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Effects of chondroitin sulphate, human serum albumin and Tamm-Horsfall mucoprotein on calcium oxalate crystallization in undiluted human urine

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Summary. The effects of physiological concentrations of chondroitin sulphate, human serum albumin and Tamm-Horsfall mucoprotein on the crystallization of calcium oxalate in undiluted, ultrafiltered human urine were investigated using particle size analysis and scanning electron microscopy. Neither the amount of oxalate required to induce detectable calcium oxalate crystal nucleation nor crystal morphology was affected by the presence of any of these macromolecules. Chondroitin sulphate had no effect on the amount of crystalline material deposited or on the size of the particles precipitated in response to a standard oxalate load. Human serum albumin slightly reduced the size of the crystal aggregates and caused a small increase in the amount of crystal matter precipitated. By contrast, Tamm-Horsfall mucoprotein significantly inhibited crystal aggregation and markedly increased the volume of matter deposited, although this could not be attributed to a promotion of solute precipitation. It was concluded that chondroitin sulphate, human serum albumin and Tamm-Horsfall mucoprotein cannot account for the inhibitory effects of macromolecules with a relative mass greater than 10 kDa in spun and filtered urine. Nonetheless, Tamm-Horsfall mucoprotein is likely to inhibit crystal aggregation in whole urine in vivo and may therefore be instrumental in preventing calcium oxalate stone formation.

Key words: Calcium oxalate urolithiasis – Chondroitin sulphate – Human serum albumin – Tamm-Horsfall mucoprotein – Urinary macromolecules – Calcium oxalate crystallization

The origin of interest in a possible role for inhibitors in calcium oxalate stone formation can be traced to the studies of Howard and Thomas in 1958 [17], which showed that urine from healthy subjects inhibited the calcification of rachitic rat cartilage, while that from stone formers did not. These early studies spawned the so-called inhibitor theory of calcium oxalate stone formation, which proposes that urolithiasis does not occur in healthy people because their urines contain substances which interdict the nucleation, growth or aggregation of calcium oxalate crystals. Stone formers, on the other hand, are susceptible to the disease because their urines lack or are deficient in these inhibitors. The potential role of inhibitors is thus a major one, for the obvious corollary of the theory is that it should be possible, at least in principle, to prevent recurrences in stone formers by correcting the inhibitory deficit. For this reason, much effort since the pioneering observations of Howard and Thomas has been directed towards identifying natural components of urine which can prevent or retard calcium oxalate crystallization, and, although a number of low-molecular-weight species, including citrate, pyrophosphate and magnesium [28, 31], have been reported to exhibit such inhibitory effects and to contribute a proportion of urinary inhibitory activity in vitro [11], it is the macromolecular constituents of urine which have attracted increasing interest in recent years. Thus, a number of reports attest to the inhibitory effects of urinary macromolecules on various aspects of calcium oxalate crystallization [2, 6, 7, 9, 10, 12, 13, 18–22, 31, 34, 35]. Although minor urinary macromolecules such as RNA [35] have been reported to retard calcium oxalate crystallization, the bulk of the inhibitory activity of urine has been ascribed principally to two classes of compounds, namely, glycosaminoglycans [1, 26, 27, 31] and proteins [20, 34, 38]. However, in virtually every case, inhibitory effects of macromolecules have been tested in inorganic solutions, rather than in human urine – the medium in which stones actually form. It is widely acknowledged that effects measured under such conditions may bear no resemblance to those which would occur in undiluted urine in vivo. Thus, it is possible that the inhibitory effects of macromolecules described in the literature may be of little or no physiological relevance, and there is, consequently, still a need to establish what influence they would have in a milieu more closely resembling that found in vivo.

Recently it was shown that removal from human urine of all macromolecules greater than 10 kDa in mass causes

a marked increase in the size of calcium oxalate particles crystallized in response to an oxalate load [6]. This was shown to be the result of a remarkable enhancement of crystal aggregation, rather than to an increase in the size of individual crystals. Whether this potent inhibitory effect of urinary macromolecules on crystal aggregation is the result of the removal of a single specific macromolecule, or of a number of them, has still to be ascertained. The object of this study was, therefore, to determine whether the enhancement of crystal aggregation caused by removing the macromolecules could be reversed or mitigated by the addition to undiluted, ultrafiltered urine of physiological concentrations of the most abundant natural glycosaminoglycan and proteins, namely, chondroitin sulphate A (CS), human serum albumin (HSA) and Tamm-Horsfall mucoprotein (THM).

