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A. L. Rodgers
University of Cape Town, South Africa

D. Ball
University of Cape Town, South Africa

W. Harper
University of Cape Town, South Africa

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EFFECT OF URINARY MACROMOLECULES AND CHONDROITIN SULPHATE ON CALCIUM OXALATE CRYSTALLIZATION IN URINE

A.L. Rodgers[1,*], D. Ball[2] and W. Harper[2]

Departments of Chemistry[1] and Physics[2], University of Cape Town, South Africa

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Abstract

After filtration and ultrafiltration (10 kD) of 24 hour urine specimens from 12 healthy male subjects, calcium oxalate crystallization was induced in the filtered (FILTD) and ultrafiltered (UF) fractions by administration of a sodium oxalate load. In addition crystallization was also induced in UF fractions to which physiological quantities of chondroitin sulphate (CHON) had been added (UF+CHON). The rate of calcium oxalate crystallization was determined by measuring the rate at which turbidity increased. Crystal numbers and sizes were measured with a Malvern particle size analyzer and by scanning electron microscopy.

Crystallization rates, crystal numbers and crystal sizes were generally lower in UF fractions than in FILTD fractions suggesting that urinary macromolecules are promoters of calcium oxalate crystallization. No increase in crystallization rate, crystal numbers or sizes occurred when chondroitin sulphate was added to UF fractions, indicating that the promoter activity of urinary macromolecules is not due to this particular glycosaminoglycan. On the contrary, crystallization rates were qualitatively lower in UF+CHON fractions than in UF fractions alone, suggesting a possible inhibitory role for chondroitin sulphate in real urine. Scanning electron microscopy revealed that while aggregates were present in UF and FILTD fractions, they were absent in UF+CHON fractions. This observation indicates that chondroitin sulphate might be an inhibitor of calcium oxalate crystal aggregation in real urine.

Key Words: Urinary macromolecules, chondroitin sulphate, calcium oxalate, crystallization, inhibitors, promoters, crystallization rate, turbidity measurements, Malvern Particle Size Analyzer.

*Address for correspondence:
A.L. Rodgers,
Department of Chemistry, University of Cape Town,
Rondebosch South Africa 7700
Telephone Number: (27)(21)6502572
FAX number: (27)(21)6503788

Introduction

The role of urinary macromolecules (UMM) in promoting or inhibiting calcium oxalate (CaOx) urolithiasis has been the subject of intensive investigation in recent years. Although several kinds of crystallization systems have been employed in such studies, two main approaches have been applied with respect to UMM in solution. The first of these has been to examine the collective effect of UMM within certain molecular weight (MW) ranges. This has been achieved by removal or concentration of UMM by methods such as ultrafiltration, followed by various crystallization experiments involving the ultrafiltrate or the retentate or both [1, 2, 5, 6, 7, 9, 12, 17, 20, 21, 25, 30]. Conclusions in these experiments are thus based on observations recorded in the absence or presence of the entire range of UMM which has been excluded or included as a result of the particular procedures employed. In the second approach, individual UMM have been added to real or synthetic urines and their effects on subsequent crystallization processes have been monitored [3, 5, 8, 10, 11, 13, 16, 18, 19, 22, 23, 24, 26, 27, 29, 34].

Unfortunately, neither approach has yielded consistent results. For example, some studies involving the collective effect of UMM have found them to be inhibitors of CaOx crystal formation [1, 2, 6, 7, 9, 17, 21] while others have found them to be promoters [5, 25]. In some cases, a dual promotion-inhibition role affecting different crystallization mechanisms (nucleation and growth) have been reported [12, 20, 30].

