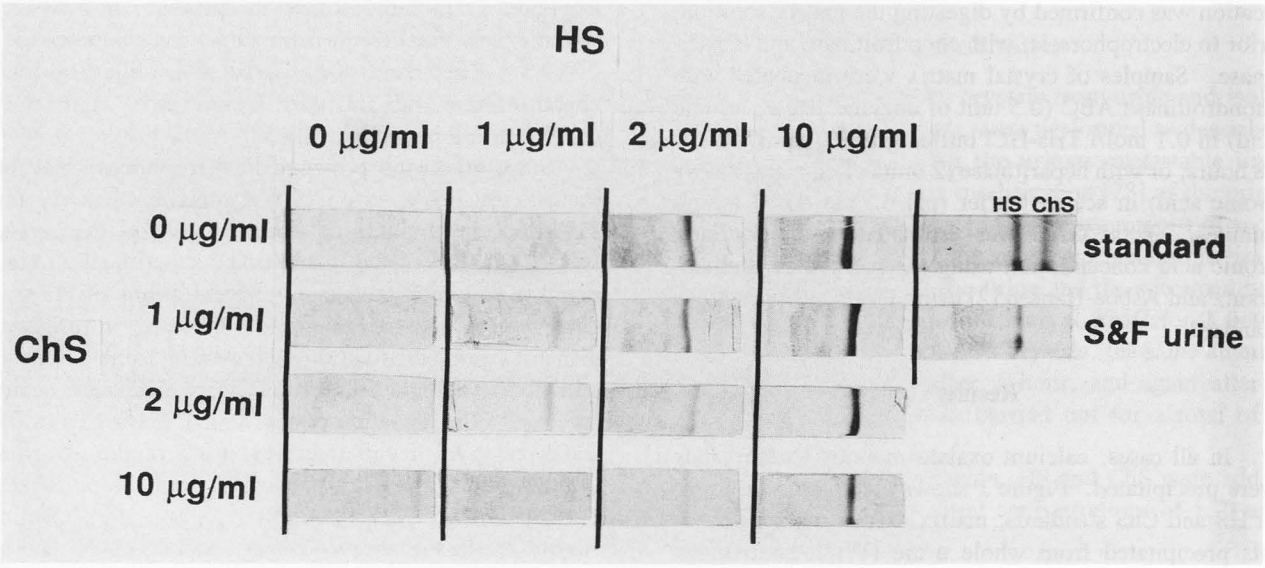

Figure 2. Cellulose acetate electrophoretograms of extracts of CaOx crystals precipitated from ultrafiltered urine supplemented with varying concentrations of ChS and HS. The concentrations refer to the final concentrations of GAGs added to the ultrafiltered urine. Standards are included for comparison (HS, 1 μg/ml; ChS, 2 μg/ml) as well as the electrophoretogram for the extract of the same urine which had been centrifuged and filtered only, and which contained no supplementary ChS or HS.

HS can also be seen at an HS concentration of 1 μg/ml in the presence of 10 μg/ml ChS, where the 10-fold greater concentration of the latter appears to have prevented the inclusion of HS, which was observed at lower concentrations of ChS.

Crystals precipitated from the same urine which had been spun and filtered, but not ultrafiltered, contained only HS, confirming the findings of the first part of this study.