Materials and methods

Collection and preparation of urine samples

Twenty-four hour urine specimens were collected simultaneously and stored under refrigeration without preservative, from 11 male members of the hospital staff (mean age 37 years) who had no previous history of kidney stone disease. The absence of blood from the individual urine samples (7) was confirmed by the use of urinalysis test strips (N-Multistix, Miles Laboratories, Australia) and the samples were then pooled and centrifuged at 8,000 × g for 10 min. Microscopic examination of the sediment revealed only cellular debris and a gelatinous pellet of precipitated THM. After filtration through 0.22 μm Millipore filters the sample was divided: one third (spun and filtered urine) was retained for later use and the remaining portion (ultrafiltered urine) was ultrafiltered using an Amicon hollow fibre bundle (H1P10-20) with a nominal cut-off at 10 kDa relative molecular mass. Urine samples were refrigerated until needed and were warmed to 37°C and refiltered (0.22 μm) just prior to use. Crystals were never recovered from the filter. Fresh pooled samples were used for each series of experiments.

Experimental technique

The method of inducing and quantifying calcium oxalate crystallization in undiluted urine has been described previously [32]. Briefly, the first part of the method involves empirical determination of the metastable limit of the urine with respect to calcium oxalate. This is defined as the minimum amount of oxalate required to produce crystals in 20 ml of the urine sample and is determined by titrating the urine with 0.2-ml portions of sodium oxalate solutions of increasing concentration, and using a Coulter Counter (Model TA II, fitted with a Population Count Accessory) to detect the presence of crystals. Results are confirmed by light microscopy. Once the metastable limit has been measured in duplicate, an amount of oxalate equivalent to a final concentration of 0.3 mmol/l in excess of this limit is added dropwise to flasks containing 100 ml urine. A zero-time particle analysis is performed before addition of the oxalate solution, and then the Coulter Counter is used to monitor changes in particle size distribution during a 90-min incubation period at 37°C in a shaking water bath.

In the present study, when the effects of the added macromolecules were being tested, an aqueous solution of HSA (Sigma Chemical Company #A-8763, USA), CS, sodium salt, type A (Sigma Chemical Company #C-4134, USA), or THM (method of preparation follows) was added to the incubation flasks in a volume of 1 ml, to give a final concentration of 20 ml/l. To take account of the possibility of precipitation of the added macromolecules during the incubation period, ultrafiltered urine controls containing the additive, but no oxalate, were routinely included.

All measurements were performed in sextuplicate, and the entire experiment was repeated on an additional two pooled urine specimens.

At the end of the incubation period portions of each of the various urine and precipitated crystal suspensions were filtered (0.22 μm) and the filter rinsed with 1 ml distilled water. The crystals and filter were dried in air, mounted on an aluminum stub and coated with gold (200 s; SEM Autocoating Unit E5200, Polaron Equipment Ltd., UK). The stubs were examined using an ETEC Auto Scan electron microscope (Siemens AG, FRG), at an operating voltage of 20 kV.

Previous experiments have demonstrated that the concentration of calcium, magnesium, phosphate, oxalate, sodium, potassium, citrate and pyrophosphate are unaltered by ultrafiltration [6].

Purification of Tamm-Horsfall mucoprotein

THM was prepared from normal human urine by a slight modification of the method of Tamm and Horsfall [36]. Solid sodium chloride was added to 20 l pooled urine to give a final concentration of 0.58 mol/l. To prevent bacterial growth, sodium azide was also added to the urine at a final concentration of 0.025%. The urine was left at 4°C for 48 h. The mucoprotein formed a white fluffy cloud at the bottom of the container and was collected by centrifugation at 8,000 × g for 20 min at 4°C. The precipitate was dissolved in 2 l water, and again centrifuged to remove cellular debris. Sodium chloride was then added to a final concentration of 0.58 mol/l and the sample left overnight at 4°C. The suspension was centrifuged at 8,000 × g for 20 min at 4°C and the pellet redissolved in water. Following a further addition of sodium chloride, as described above, the material was dissolved in the minimum amount of water and extensively dialysed against distilled water at 4°C until free of chloride. The clear, viscous solution remaining after dialysis was lyophilized and stored at −20°C until required.

Statistical analysis

For the sake of clarity, only mean values of results are depicted. However, where statistical comparisons of data were performed, the Wilcoxon test for matched pairs was performed using median values of the replicates, by pairing observed particle volumes at each incubation time point.

