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INFLUENCE OF AGEING, pH AND VARIOUS ADDITIVES ON CRYSTAL FORMATION IN ARTIFICIAL URINE

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Abstract

In order to investigate the effect of various factors on urinary crystallization processes, a series of five experiments was carried out using an artificial urine (AU) in a rotary evaporator. The influence of ageing, pH and organic, inorganic and potential inhibitory additives formed the basis of the study. Precipitates were characterized by X-ray powder diffraction, scanning electron microscopy and energy dispersive X-ray analysis. In the ageing experiment, AU aliquots, adjusted to various pH values, were allowed to stand for several days and were not evaporated. Calcium oxalate monohydrate (COM) was formed at low pH, while whitlockite, apatite and struvite occurred at pH > 7. In the second experiment, AU aliquots at various pH values, were evaporated. Similar results to those of series 1 were recorded but, in addition, calcium oxalate trihydrate (COT) precipitated in the pH range 3 to 6.5 and brushite at pH > 5.5. In series 3, uric acid, creatinine and urea were included in AU aliquots (pH 5.5) which were subjected to evaporation. Uric acid promoted the formation of uric acid dihydrate; however, when present with creatinine, dihydrate formation was inhibited. Urea appeared to inhibit precipitation. In the fourth experiment, MgO, methylene blue and chondroitin sulphate A were independently included in the AU (pH 5.5). Precipitates of calcium oxalate mono-, di- and trihydrates were obtained. In the final experiment fluoride aliquots of variable concentrations were included in the AU (pH 5.5 and 6.5). COT crystals of superior quality to those observed in control solutions were obtained.

KEY WORDS: Calcium oxalate, artificial urine, ageing, pH, inhibitors, promotors.

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Introduction

Many different methods have been employed in the study of the physico-chemical factors governing calcium oxalate crystallization. Several workers have examined diffusion-limited systems in which component ions are permitted to diffuse into a stagnant urine solution causing encrustations to grow on fibres suspended in the medium [43]. Chemical supersaturation is reached by the gradual addition of ions. This may be achieved using a simple 'paper wick technique' [42] or more sophisticated gel-systems [1]. Others have examined the inhibitory effects of certain ions and compounds, again employing static supersaturated systems [29]. On the other hand, the concept of the urinary tract as a biological analogue of a sequence of continuous crystallizers has enjoyed much support since first described by Finlayson [10]. Many studies have been conducted in such mixed suspension mixed product removal (MSMPR) crystallizers [26,34,35,38]. Use of a Coulter counter has permitted the study of the qualitative and quantitative effect of inhibitors on nucleation and particle growth [7]. As a result, *in vitro* measurements of crystal size distributions have enabled comparisons to be made between the urines of stone formers and controls and have also permitted experimental examinations of changes in standard synthetic urines after the addition of inhibiting substances [36]. Hallson and Rose employed another approach in which human urine samples were subjected to rapid evaporation at 37°C [17]. They suggested that this is analogous to the concentration of urine in the renal tubules where water is removed and a state of supersaturation is maintained.

In the present study a series of crystallization experiments using an artificial urine (AU) in a rotary evaporator was carried out. The purpose was to investigate the influence of certain physico-chemical factors (ageing, pH, AU composition, presence of potential inhibitors and promoters) on calcium oxalate crystallization. In certain cases, non physiological conditions were employed in the hope of observing trends which might otherwise be obscured.