Discussion

More than twenty years have elapsed since Crawford *et al.* [7] showed that the crystallization of calcium oxalate (CaOx) is inhibited by synthetic and natural GAGs. Their observation, and the fact that GAGs occur naturally in urine, have since prompted numerous workers to investigate the possibility that these molecules may play a determinant role in stone pathogenesis. This possibility was later strengthened by the detection of GAGs in urinary stones [16, 19, 34], but was confused somewhat by the observation of a mismatch between the relative amounts of specific GAGs in urine and calculi. The GAGs present in CaOx stones, HS and HA [16, 19, 34], are only minor urinary constituents, while the most abundant urinary GAG, ChS, has not been detected in stone matrix. One explanation for this discrepancy is that matrix GAGs, like proteins [9], may possibly derive from two distinct sources. First, normal urinary GAGs may bind to the surface of newly formed crystals in the renal collecting system; provided the urine remains sufficiently supersaturated, they will then become embedded within the crystal architecture as new mineral is deposited upon the crystal surface. Second, it is possible that once crystals have formed, their very presence in the urinary tract may alter the GAG content of urine by damaging the urothelium and causing the release of GAGs in relative quantities quite different from their normal proportional complement in urine. These GAGs may now bind to the surface of the crystals, and as stone development occurs, these too will become incorporated within the stone structure. It is possible therefore that, despite its low concentration in urine, HS may be present in stones because large amounts of it are released in response to urothelial injury, not because it plays some active role in the precipitation of CaOx crystals from urine. It is important to distinguish between the two mechanisms of GAGs inclusion because in one case the GAGs may have contributed to the formation of the calculus, while in the other they are a product of it.

In the present study, the complicating factor of GAGs derived from tissue trauma was avoided by studying the GAG component of CaOx crystals freshly precipitated from human urine, an approach that has been successfully adopted to study the role of urinary proteins in matrix [9, 15]. It was found that GAGs are present in CaOx crystals even when precipitation takes place in the presence of other urinary macromolecular competitors such as proteins, suggesting that binding takes place on specific sites on the crystalline surfaces. Only one urinary GAG, HS, was included into the crystals. GAGs in CaOx crystals are therefore identical to those in CaOx stones: Nishio *et al.* [16] showed that the soluble matrix of CaOx dihydrate stones contained HS and HA, and similar results were later reported for CaOx monohydrate stones [19] although more recent findings [34] showed that HA is not present in stones. Thus, even if GAGs *are* released into urine in response to urothelial trauma, their qualitative incorporation into stones is identical to that into freshly deposited crystals, indicating that their presence in matrix is more a function of selectivity than of quantity. This selectivity can be ascribed to the fact that different GAGs possess different binding affinities for the CaOx crystal surface. Fellström *et al.* [11] measured the relative binding affinities of a number of GAGs to calcium oxalate crystals in inorganic media and reported that, in addition to ionic strength, the binding affinity of each GAG was directly related to its charge density in the order: pentosan polysulphate (a synthetic GAG) > heparin (not present in human urine) > ChS. They also demonstrated that the inhibitory potency of these GAGs decreased in the same order [10], and concluded that the inhibitory activity of GAGs is largely a function of their charge density. Later, Angell and Resnick [1] also showed that GAGs differ in their binding affinity for CaOx, but obtained different results: heparin > HA > ChS > pentosan polysulphate. The discrepancy in the order of binding affinity may perhaps result from differences in methodology used. Nonetheless, both studies confirm that different GAGs do not bind with equal strength and this may explain the selective incorporation of HS into stones and CaOx crystals at the expense of ChS.

It is noteworthy that the selectivity of GAGs inclusion into stones, observed independently by three different research groups and now confirmed in this study using CaOx crystals, was ascribed to completely opposite causes. The fact that ChS could not be detected in stone matrix, coupled with earlier observations that it inhibits CaOx crystallization, prompted Nishio *et al.* [16] to conclude that HS and HA, which **were** present in the matrix, must be promoters of stone formation. Paradoxically however, Roberts and Resnick [19] on the basis of identical findings, suggested the contrary interpretation. They attributed the presence of HS and HA in stone matrix to their strong binding affinity for the surface of the crystals comprising the stone, and since it is generally accepted that the magnitude of an inhibitor's effect is a function of the efficiency of its binding to the crystal surface, they implied in their discussion that HS and HA normally fulfil an inhibitory role in urine. Yamaguchi *et al.* [34] arrived at the same conclusion; they inferred from their results that HS strongly inhibits CaOx crystal growth and aggregation, since HS was the only GAG present in soluble stone and crystal matrices, which exhibited strong inhibitory effects in their study. More recently, however, evidence has been obtained that urinary GAGs can exert a dual effect on CaOx crystallization,