Similarly, contradictory roles have been advocated for individual macromolecules. For example, Tamm-Horsfall mucoprotein has been reported as being a promoter [5, 12, 24, 34] and inhibitor [8, 12, 29, 34] of CaOx crystallization and is the subject of a review which addresses this very anomaly [14]. Another example is afforded by chondroitin sulphate (CHON) which is the most abundantly occurring glycosaminoglycan in urine [32]. Most studies involving this urinary macromolecule are in agreement that it is an inhibitor of CaOx crystal growth [3, 8, 18, 22, 23] and an inhibitor of CaOx crys-

tal aggregation [3, 10, 22, 23, 27]. However, there have been other reports showing that CHON is not an inhibitor of aggregation [18], but is, a promoter of growth [16], an inhibitor of nucleation [16], and a promoter of nucleation [23]. Moreover, it is noted that all of these studies, including those which are in agreement that CHON is an inhibitor of growth and aggregation have been limited to investigations in inorganic solutions. It is interesting that studies using real urines have failed to find any inhibitory activity that could be attributed to this urinary macromolecule [11, 26]. However, a recent study involving urine-derived CHON has demonstrated enhancement of CaOx crystal nucleation in urine ultrafiltrates [31].

In the present study, aspects of both the "collective" and "individual" approaches were applied to a series of real urines. The first objective was to examine how the crystallization of CaOx in real urine would be affected by removal of UMM of MW > 10 kD (kilo-Daltons). The second objective was to investigate the extent to which the addition of CHON to ultrafiltered (UF) fractions could restore the properties of the latter to those of the original filtered (FILTD) urine.

Materials and Methods

Twenty-four hour urines were collected by each of 12 healthy male subjects in the age group 23 to 46 years (mean age 26 years). Urines were collected in glass bottles and were stored at 4°C during the 24 hour period, a method that has been widely used in numerous studies [2, 6, 12, 21, 26, 27, 30]. No preservative was added. Particulate matter was removed by passing each specimen through a 74 μm sieve, a Millipore AP prefilter and a Sartorius cellulose acetate 0.45 μm filter. This urine fraction is henceforth referred to as FILTD.

The limit of calcium oxalate metastability in each urine was determined by titration with sodium oxalate. In this method, successive samples of the particle-free urine (FILTD) at 38°C were inoculated with increasing concentrations of aqueous sodium oxalate (NaOx) until detectable crystallization occurred. The extent of crystallization after each inoculation was measured by means of an AQUALYTIC AL 1000 turbidimeter while the metastable limit (MSL) itself was taken as the NaOx concentration at which the sudden increase in turbidity occurred (turbidity measurements are reported in terms of "turbidity units", tu).

Particle-free urine specimens were ultrafiltered using an Amicon stirred ultrafiltration cell containing a Diaflo Type YM10 membrane with a MW cut off of 10 kD. Nitrogen gas was used to maintain a pressure of 3.7 atm (2812 torr) within the cell. Concentrations of low molecular mass ions and inhibitors were assumed to have been unaffected by ultrafiltration since Edyvane *et al.* [6] have established that calcium, magnesium, phosphate, oxalate, urate, pyrophosphate and citrate concentrations remain unchanged after this procedure.

Calcium oxalate crystallization was induced in the FILTD and UF fractions at 38°C by inoculation with NaOx corresponding to the previously determined MSL of the former. By administering the same NaOx load to each fraction in this way, we could effectively compare the crystallization response of each to this challenge. In addition, CHON (sodium salt, type A; Sigma Chemical Co.) was added to UF fractions which were then inoculated with NaOx as before; the amount of CHON added was such that the final concentration of the salt was 10 mg l^{-1} which is within the normal concentration range reported by Ryall *et al.* [27] for glycosaminoglycans in males.

After inoculation with NaOx, the rate of calcium oxalate crystallization in each of the fractions FILTD, UF, and UF+CHON was measured by continuously monitoring the turbidity as a function of time. The rate at which turbidity increased (as determined from the gradient of the linear portion of turbidity versus time graphs) was taken as the rate of CaOx crystallization. This is similar to the approach adopted by Ryall *et al.* [28] for their plots of particle volume vs time. Best fit straight lines were calculated by linear regression analysis using interactive outlier rejection (Statgraphics, Version 4, Statistical Graphics Corporation, U.S.A.). The Wilcoxon signed rank test was used to compare the crystallization rates obtained from the slopes of these lines.

A further assessment of the extent to which crystallization occurred in each fraction was achieved by Malvern particle size analysis [4]. The principle upon which this technique is based is that of light scattering by particles in a suspension. In the present study, the various fractions of each urine were inoculated with NaOx as previously described and particle numbers (percentage volume concentration - pvc) and particle sizes (volume mean diameter - vmd) were determined 120 minutes thereafter. A MALVERN 3600 EC particle size analyzer was used for this purpose.