Materials and methods

Stock solutions of an AU (volume 1 dm^3) were prepared according to the recipe of Burns and Finlayson [5] by dissolving the appropriate mass of inorganic salts in the sequence given in Table 1. The pH was adjusted to 6.5 by dropwise addition of 5 molar ammonia solution. Prior to evaporation, components of interest were added to the stock solution and/or urinary pH was adjusted to the desired value. The solution was then allowed to equilibrate at 37°C. Aliquots were evaporated at 37°C in a rotary evaporator (ROTAVAPOR-R, Buchi Laboratoriums-AG, Switzerland) at a pressure of approximtely 25 Pa (~0.2 mm Hg). The rate of evaporation was 2 ml min^{-1}. Precipitates formed in the experiments were filtered using 0.20 or 0.45 μm PTFE filters (Sartorius SM 11807) preconditioned with methanol and were washed with 0.01M HCl, distilled water and methanol. After drying, the mass of each precipitate was determined using a 4-figure balance. X-ray powder diffraction (XRD) patterns were recorded using a Debye-Scherrer powder camera of radius 28.65 mm with Ni-filtered CuK_α radiation (wavelength λ = 1.5418 Å). Exposure times were 4 hours with the X-ray generator (Philips model 1008) settings at 40 kV and 20 mA. For scanning electron microscopy (SEM), sections of the filters measuring 1cm x 1cm were cut and glued to aluminium stubs which were then coated with a thin film (100nm) of carbon. Specimens were bombarded with a 15 kV, 100 μA electron beam in a Cambridge S 180 scanning electron microscope. An energy dispersive x-ray analyzer (KEVEX) was used for the qualitative identification of elements.

Table 1. Composition of artificial urine

Component	concentration (mols dm^{-3})
NaCl	0.1054
$NaH_2PO_4 \cdot 2H_2O$	0.0323
$Na_3C_6H_5O_7 \cdot 2H_2O$	0.0029
$MgSO_4 \cdot 7H_2O$	0.0039
Na_2SO_4	0.0170
KCl	0.0637
$CaCl_2 \cdot 2H_2O$	0.0059
$Na_2C_2O_4$	0.0003
NH_4Cl	0.0276

A series of 5 experiments was conducted. In series 1 the effect of ageing was investigated. AU aliquots, the pH values of which were varied from 3.0 to 9.0 in steps of 0.5 pH units, were not evaporated but were thermostated at 37°C in closed containers with no preservative and allowed to stand for 4 days. The pH adjustments were achieved by addition of either 5M HCl or 5 M NH_4OH. The maximum volumes required in each case constituted negligible changes of 0.7% in the chloride concentration (pH 3) and 3.8% in the ammonia concentration (pH 9). The pH and volume were not recorded at the end of the 4 day period. Samples were filtered and the precipitates analysed.

In the second set of experiments, the pH of the AU was varied as in series 1. 50 ml aliquots of the AU were evaporated until 25 ml of distillate had been collected. Precipitates were retrieved by filtration as described earlier. These experiments were repeated in duplicate.

In series 3, the composition of the urine was varied to investigate the role of individual constituents in determining the crystallization characteristics. The effect of uric acid (0.0028M), creatinine (0.0172M) and urea (0.4163M) were individually examined. Thereafter, uric acid, together with each of the other two components in turn, was included in the AU to examine their synergistic effect. In order to focus attention on calcium oxalate formation, the pH of the solution was adjusted to 5.5 in most cases. In an attempt to increase the amount of material which precipitated, the initial AU volume was raised to 150 ml, of which 100 ml was evaporated.

In the fourth series of experiments, the potential inhibitors, magnesium (as MgO, (0.0010M)), methylene blue (7.8×10^{-5}M) and chondroitin sulphate A (20 mg l^{-1}) were individualy investigated. The pH was kept constant at 5.5 and two thirds of 150 ml initial volumes were evaporated.

In series 5, the influence of fluoride on crystallization was investigated since some studies have suggested that the fluoride content of drinking water might be of importance in urinary stone formation [22]. AU's containing fluoride concentrations of 20, 50 and 100 mg l^{-1} at pH values 5.5 and 6.5 were used in these experiments. In each case two thirds of 100 ml initial volume was evaporated.

Results

The chemical formulae of the precipitated solids are given in Table 2.

Series 1. In the acidic pH range, calcium oxalate monohydrate (COM) was detected as the major component in the precipitate. Between pH 6 and 7 whitlockite (WHI) was identified while apatite (APA) appeared at pH values close to 7. Struvite (STR) was found to be present in the alkaline pH range.