Results

The amount of oxalate required to elicit spontaneous nucleation of calcium oxalate crystals was not affected by ultrafiltration, or by the addition of CS, HSA or THM to the ultrafiltered urine samples. Therefore, within any one experiment, identical amounts of oxalate were added to each flask to induce precipitation during the 90-min incubation period.

Figure 1 shows the accumulation of particle volume with time in spun and filtered urine, the same urine after ultrafiltration, and in the ultrafiltrate containing CS. There was no discernible difference between the values recorded in the ultrafiltered urine in the presence or absence of CS, but both of these showed a crystal volume significantly less than that observed in the spun and filtered urine ($p < 0.01$).

In Fig. 2 are shown the volume-size distribution curves of the crystalline particles deposited in the samples at the

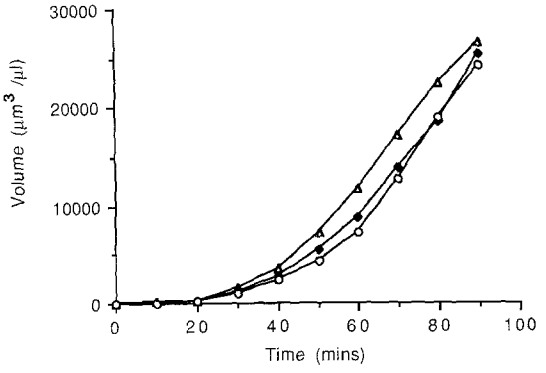

Fig. 1. Increase in particle volume with time in spun and filtered urine (–△–, SF) in the same urine after 10-kDa ultrafiltration (–♦–, UF) and in the same ultrafiltered urine to which CS had been added, at a final concentration of 20 mg/l (–○–, UF + CS).

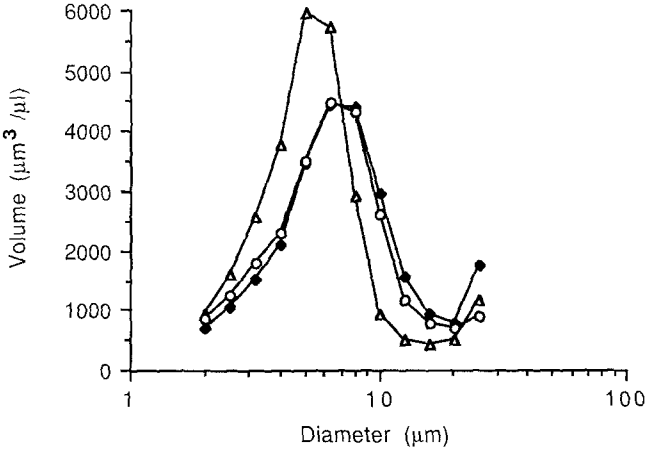

Fig. 2. Volume-size distribution curves of the particles precipitated from spun and filtered urine (–△–, SF), in the same urine after 10-kDa ultrafiltration (–♦–, UF) and in the same ultrafiltered urine supplemented with 20 mg/l CS (–○–, UF + CS)

end of the 90-min incubation period. Although the modes of the curves for the crystals precipitated from the two ultrafiltered samples are indistinguishable, a slight shift in the curve to smaller diameters in the presence of CS is evident. There appear to be fewer particles larger than

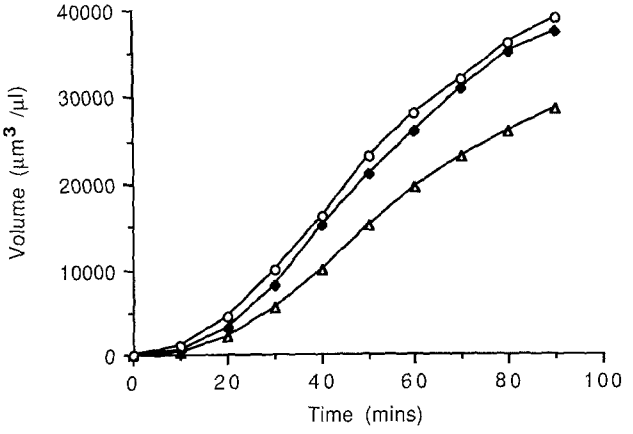

Fig. 4. Increase in particle volume with urine in spun and filtered urine (–△–, SF), in the same urine after 10-kDa ultrafiltration (–♦–, UF) and in the same ultrafiltered urine containing HSA at a final concentration of 20 mg/l (–○–, UF + HSA)