At the completion of each kinetic experiment (about 90 minutes after administration of the NaOx load), each urine fraction was spun for 30 minutes in a Labofuge 200 centrifuge (Heraeus) operating at 3500 revolutions per minute. The deposited crystals were removed by repeated aspiration using a Pasteur pipette. Drop amounts were filtered through a 0.2 μm Nucleopore filter (13 mm diameter) supported in a Sartorius membrane filter clamp. Thereafter, filter papers, with deposited crystals, were pasted onto aluminium stubs. These were coated with approximately 100 nm of Au/Pd at a pressure of about 1.3 mPa in a Balzer's vacuum coater equipped with planetary sample rotator. Specimens, tilted at 35°

Table 1. Calcium oxalate crystallization rates (tu min^{-1}) after addition of sodium oxalate.

Sample no.	UF[1]	FILTD[1]	UF+CHON[2]
1	0.03	0.13	0.03
2	0.03	0.02	0.06
3	0.02	0.02	0.01
4	0.13	0.54	0.07
5	0.09	0.44	0.04
6	0.03	0.05	0.05
7	0.19	0.34	0.04
8	0.04	0.06	0.06
9	0.12	0.42	0.04
10	0.10	0.14	0.08
11	0.01	0.06	0.01
12	0.06	0.05	0.02

[1]UF: ultrafiltrate; FILTD: Filtered;
[2]UF+CHON: ultrafiltrate + chondroitin sulphate

FILTD significantly greater than UF ($p < 0.005$);
FILTD significantly greater than UF+CHON ($p < 0.005$);
UF versus UF+CHON: no significant difference.

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to the collector, were examined using a Cambridge S200 scanning electron microscope operating at an accelerating voltage of 10 kV and a beam current of 100 μA.

Results

Calcium oxalate crystallization rates (as reflected by the rate at which turbidity increased) in UF, FILTD and UF+CHON fractions are given in Table 1. It is noted that rates in FILTD fractions are significantly higher than in UF ($p < 0.005$) and UF+CHON ($p < 0.005$) fractions. There is no significant difference between UF and UF+CHON rates.

For Malvern measurements, particle numbers (Table 2) and particle sizes (Table 3) were indeterminately low in five UF and in six UF+CHON samples. However, finite values were obtained in all FILTD fractions. Because of the indeterminately low values, statistical analyses of the Malvern data could not be performed. However, when fractions from the same urine are individually compared, it is seen that particle numbers in all FILTD samples are greater than those in the corresponding UF and UF+CHON fractions. No obvious trend in particle sizes (vmd values) were observed.

Scanning electron microscopy (SEM) revealed several noteworthy features in the various fractions. FILTD specimens were characterized by small quantities

Table 2. Malvern percentage volume concentrations (pvc) after addition of sodium oxalate.

Sample no.	UF	FILTD	UF+CHON
1	-	0.0035	-
2	-	0.0019	-
3	-	0.0033	-
4	0.0071	0.0209	0.0118
5	-	0.0387	0.0146
6	0.0039	0.0066	0.0027
7	0.0002	0.0087	-
8	0.0037	0.0079	0.0124
9	-	0.0064	-
10	0.0031	0.0125	0.0008
11	0.0004	0.0029	0.0001
12	0.0003	0.0053	-

- : indeterminately low value

Table 3. Malvern volume mean diameters (μm) after addition of sodium oxalate.

Sample no.	UF	FILTD	UF+CHON
1	-	21	-
2	-	26	-
3	-	19	-
4	35	14	33
5	-	12	19
6	3	3	3
7	10	18	-
8	8	14	21
9	-	8	-
10	27	33	13
11	4	14	3
12	8	7	-