Series 2. COM was formed in the pH range 3 to 5.5, together with calcium oxalate

Table 2. Chemical composition of precipitated solids

Abbreviation	Chemical or mineralogical name	Chemical Formula
APA	apatite	$Ca_{10}(PO_4)_6(OH)_2$
BRU	brushite	$CaHPO_4 \cdot 2H_2O$
COD	calcium oxalate dihydrate	$CaC_2O_4 \cdot 2H_2O$
COM	calcium oxalate monohydrate	$CaC_2O_4 \cdot H_2O$
COT	calcium oxalate trihydrate	$CaC_2O_4 \cdot 3H_2O$
OCP	octacalcium phosphate	$Ca_4H(PO_4)_32 \cdot 5H_2O$
STR	struvite	$MgNH_4PO_4 \cdot 6H_2O$
WHI	whitlockite	$Ca_3(PO_4)_2$
UA	uric acid	$C_5H_4N_4O_3$
UAD	uric acid dyhydrate	$C_5H_4N_4O_3 \cdot 2H_2O$

trihydrate (COT), the latter being the major constituent. At pH values above 5.5, brushite (BRU) and apatite (APA) were detected as well. COT (but not COM) continued to precipitate with BRU and APA at higher pH levels but finally disappeared at pH values above 6.5. Calcium oxalate dihydrate (COD) was not detected at any pH by XRD. However, small amounts were identified by SEM at pH 5.5 (Fig.1). At pH values greater than 7.5, STR was identified with BRU and APA. An example of a large STR crystal is shown in Fig. 2. The possible presence of octacalcium phosphate (OCP) was suspected at pH values 5.5 and 6 as a weak reflection at a "d" spacing of 3.20 Å was observed. (Other OCP reflections, with the exception of the 18.77 Å line, would have been swamped by reflections from APA, BRU and COT, all of which were present). However, due to the limited sensitivity of the camera and its inability to detect the low 2θ reflection at 18.77 Å, its presence could not be verified from XRD powder diffraction patterns. The total mass of precipitate formed in each experiment of this series was relatively low in the pH range 3 to 6 (< 1 mg) but thereafter increased dramatically, reaching a maximum at pH 8 (150 mg). The relative mass of COT within each precipitate decreased with increasing pH while that of COM remained fairly constant. The masses of BRU and APA deposits within the particular mixtures were greatest at pH values 6 and 7, respectively.

Series 3. The inclusion of uric acid (UA) in the AU resulted in the deposition of uric acid dihydrate (UAD). Small amounts of COD and COT were also detected. When creatinine was included in the AU, COD and COM were identified in the precipitates. However, SEM studies revealed the presence of small COT deposits (~10 μm) interspersed with small envelope-shaped COD crystals (~4 μm) and ill-defined deposits (Fig. 3) which we suspect to be COM. Evaporation of the AU solution to which both UA and creatinine had been added appeared to inhibit the formation of UAD. COD

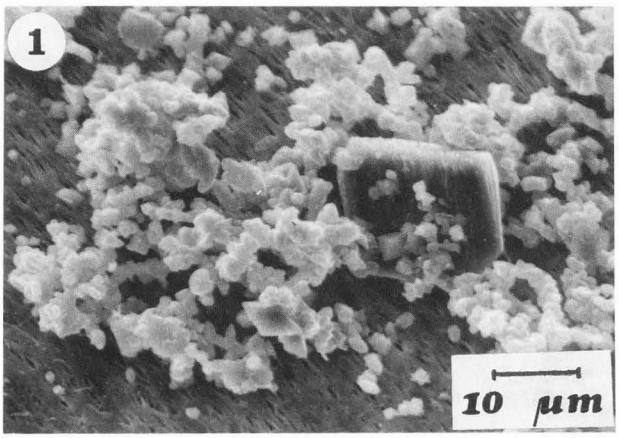

Figure 1. Large COT crystals surrounded by smaller COM and COD crystals : (pH 5.5, Series 2).