8 μm in diameter and slightly more with diameters of 4 μm and less in the urine containing the CS. As is always observed in this experimental system, the mode of the distribution curve for the crystals from the spun and filtered urine corresponds to a smaller diameter (approximately 5 μm) than the mode of the curve describing the distribution of the crystals precipitated from the ultrafiltered urine. This difference is reflected in the scanning electron micrographs shown in Fig. 3, which show typical crystals precipitated from each of the urine samples. Those deposited from the spun and filtered urine consisted of single crystals of both calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD), together with small aggregates and twins composed of a mixture of both crystal habits. Mixed aggregates and crystal twins were also observed in the ultrafiltered urine, but as is always observed, the degree of aggregation was considerably greater than was seen in the spun and filtered urine. Crystals precipitated from the ultrafiltered urine supplemented with CS bore a remarkable resemblance to those from the ultrafiltered urine: no obvious qualitative differences were apparent.

Figure 4 shows the time course of particle volume accumulation for the experiment testing the effect of HSA. Here, the volume deposited in the spun and filtered

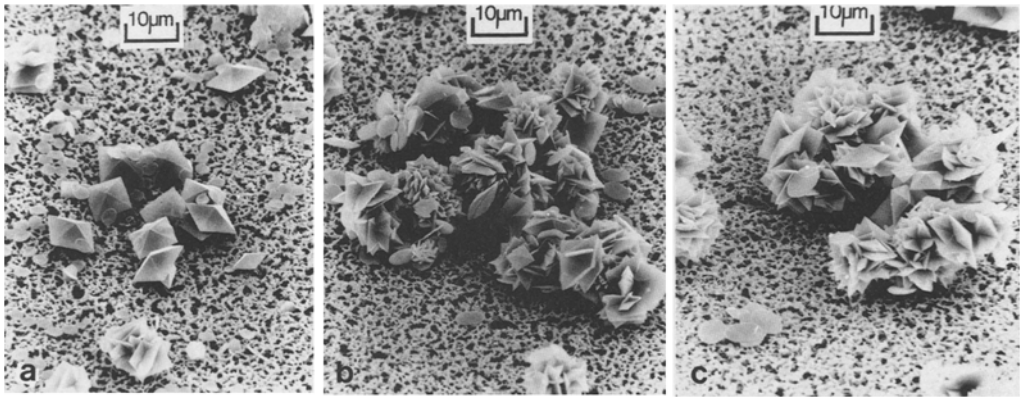

Fig. 3. Scanning electron micrographs of the crystals precipitated from the urine samples whose particle volume data are depicted in Figs. 1 and 2 **a** SF, **b** UF, and **c** UF +CS

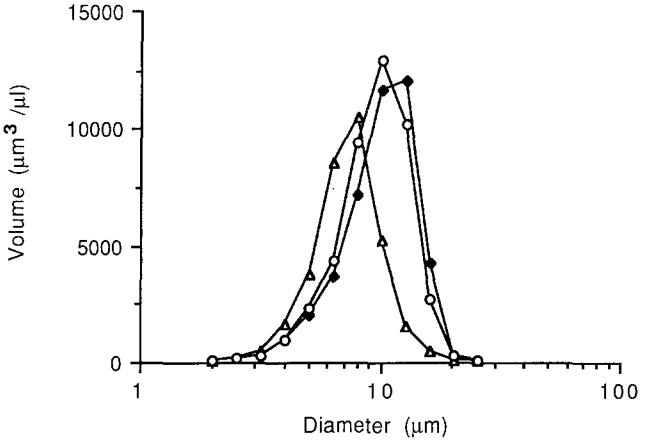

Fig. 5. Volume-size distribution curves of particles precipitated from spun and filtered urine (—△—, SF), the same urine after 10-kDa ultrafiltration (—♦—, UF) and the same ultrafiltered urine containing HSA at a final concentration of 20 mg/l (—○—, UF + HSA)

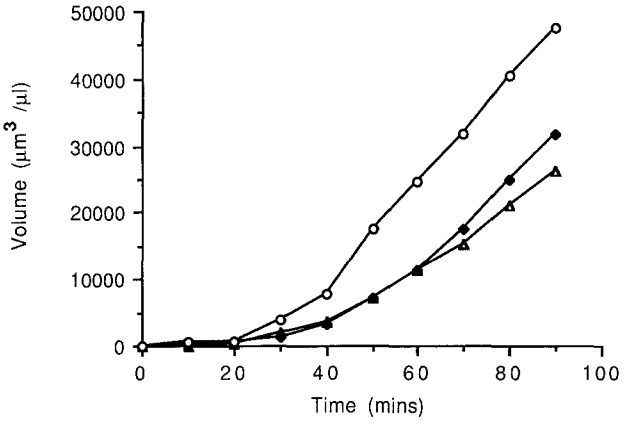

Fig. 7. The increase in particle volume with time in spun and filtered urine (—△—, SF), in the same urine after 10-kDa ultrafiltration (—♦—, UF), and in the same ultrafiltered urine to which 20 mg/l THM had been added (—○—, UF + THM)