- : indeterminately low value

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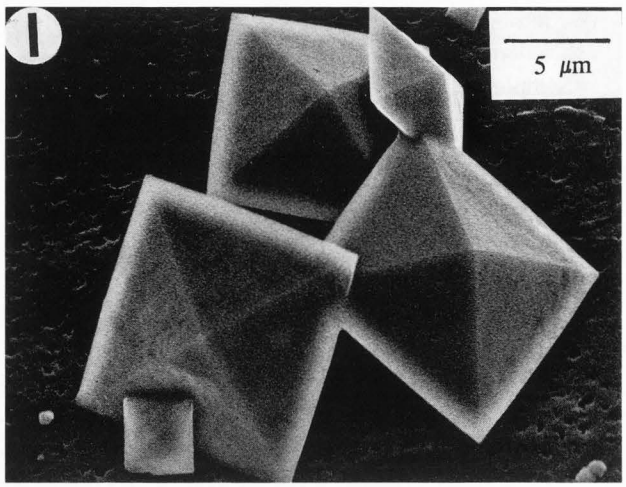
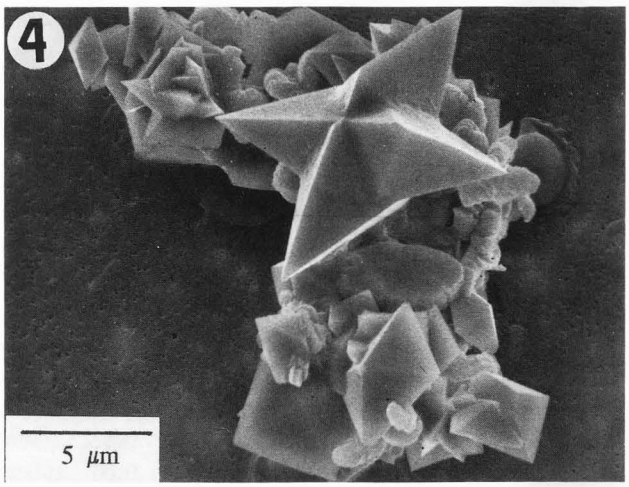
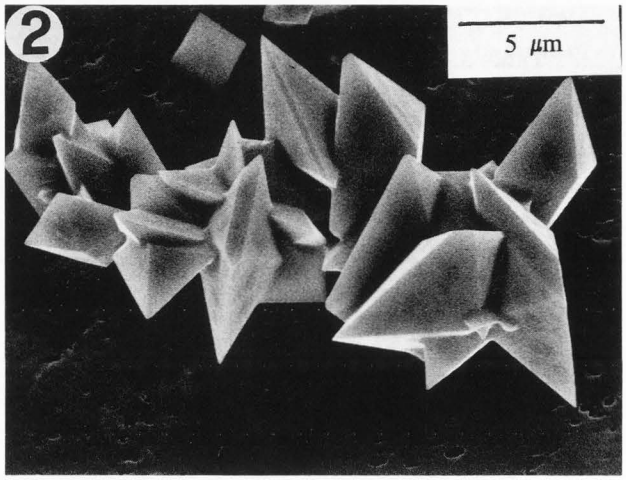
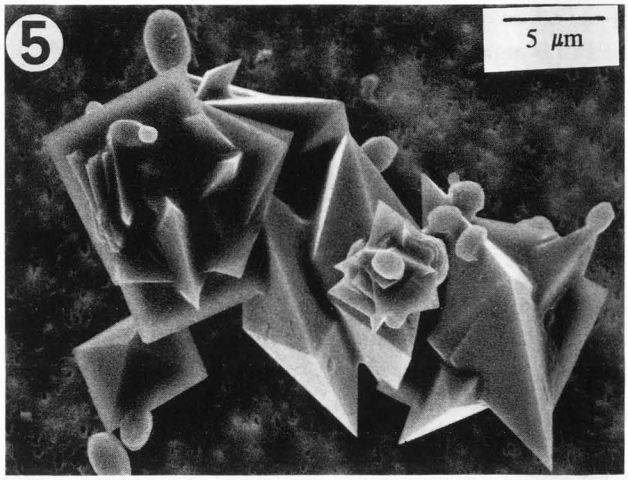
of small, single, calcium oxalate dihydrate (COD) crystals in the size range 5-10 μm (Fig. 1). Small aggregates of COD crystals were occasionally observed (Fig. 2). No calcium oxalate monohydrate (COM) deposits were detected. The quantity of COD crystals in UF fractions was either equal to or less than those in FILTD fractions. Crystal sizes were similar. However, aggregates appeared more frequently in UF relative to FILTD; these consisted of crystals which were less than or equal to 10 μm in cross-section. Examples are shown in Figures 3 and 4. Another feature of UF fractions was the presence of small, oval-shaped COM deposits (Fig.