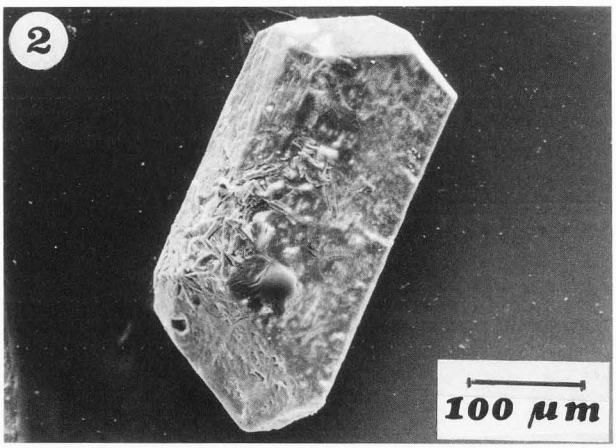

Figure 2. Large STR crystal : (pH 7.5, Series 2).

and COM were the major phases precipitated in this experiment.

When urea was included as a component of the AU, COM, COT, and a small amount of COD, were formed. This is clearly shown in Fig. 4 where layered COT deposits are surrounded by small COD crystals and debris of similar appearance to that observed in Fig. 3. The addition of urea to the UA/AU solution yielded very little precipitate. The latter gave a very weak XRD photograph, the faint reflections of which suggested the possible presence of COD.

Series 4. Evaporation of the AU containing MgO yielded no visible precipitate. However, SEM revealed the presence of a few small COD crystals (~6 μm). All three calcium oxalate hydrates were identified by XRD.

With methylene blue, no qualitative or quantitative differences in the precipitate relative to the MgO experiments were observed by XRD, while only a few tiny isolated COD crystals (~1 μm) were detected by SEM.

The addition of chondroitin sulphate A to the AU yielded a very fine precipitate of only COM as identified by XRD. However, isolated COT crystal clusters could be seen using SEM (Fig. 5). Because the only element detected by energy dispersive x-ray analysis (EDX) was Ca, it was concluded that the small deposits seen in Fig. 5 were probably COM, although very many of the typical COT crystals were seen among the smaller debris.

Series 5. The experiments conducted at pH 5.5 yielded COT and COM only. SEM showed that both crystal types were generally smaller (Fig. 6) than those precipitating from the AU (Fig. 1).

At pH 6.5, COT and COM were again precipitated. However, amorphous halos were often observed in the diffraction patterns. SEM revealed that at 20 mg l^{-1} F^- concentration, COT crystals (~14 μm) occurred in small groups. These were generally admixed with a fair amount of non-descript COM crystals and small (apatite) deposits containing Ca and P only (Fig. 7).

At F^- concentrations of 50 and 100 mg l^{-1}, the amount of APA was much greater (Fig. 8) while the size of the COT crystals remained unchanged (Fig. 9).

Discussion

The formation of COT in the evaporation experiments of the present study is in good agreement with the findings of Gardner [13] who reported COT to be the initial phase precipitating from solutions supersaturated with respect to calcium oxalate. Concomitant formation of COM through nucleation of the latter on the surface of the COT crystals has also been previously reported [39,41]. This was also observed in the present study (Fig. 10). The absence of COT in the experiments of series 1 is thought to be due to the fact that initially formed COT readily undergoes transformation to COM [14]. This transformation is completed in approximately

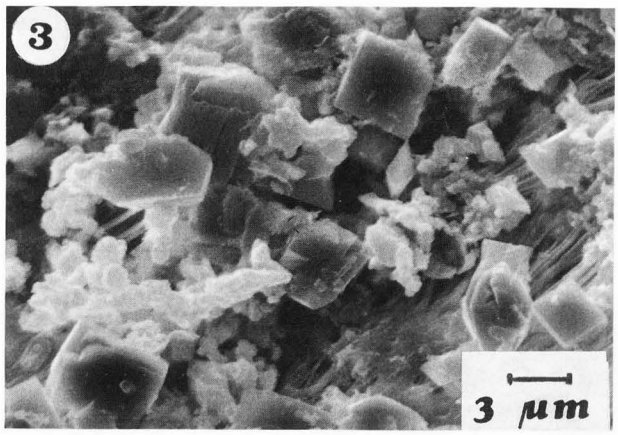

Figure 3. COT deposits interspersed with small envelope- shaped COD crystals and ill-defined COM crystals : (creatinine + AU, Series 3).