urine was significantly less than that observed in both ultrafiltered specimens ($p < 0.01$). Furthermore, the particle volume precipitated from the ultrafiltered urine in the presence of HSA was significantly greater than that deposited in its absence ($p < 0.01$). The particle volume precipitated from the no-oxalate controls was negligible.

The particle volume-size distribution curves for the material deposited from the urines are presented in Fig. 5. As was observed in the previous experiment, the mode of the distribution curve for the particles deposited from the ultrafiltered urine corresponded to a larger diameter (approximately 11 μm) than that recorded for the spun and filtered urine (8 μm). The presence of HSA caused a slight shift of the mode to a diameter of 10 μm. These findings were confirmed in the corresponding scanning electron micrographs presented in Fig. 6. Once again, the morphology of the crystals precipitated from the ultrafiltered urine was the same as that seen in the spun and filtered, with particles consisting principally of COD and the occasional COM. And as before, those deposited from the ultrafiltered urine were considerably more highly aggregated than those from the spun and filtered urine. Crystals precipitated from the ultrafiltered urine in the presence of added HSA were indistinguishable from those sedimented in its absence.

Figure 7 shows the change in particle volume with time for the experiment testing the effect of THM. There was no significant difference between the spun and filtered urine sample and its ultrafiltered counterpart. However, the volume of material precipitated in the ultrafiltered urine which had been supplemented with THM was significantly increased ($p < 0.01$): at 90 min the mean volume recorded in the presence of THM was 50% greater than the corresponding value observed in the spun and filtered urine and 80% greater than that in the ultrafiltered urine. If the 90-min volume of the ultrafiltered urine plus THM was corrected for the particle volume recorded in the control ultrafiltered urine to which THM, but no oxalate, had been added, then the volume increase was reduced to 63%.

Despite the fact that a greater volume of material was deposited in the presence of THM, it can be seen from Fig. 8 that the size of the particles was reduced. A progressive increase in size of crystals precipitated, from the spun and filtered to the ultrafiltered urine, was again observed: the mean particle size increased from approxi-

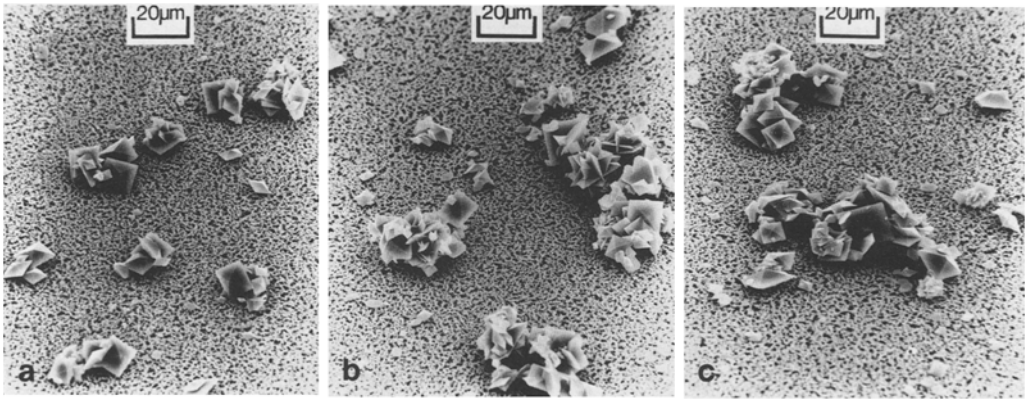

Fig. 6. Scanning electron micrographs of the crystals precipitated from the urine samples whose particle volume data are shown in Figs. 4 and 5 **a** SF, **b** UF, and **c** UF + HSA