promoting crystal nucleation, while at the same time reducing the final size of the crystals produced. Thus, Shum and Gohel [26] showed that, at the same uronic acid concentration, both ChS and HS enhanced CaOx nucleation from frozen ultrafiltered urine, whereas only HS derived from controls exhibited the effect. Similarly, they reported that crystals precipitated from frozen ultrafiltered urine samples to which HS had been added were smaller than those deposited in the presence of ChS, irrespective of whether the GAGs were isolated from stone formers or controls. Those findings are consistent with those reported here. The greater inhibitory effect of HS is not unexpected, since it bears structural similarities to heparin [10, 11], unlike ChS, which has no measurable effect on CaOx crystallization in ultrafiltered, undiluted human urine [23]. Moreover, it can be argued that enhancement of crystal nucleation can be beneficial, since it is well recognised that rapid nucleation gives rise to smaller crystals; such crystals would be more easily and quickly flushed from the urinary tract and thereby reduce the probability of crystal retention and stone formation.

In addition to demonstrating that the inclusion of urinary GAGs into CaOx crystals is a selective phenomenon, the present study also represents the first attempt to examine the relative binding affinities of HS and ChS in undiluted human urine, a medium in which their binding properties are more likely to reflect their binding properties *in vivo*. This was done by systematically increasing the relative amounts of HS and ChS added to ultrafiltered urine before inducing CaOx crystallization. The data indicated that when binding does occur, the amount of GAG bound is directly proportional to the ambient concentration; for instance, although not quantified, it was apparent that the amount of HS incorporated into the crystals increased in response to increasing concentrations of exogenous HS in the ultrafiltered urine to which it was added. It was also apparent that the binding affinity of ChS was very much less than that of HS; ChS was only detectable in the crystals when its concentration in the ultrafiltered urine was 10 μg/ml, in contrast to HS, which was visible in crystals generated in the presence of a concentration of 2 μg/ml. The relative binding affinities of ChS and HS in undiluted urine are therefore the same as those reported for inorganic solutions [1, 10]. The present studies also demonstrated that ChS can bind to CaOx crystals and become included into crystals precipitated from urine. The fact that this only occurs in the absence of HS suggests that HS and ChS compete for the same binding sites on the crystal surfaces. Since evidence would indicate that HS may fulfil an inhibitory role in CaOx stone pathogenesis, as discussed above, it is attractive to speculate that *in vivo* ChS may compensate for a deficiency of HS in the urine. However, this is doubtful in view of the fact that ChS does not affect CaOx crystallization in undiluted, ultrafiltered human urine [23] at a final concentration of 20 μg/ml, a concentration double that used in the present study.

In summary therefore, the present study has demonstrated that urinary GAGs are involved in the critical crystallization stage of stone formation. The inclusion of GAGs into CaOx crystals precipitated from undiluted human urine is a selective process and, as with urinary stones, HS is incorporated in preference to ChS, despite the fact that ChS is present in urine at a concentration approximately 4 times greater than that of HS [13, 30, 32]. Furthermore, even if a proportion of GAGs in stones is derived from those released into urine as a result of tissue damage caused by the stones themselves, it is apparent that only HS binds strongly enough to be included in the fully developed structure. The routine measurement of **total** urinary GAGs excretion is therefore unlikely to be of any practical benefit in the diagnosis and management of urolithiasis, a point made previously by Fellström *et al.* [10]. However, the mere presence of HS in kidney stones does not presuppose a critical function for it in calculus formation; it may simply be a passive adsorbent that binds non-specifically to the inorganic crystal phase, rather than an active protagonist in the crystallization process. Furthermore, even if the involvement of HS in the crucial crystallization stage of stone formation **is** directive, paradoxically, it may be either as an inhibitor or promoter. Future studies should therefore be aimed at determining whether the presence of HS in crystals and stones is a consequence of its acting as an inhibitor or promoter of CaOx crystallization, or whether it is simply a disinterested participant in the process.