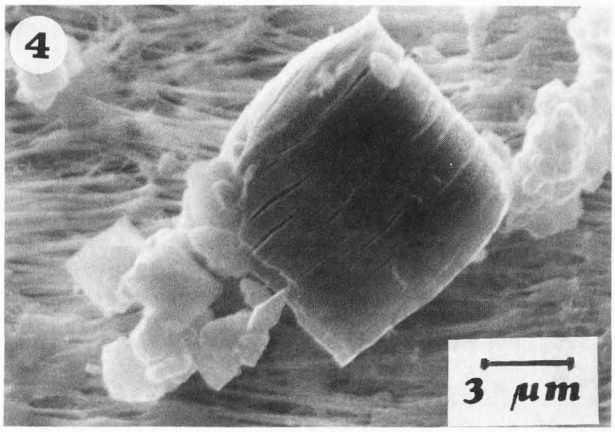

Figure 4. Layered COT crystals surrounded by COD and COM deposits : (urea + AU, Series 3).

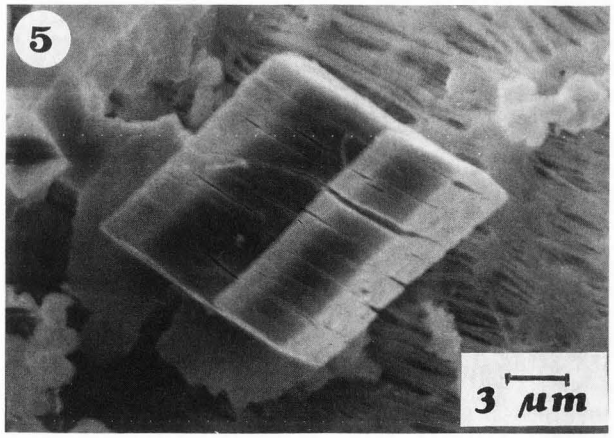

Figure 5. Isolated COT crystals : (chondroitin sulphate + AU, Series 4).

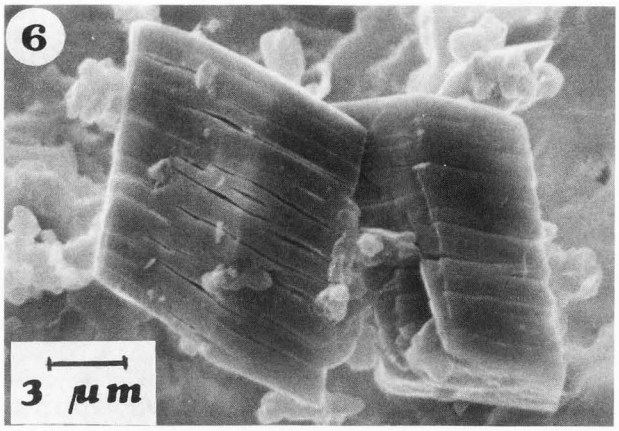

Figure 6. COT and COM crystals : (F^- + AU, pH 5.5 Series 5). Note that the crystals are smaller than those precipitating from the AU (Figure 1).

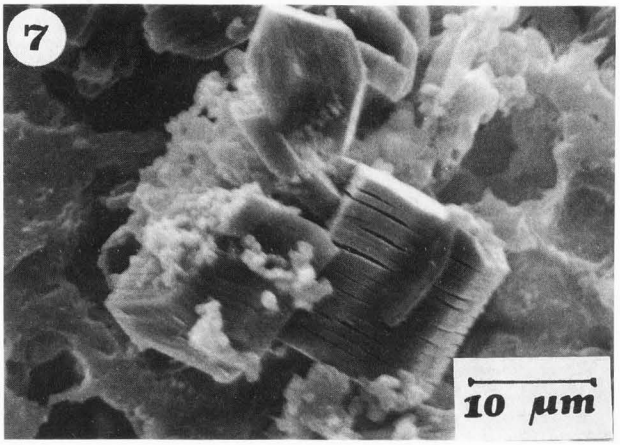

Figure 7. Small groups of COT crystals admixed with COM and APA deposits : (F^- + AU, pH 6.5, Series 5). Note the "clean" COT surfaces.