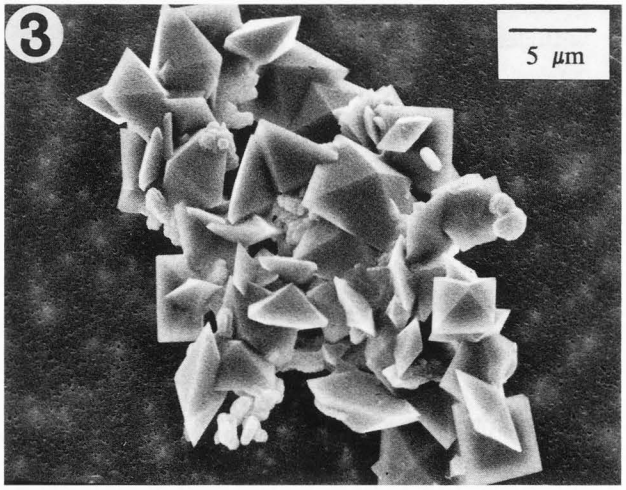


Figure 1. Single COD crystals, typical of those observed in FILTD urines after inoculation with NaOx.

Figure 2. Small aggregate of COD crystals, typical of those observed in FILTD fractions after inoculation with NaOx.

Figure 3. Aggregate of COD crystals, typical of those which were frequently observed in UF fractions after inoculation with NaOx.

Figure 4. Star-shaped aggregate of COD crystals in contact with an aggregate of smaller COD crystals observed in UF fraction after inoculation with NaOx.

Figure 5. Individual and aggregated COD crystals as well as small oval-shaped COM crystals typical of those observed in UF fractions after inoculation with NaOx.

5). UF+CHON fractions showed the presence of fewer crystals than in the other fractions; these were small, single and COD.

Discussion

The turbidity data clearly show that removal of UMM (MW > 10 kD) from undiluted urine results in decreased crystallization rates in UF fractions and that addition of CHON to the latter does not restore its crystallization potency. It is therefore concluded that, collectively, UMM (MW > 10 kD) are promoters of calcium oxalate crystallization (in agreement with the findings of other workers [5, 20, 25, 30]) and further, that CHON is not independently responsible for this promotion. Of course, CHON might behave in a totally different manner if other UMM's were present. Further experiments involving the re-addition of all removed UMM are required to resolve this question.

In addition, the potential role of Tamm-Horsfall mucoprotein (THM) must be considered. Although our filtration procedures will have removed the polymerized form of the mucoprotein [33], the possibility exists that other forms of THM might have been allowed to pass through. However, it is noted that other workers [12, 26], like ourselves, have not taken this possibility into account. The role of the THM which might not have been eliminated by filtration will also have been influenced by the ionic strength of our urine samples [14]. Since the latter were all obtained from normal male subjects and were not chemically tampered with, their ionic strengths are likely to have been fairly constant [15].

Further consideration of the results in Table 1 reveals that in 9 of the 12 samples, crystallization rates in UF+CHON fractions are either equal to, or less than those in UF fractions. Although these differences are not statistically significant, the qualitative trend suggests that CHON is an inhibitor of CaOx crystallization. The fact that particles could not be detected by Malvern techniques in several UF and UF+CHON samples, while finite counts (Table 2) and sizes (Table 3) were registered in FILTD samples, may be interpreted as qualitative support for our conclusions that UMM (MW > 10 kD) are promoters of CaOx crystallization and that CHON does not independently contribute towards this process.

Our SEM studies indicate that the number of single crystals deposited in the various fractions increased in the sequence UF+CHON < UF < FILTD. This is in agreement with our Malvern data which showed that FILTD fractions contained detectable particle numbers while the other fractions (UF and UF+CHON) did not (Table 2) and thus supports our hypotheses that UMM are promoters of CaOx crystallization and that CHON macromolecules are inhibitors. Crystal sizes, as observed by SEM were found to be similar in all fractions and is thus in qualitative agreement with our vmd data.