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Discussion with Reviewers

S. Yamaguchi: In general, both glycosaminoglycans and urinary proteins adsorb on CaOx crystals in whole urine. Do these proteins and heparan sulphate compete for the same binding sites on the crystals?
Authors: We have not performed competitive binding studies between GAGs and proteins and so cannot provide a definite answer to this question. However, even if they do compete, it is apparent that binding of neither type of macromolecule completely prohibits adsorption of the other, since the organic matrix of calcium oxalate crystals precipitated from whole urine, contains both HS (as shown in this paper) and proteins (as shown in reference 9). This could suggest that HS and proteins do not compete for the same binding sites on the crystals. Alternatively however, it is possible that either HS or proteins could first be adsorbed in the early stages of crystallization, but that subsequent deposition of CaOx is sufficient to bind the other in the later stages of crystal growth.

S. Yamaguchi: What do you think of the selective incorporation of heparan sulphate into CaOx crystals? Is it simple incorporation or specific incorporation?
Authors: The fact that HS is incorporated into CaOx crystal matrix is evidence that the GAG binds to the crystal surface, but whether this binding is simple (non-specific) or occurs at specific sites cannot be stated with certainty from our data. Nonetheless, the fact that HS is incorporated to the exclusion of ChS, suggests that the process is competitive, and this in turn implies that the binding is specific, rather than indiscriminate.

S.R. Khan: Since crystalluria is common in both stone formers and non-stone formers, why do you consider crystallization a critical event in stone formation?
Authors: In so far as stone formation can never occur without crystal nucleation, this event may be regarded as the critical step in the crystallization process . That is not to say, of course, that subsequent steps are not also crucial; there is good evidence that the process of crystal aggregation is also vital in determining whether or not crystalluria will proceed to stone formation.

S. Nishio: The present study showed that heparan sulfate was selectively included into calcium oxalate crystals in the presence of both heparan sulfate and chondroitin sulfate. Did you examine the influence of urinary pH?
Authors: No, we did not examine the influence of pH. All urines were used at their native pH.

J.P. Binette: Papain digestion, had it displaced the HS band, would have complicated matters and cannot exclude the peptide moiety in this system. Why were GAGs not expected from CaOx crystals deposited from UF urines?
Authors: Had the HS band been altered after heparitinas treatment, it might have indicated that the binding affinity of HS for the crystal surface was dependent upon its being linked to a protein moiety. GAGs were not expected from crystals precipitated from ultrafiltered urine because all macromolecules with molecular mass $>$ 10 kDa were removed by the ultrafiltration process.

J.P. Binette: ChS is detected in the matrix of some stones. The statement that GAGs are a product of the calculus is succinct and incorrect. GAGs maybe dislodged from tissue by sharp crystals or aggregates to which they may bind and thus rejoin the formation of the calculus.
Authors: I am aware of no study that has conclusively demonstrated the presence of ChS in calcium oxalate stone matrix, although it has been detected in magnesium ammonium phosphate stones [19] and is present in small amounts in apatite calculi [16]. The statement that there are two possible mechanisms of GAGs inclusion into the matrix of stones may well be succinct, but it is not incorrect. In fact, Dr. Binette's remark that GAGs "may be dislodged from tissue by sharp crystals or aggregates to which they may bind and thus rejoin the formation of the calculus" completely supports the argument. Normal urinary GAGs may be present in crystals/stones because they have been involved in the crystallization process: they are forerunners to nucleation. On the other hand, GAGs not normally present in urine, but which are released by tissue injury caused by crystals or the growing stone, may also then bind to the crystals and become part of the final stone structure, these GAGs are products of the **developing** stone which later become part of the **final** urolith.