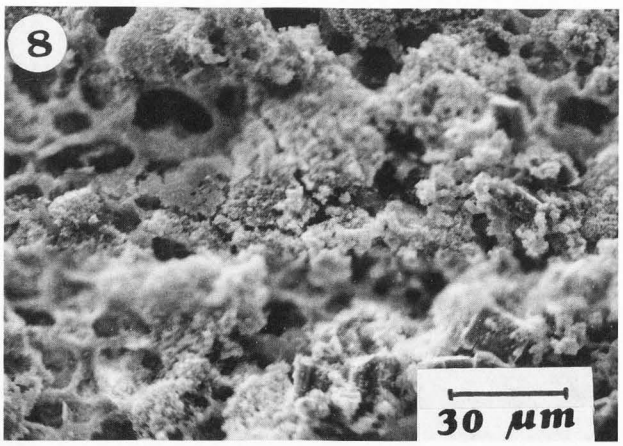

Figure 8. Survey micrograph showing profuse APA deposits: (F^- + AU, pH 6.5, Series 5).

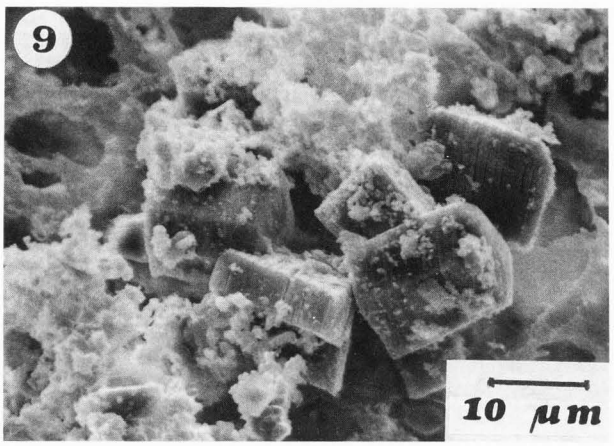

Figure 9. COT crystals encrusted with APA deposits : (F^- + AU, pH 6.5, Series 5). Note that the amount of APA is greater than that shown in Figure 6; COT crystals are the same size.

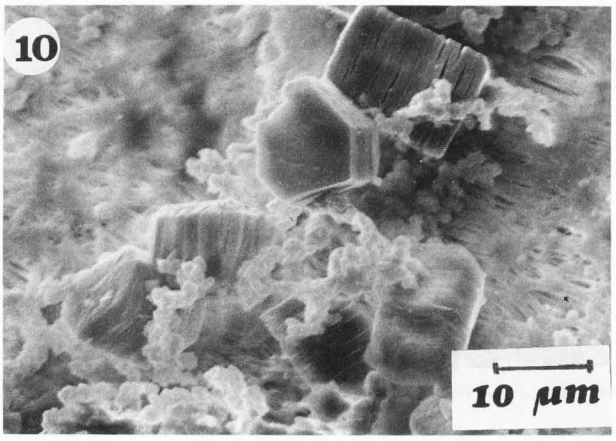

Figure 10. COT crystals surrounded by COM deposits : (chondroitin sulphate A + AU, pH 5.5, Series 4).

one day if the precipitate is allowed to stand in contact with the mother liquor and occurs much faster at elevated temperatures [31]. Finlayson [11] reported a transformation half-time for COT to COM (mediated by dissolution) of ~4.5 hrs at 37°C . The precipitation of COT over almost the entire acid range in series 2 and its absence in series 1 again suggests that this species might be a thermodynamically unstable precursor of COM [39,40].

The significance of the formation of whitlockite (WHI) in series 1 (pH range 6-7) is unclear. Although WHI is more stable than BRU or OCP, the latter compounds are favoured under most physiological conditions. However, since the conditions of series 1 more closely simulate the achievement of thermodynamic equilibrium than those of series 2, it is conceivable that kinetic factors are not

significant and that the thermodynamically more stable phases should form. It has been suggested that WHI is a precursor of HAP formation [12]. The WHI is stabilized by magnesium ions and other small bivalent cations which substitute for calcium in the crystal lattice [2]. In such cases, WHI might be even more stable than the microcrystalline HAP usually found in biological concretions. Hence its appearance in series 1 may be explained in this way.