The appearance of aggregates in both UF and FILTD fractions is noteworthy since, on one hand, it contradicts studies which have reported that UMM, collectively, are inhibitors of aggregation in inorganic solutions [17] and real urine [6, 26]. The latter studies, both from the same laboratory, also provide supportive SEM evidence. On the other hand, however, aggregates did appear more frequently in the UF fractions thereby lending support to these studies.

Of some importance in our SEM studies is the observation of single (as opposed to aggregated) crystals in the UF+CHON fractions as it indicates that CHON may be an inhibitor of CaOx aggregation in real urine. Mention has already been made of the fact that such a role for CHON has been demonstrated in inorganic solutions [3, 10, 22, 23, 27] but not in real urine [11, 26]. However, our results in this context are merely speculative as it can be conversely argued that since crystal numbers decrease in the presence of CHON, the probability of secondary aggregation will also decrease. Thus, the absence of aggregates is not necessarily a direct consequence of inhibition of aggregation.

Another SEM feature worthy of comment is the appearance of COM crystals in UF fractions. We are not sure of the significance of this observation but suggest that it might be related to slower crystallization rates in these fractions culminating in the formation of the thermodynamically most stable hydrate. It is noted that, although COM crystals have been observed in other studies [6, 26], no inferences were drawn by those authors.

An aspect of the present study that deserves comment concerns our use of a commercial preparation of CHON. Since degree of sulphation and charge density can influence the role played by this glycosoaminoglycan in CaOx crystallization processes, it is recognized that it would be more meaningful to use chondroitin fractions isolated from fresh human urine. However, despite this, it is noted that commercial products have been widely used by other workers [10, 11, 19, 27, 29].

The results of this study have thus shown that UMM (MW > 10 kD) are promoters of CaOx crystallization and that CHON does not contribute towards this promotion. On the contrary, there is independent evidence (turbidity, Malvern, and SEM) to suggest that this glycosaminoglycan may play an inhibitory role in CaOx crystallization processes in real urine.

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Discussion with Reviewers

W.G. Robertson: What are the counting limitations of the Malvern 3600 EC Particle Sizer both in terms of particle diameter and slurry density?

Authors: The lower detection limit for particle diameter is 1.9 μm. Thus, "indeterminately low values" referred to in Tables 2 and 3 correspond to particles smaller than 1.9 μm. The Malvern does not give values for the slurry density. However, obscuration values are directly related to the latter and analyses are restricted to suspensions in which this value lies between 0.05 and 0.3. In this way, multiple scattering can be regarded as insignificant.

W.G. Robertson: Why was calcium oxalate dihydrate the predominant crystal formed since, under conditions of high oxalate concentration, it is usually calcium oxalate monohydrate that is formed?

Authors: In general, the products of crystallization depend on a multitude of factors. Although high (endogenous) oxalate concentrations produce the monohydrate, our experiments involved administration of an acute oxalate challenge which produced the dihydrate. Indeed, our experience is that this method of crystal induction always produces the dihydrate (as well as the trihydrate) while the monohydrate is seldom observed. Thus, the formation of the higher hydrates occurs very quickly but may transform to the thermodynamically more stable monohydrate with the passage of time. Perhaps, the transformation had not commenced by the time we conducted our SEM investigation.

F. Grases: Is it possible to relate inhibitory effects of CHON with some particular value of urinary pH? If affirmative, how is this relation explained?

Authors: We did not investigate the inhibitory potential of CHON as a function of pH. Indeed, it is likely that some relationship does exist between these two parameters. However, we elected to use each urine at its natural pH without any adjustment thereof. pH values for our subjects did not show significant variation thereby precluding a retrospective investigation of this point.

F. Grases: Can the authors assign the inhibitory action of CHON to nucleation processes rather than to crystal growth inhibition?

Authors: Yes. Since crystal numbers are lower when CHON is present, it can be postulated that CHON is an inhibitor of nucleation. In addition, turbidity is strongly dependent on particle number as opposed to particle size. Thus, our kinetic data also point to inhibition of nucleation.