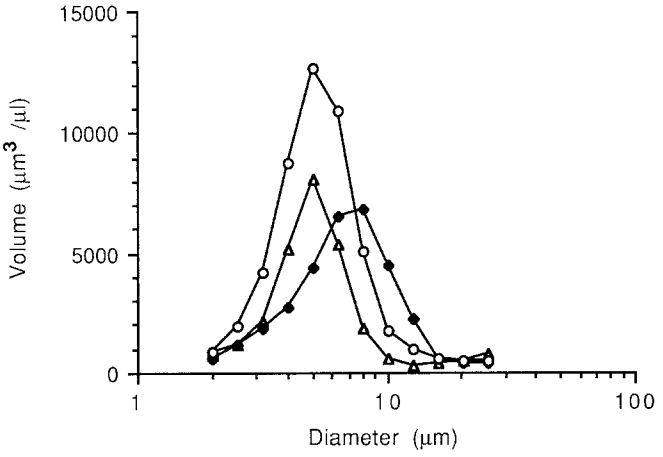

Fig. 8. Volume-size distribution curves of particles precipitated from spun and filtered urine (–△–, SF), the same urine after 10-kDa ultrafiltration (–♦–, UF) and the same ultrafiltered urine supplemented with 20 mg/l THM (–○–, THM)

mately 5 μm to 7.8 μm. However, in the presence of THM the mean particulate size was 5 μm – the same as that observed in the spun and filtered urine. As before, both COD and COM crystals were deposited, although there appeared to be a greater preponderance of the monohydrate habit in the spun and filtered urine. As was suggested by the volume distribution curves, the particles precipitated from the ultrafiltered urine in the presence of THM were more like those from the spun and filtered urine than from the ultrafiltrate. Large aggregates were not observed: small clusters of both COD and COM, as well as single crystals of each type, were typically seen (Fig. 9). Precipitated organic material, presumably THM, was clearly evident on the surface of the filter.

Discussion

The results from this study confirm the previous report from our laboratory [6] that removal of all macromolecules greater than 10 kDa in mass has no effect on the amount of oxalate required to induce detectable calcium oxalate nucleation, but causes a dramatic increase in the degree of aggregation of crystalline particles precipitated from human urine. On the other hand, ultrafiltration has an unpredictable influence on the volume of material deposited: the volume precipitated from the ultrafiltered urines used in the present study was variously greater than, indistinguishable from, and less than that from the corresponding spun and filtered sample. This variability can probably be ascribed to modulations in the concentrations of one or more urinary inhibitors from one urine specimen to another. For example, it is now well recognized that the amount of crystalline material precipitated in the presence of a given inhibitor is contingent directly upon that inhibitor's effect on crystal growth (i.e. laying down of new crystalline material) and indirectly on crystal aggregation. Thus, if it is an efficient interdictor of aggregation, but has little or no effect on crystal growth, it can increase the area of crystal surface available for solute deposition and thereby increase the total amount of crystal matter deposited in a given time [33]. The effect of ultrafiltration on the mass of material deposited from any one urine sample will therefore depend on the balance between the relative concentrations of its component high- and low-molecular-weight species and their direct inhibitory influences on growth and on aggregation. For this reason, small increases in crystal volume deposition wrought by an inhibitor provide no conclusive information regarding that inhibitor's direct effect on crystal growth [33]. On the other hand, except in the unlikely event of a promotion of aggregation, any decrease in volume relative to a control must reflect a true inhibition of growth. These considerations must be borne in mind when considering the effects of the macromolecules tested in the present study.

In the experiment examining the effect of CS, the particle volume deposited from the ultrafiltered urine was less than that from its spun and filtered counterpart and, as is always seen with this experimental technique, Coulter Counter analysis indicated a shift of particle size to a larger diameter. This was confirmed by scanning electron microscopy which showed that the increase in size was a consequence of a greater degree of crystal aggregation. In this case, therefore, ultrafiltration must have removed macromolecules which inhibited aggregation, but which had little effect on crystal growth. Addition of a physiological concentration of CS to the ultrafiltered urine did