Although COD is frequently reported as a stone constituent, it could not be detected by XRD in any precipitate in series 2. However, small amounts of tiny COD crystals were observed using SEM (Fig. 1). Other studies have reported precipitaion of COD from an artificial urine in a MSMPR crystallizer at much higher calcium and oxalate concentrations than those employed in the present study [30]. These observations are in agreement with reports by Gardner et al. that COT is the important growth phase at low and medium relative supersaturations whereas at high supersaturations the COD form is the principle growth phase [15,16].

The precipitation of calcium phosphates at pH values greater than 5.5 and magnesium phosphate at pH 7.5 (series 2) is in accordance with the finding of other workers [9,31]. According to Lagergren [24], brushite is easily precipitated from solutions at pH below 6. Spontaneous precipitation experiments by Pak [32] showed that when the pH of the supernatant fluid is less than or equal to 6.9, the solid phase is BRU. These results are confirmed by those of the present study (series 2).

Although BRU is often the first phosphate phase which precipitates over almost the entire physiological pH range, it is not a common constituent of urinary stones, its frequency of occurrence varying between 2.0 and 2.5 % [19, 25, 33]. The observation that BRU also forms a thin surface layer around some APA / STR calculi [24] led to the suggestion that BRU may serve as the crystal nidus and precursor of calcium phosphate calculi [32]. It is only at high pH values, i.e. above 7.5, that the conversion of BRU to HAP is so rapid that little or none of the former is detected [12].

Apatite, which is the most stable of all calcium phosphates, was found to precipitate over a wide pH range in the experiments of series 2. As early as 1932, Schleede et al. showed that all calcium phosphates will eventually transform into HAP when water is allowed to flush out excess phosphate radicals from the precursors' lattices [37]. This early observation might explain why APA is usually the only calcium phosphate which is co-precipitated with struvite, since the latter captures most of the phosphate, leaving little for the formation of other calcium phosphates. Since APA is often found admixed with calcium oxalate and STR, it is not surprising that it occurred over a wide pH range in the present study.

In the urinary tract STR crystals form primarily as a result of urease-induced alkalinity in infected urines. However, the formation of these crystals in the absence of infection has been shown to occur in the urine of male Sprague-Dawley rats, but their precipitation requires high urinary pH and ammonium [23]. The precipitation of STR under these conditions has been clearly demonstrated in the present study where it formed only at pH values greater than 7.5 (series 2). The increase in precipitated mass with increasing pH suggests that all the available ions are incorporated into the respective lattices after the formation of STR and APA has been initiated. It is interesting to note that STR may even form in acidic conditions [3].

In series 3 where uric acid was added to the AU, the high concentration of the former as well as the acidic pH fulfil the conditions for *in vivo* uric acid stone formation. That the dihydrate and not the anhydrous UA is formed is confirmation of the hypothesis that hydrated species precipitate more readily from aqueous solutions. However, UAD is seldom found in calculi where its detection is hampered by its spontaneous dehydration to the thermodynamically more stable anhydrous acid [20]. Borner et al. reported that high molecular weight (M > 20000) organic substances decrease the incidence of UAD in stones [4]. Since no organic substances (besides UA) were present in the AU, the sole precipitation of UAD in series 3 tends to support this idea.

The effect of urea on calcium oxalate crystallization is not yet clear. From the general SEM observation in the present study that COT crystals were smaller and occurred only singly when compared with control experiments (series 3), it is concluded that urea might show slight solubilizing action. This is in agreement with the results of Hartung *et al.* who used Coulter counter techniques to show that urea has an inhibitory effect on calcium oxalate crystal growth [18]. On the other hand, Finlayson et al. suggested that the effect of urea on divalent ion activity is sufficiently small to justify its omission from first order considerations [10]. Further experiments are therefore needed to clarify the extent to which urea plays a role in calcium oxalate formation.

In the present study, COD was found to be the major phase precipitating when creatinine was included in the AU (series 3). This might be explained by consideration of the results of Gardner and co-workers who found that various polyelectrolytes retard the growth of COM whereas compounds of high molecular weight and charge density such as heparin, inhibit COT growth [15,16]. These workers also found that growth of COD was only initially retarded by these compounds. It is perhaps not unreasonable to suggest that creatinine might act in a similar way to heparin.