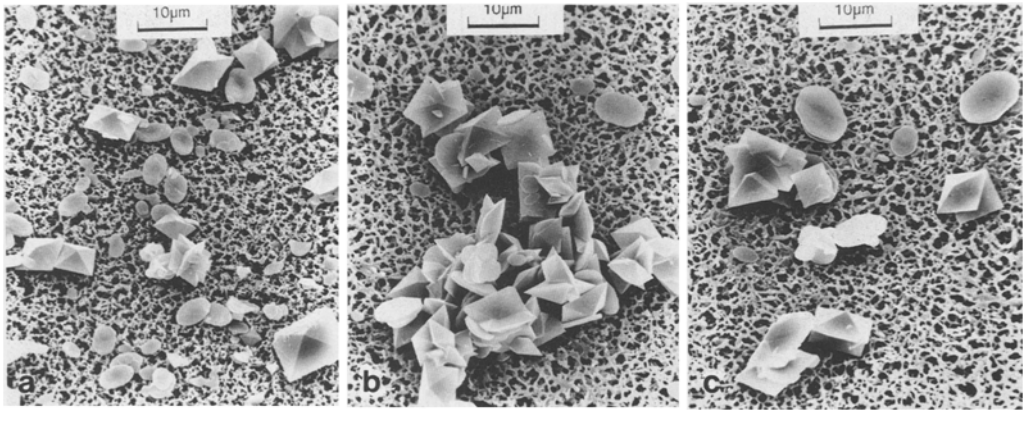

Fig. 9. Scanning electron micrographs of the crystals precipitated from the urine samples whose particle volume data are depicted in Figs. 7 and 8. **a** SF, **b** UF, and **c** UF + THM

not compensate for the removal of these macromolecules: the volume of material deposited in the presence of this glycosaminoglycan did not differ significantly from that deposited in the ultrafiltrate. Similarly, CS had only a minor effect on crystal aggregation: there was only a very slight shift in the position of the volume distribution curve to the left, and this was not sufficient to be qualitatively apparent on the scanning electron micrographs. It is reasonable to conclude on the basis of these observations that CS is unlikely to contribute a significant proportion of urinary inhibitory activity in vivo. This conclusion is noteworthy, in view of previous reports that CS is a potent inhibitor of calcium oxalate crystal growth [3, 29, 31] and aggregation [31] in defined inorganic media. This discrepancy highlights a problem that has been widely recognized for a number of years [11] namely, that an inhibitor's effect in a dilute, aqueous medium may bear no resemblance to its influence on crystallization in undiluted urine, and may therefore, as a consequence, provide no information as to its likely relationship to stone formation in vivo.

A similar discrepancy was observed with HSA. It has previously been shown that HSA is an efficient inhibitor of calcium oxalate crystal aggregation in an aqueous inorganic medium [7], with a concentration as low as 5 mg/l causing complete inhibition. Its effect on crystal growth was less marked: the same concentration inhibited growth by approximately 15%. In the present study, HSA at a concentration of 20 mg/l had a relatively minor effect on the size of crystals and aggregates precipitated from ultrafiltered urine, reducing the average diameter from approximately 11 μm to 10 μm. This finding and the corroborative scanning electron microscopic evidence indicate that HSA accounts for only a portion of the inhibitory effect of urinary macromolecules on crystal aggregation, since the mean diameter of the particles precipitated from the corresponding spun and filtered urine was approximately 6.4 μm. At the same time, the volume of crystalline material deposited in the presence of HSA was slightly, but nonetheless significantly, increased. These observations suggest that in undiluted urine HSA exerts little or no direct influence on the deposition of crystalline calcium oxalate. In fact, its slight inhibitory effect on crystal aggregation results indirectly in a small increment in the amount of calcium oxalate precipitated. This increase could not be attributed to precipitated protein, since controls which contained HSA, but which lacked any additional oxalate, showed no increase in particle volume during the incubation period. A further indication that the increase in volume in the presence of HSA reflected a true enhancement of solute deposition can be found in a similar previous study using human serum [8]. In that case, the small augmentation of particle volume was shown, by concurrent analysis for calcium depletion, to be the result of an increased precipitation of crystalline calcium oxalate. More importantly, this observation indicates clearly that HSA was not responsible for the effect that the component macromolecules had on particle volume deposition in this particular urine specimen: ultrafiltration in this case caused an increase in particle volume – a sure sign that, collectively, the macromolecules removed were inhibitors of crystal growth.

Taken together, these findings, like those from the CS experiments, strongly suggest that HSA accounts for little, if any, of the inhibitory effects of urinary macromolecules on calcium oxalate crystallization in undiluted urine. This conclusion is further supported by the observation of Morse and Resnick [25], confirmed in our own laboratory (unpublished observation), that despite its abundance in human urine, HSA is found in only very small amounts, and often not at all, in calcium oxalate crystals freshly generated from human urine. It is therefore unlikely to be of aetiological significance in vivo, notwithstanding its potent inhibitory effects in inorganic media.

Unlike CS and HSA, which have previously been regarded only as inhibitors of calcium oxalate crystallization, THM has been reported to have contradictory effects. Thus, Hallson and his colleagues [15, 16, 30] reported that the addition of THM to ultrafiltered urine concentrated by evaporation promoted the formation of calcium oxalate crystals. On the other hand, results of studies based on inorganic solutions have indicated that THM is an inhibitor of calcium oxalate crystal nucleation [19], growth [10, 19, 35] and agglomeration [35]. The disagreement in the findings relating to THM may possibly be a consequence of differences in the methodology used: to date, promotion has been reported in concentrated ultrafiltered urine and inhibition in aqueous solutions. In the present study, using ultrafiltered urine which had not been concentrated by evaporation, THM markedly increased the volume of material precipitated and significantly inhibited the formation of crystal aggregates. Before discussing these findings, it is necessary to emphasize the difficulties associated with measuring crystal volume in the presence of THM.

In the experiments reported here, controls supplemented with the macromolecule being tested, but to which no oxalate was added, were routinely included to check for non-crystalline particle precipitation during the incubation period. Such controls containing HSA and CS recorded only negligible counts. However, those containing THM registered relatively high volumes which accounted for approximately 17% of the total volume increase occurring in the presence of both THM and oxalate. This background volume can be attributed to particles of THM in the ultrafiltered urine. THM is a novel molecule whose physical state in urine is known to be affected by pH, ionic strength and its own concentration [24], which cause it to polymerize and in more extreme conditions to flocculate into large particles visible to the naked eye. Urinary particle content has been shown [5] to correlate well with THM concentration, and it has been known for some years that THM is effectively removed from human urine by low-speed centrifugation [4] and Millipore filtration [37]. THM particles present a major problem to the estimation of crystal size and volume by Coulter Counter analysis, since the instrument is unable to distinguish between different types of particles. Furthermore, although it is theoretically possible to account for the volume of the THM particles by the inclusion of the

type of control used here, it is apparent that values so corrected do not reflect true crystal deposition. Grover et al. [14] have shown by using [^{14}C]-oxalate analysis that THM has no effect on crystalline calcium oxalate deposition in ultrafiltered urine, despite the fact that parallel Coulter Counter measurements indicate a significant increase in particle volume. It may be concluded, therefore, that the augmented deposition of particle volume caused by the addition of THM to ultrafiltered urine in the present study cannot be ascribed to an increased precipitation of crystalline calcium oxalate: one possibility is that the precipitation of THM from solution is encouraged in the presence of calcium oxalate crystals [14]. Thus, THM does not appear to promote calcium oxalate crystal formation under normal urinary conditions. Furthermore, despite the problems associated with the counting of particles, Coulter Counter analysis demonstrated clearly that the size of the particles precipitated in the presence of THM, whether organic or crystalline or composites of the two, were considerably smaller than those deposited in the corresponding ultrafiltrate. This observation was supported by scanning electron microscopy which showed that the reduction in size reflected a decrease in the extent of crystal aggregation. In fact, crystal particles deposited from the ultrafiltered urine in the presence of THM were indistinguishable from those precipitated from the spun and filtered urine. However, despite this, THM cannot account for the general observation that crystals deposited from ultrafiltered urine are more highly aggregated than those from spun and filtered, since THM is not present in the latter [4, 37]. Nonetheless, its effects observed here, and its high natural concentration in urine, suggest that it should still affect the size of calcium oxalate crystal particles formed in vivo, by interdicting the aggregation of individual crystals into large clusters. The fact that THM is not incorporated into calcium oxalate crystals generated from whole human urine (our own unpublished data) demonstrates that, unlike HSA and CS [23], it does not adsorb strongly on to the surface of these crystals. It is probable, therefore, that its strong inhibitory influence on crystal aggregation can be ascribed to its physical characteristics in urine, which by steric hindrance, rather than by physicochemical adsorption, prevent newly formed single crystals from becoming close enough to allow aggregate formation. It is thus reasonable to conclude that in vivo THM may fulfil an important role in preventing calcium oxalate urolithiasis, although under conditions of extreme dehydration its tendency to flocculate could perhaps initiate the disease by promoting the deposition of calcium oxalate from urine [14–16, 30], or by trapping calcium oxalate crystals in its large gel-like flocs, thereby causing tubule blockage.

In summary, therefore, the results of this study have confirmed that, collectively, macromolecules of molecular mass greater than 10 kDa strongly inhibit the aggregation of calcium oxalate crystals formed in undiluted human urine in response to an oxalate load. Further, they have shown that this inhibitory effect cannot be ascribed to CS, HSA or THM – the most abundant macromolecules present in human urine. Whether the inhibition of crystal aggregation depends on the activity of a single, untested macromolecule, or on the combined action of a number of them remains to be seen. Whatever the case, it is apparent that macromolecules whose identities as yet are not established are likely to fulfil an important function in vivo; further studies should be aimed at identifying them and determining their anatomical source.

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