In series 4, the SEM study of crystals from the AU containing methylene blue showed the presence of a few particles only. It is therefore tentatively suggested that methylene blue may be a nucleation inhibitor. This is in agreement with observations reported by others [8,30].

Although chondroitin sulphates belong to the wide range of mucopolysaccharides (glycosaminoglycans) which are thought to posses inhibitory activity in urine, no particular effect on calcium oxalate growth could be established in the present study. However, total precipitated mass was somewhat lower when compared with control experiments.

The addition of MgO to the AU also decreased the amount of precipitate. In addition, crystals were somewhat smaller than in other precipitates. These results confirm earlier reports that Mg considerably decreases nucleation and growth rates of COT [26]. The mechanism involves complexation of oxalate by means of which the activity of the latter is lowered. Since oxalate has been shown to be about 16 times more effective in increasing calcium oxalate supersaturation than calcium ions in urine-like solutions, a small decrease in free oxalate has a pronounced effect on calcium oxalate precipitation [27]. As far as the occurrence of COD itself is concerned, it is noted that urinary trace elements, amongst them Mg in particular, are known to stabilize COD. Therefore, its presence in this experiment is not surprising.

A general point about series 3 and 4 that is worth noting is that the amounts of crystalline product which formed after threefold increases in concentration were relatively small when compared to series 1 where no evaporation was carried out. This observation serves to emphasize the inhibitory role of the various additives (in series 3 and 4), even at very high supersaturation levels of the different potentially precipitating substances.

The observation that COT and COD crystals in the fluoride-spiked AU solutions (series 5) were smaller than those in the control AU suggests a growth retardation process. However, these crystals were larger than those observed in series 4. Therefore, although growth inhibition may be occurring, it is probably weaker than that produced by the inhibitors in series 4.

On the other hand, SEM revealed that the COT crystals in series 5 were generally of a superior quality to those occurring in the control-AU. This observation is of interest as it has been shown that high fluoride concentrations increase the crystallinity of bone apatite [44] and carbonate apatite [21].

The amorphous halos observed by XRD at pH 6.5 may be due to the presence of APA which was detected by SEM. Our experiments indicate that the amount of APA precipitating increases with increasing fluoride concentration. This is illustrated by comparison of Figs. 7 and 9. The former shows the typically 'clean' surfaces observed at 20 mg l^{-1} F^- concentration, while the latter is typical of the surfaces encrusted with APA at higher F^- concentrations. It is therefore suggested that fluoride favours APA deposition with increasing concentrations (at pH 6.5). This agrees with the observation that the orderly deposition of apatite is enhanced at greater fluoride concentrations [28]. However, it contradicts the findings of Christoffersen et al. who demonstrated that there exists a maximum value of fluoride concentration above which partly demineralized tooth enamel cannot be successfully repaired [6]; since the fluoride concentrations of the present study were very high, it is likely that this maximum was exceeded.

Finally, we wish to point out that a shortcoming of the evaporation system, as used in the present study, is that it is difficult to repeat from one experiment to another precisely the same evaporation rate and extent of solution concentration. Therefore our approach has been solely qualitative. Obviously, the use of a particle size analyzer such as a Coulter counter would have permitted quantitative assessments to be made. Nevertheless, despite these shortcomings, the present study has confirmed, in many instances, the results of several other workers and has produced some original and interesting results in other instances.

Acknowledgements

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Discussion With Reviewers

S.R. Khan: What gas was used during evaporation?
Authors: No gas was used. Samples were evaporated in a rotary evaporator by evacuating the chamber to a pressure of about 25 Pa (~0.2 mm Hg).

S.R. Khan: Why was creatinine tested in the crystallization system?
Authors: The composition of the artificial urine was varied to investigate the role of individual constituents as well as their synergistic effect on crystallization processes. Creatinine was one such constituent.

S.R. Khan: How did you judge the quality of calcium oxalate trihydrate crystals by SEM? I cannot tell the difference between COT crystals in various illustrations. Do you think that fluoride is incorporated into the COT lattice?
Authors: The COT crystals observed in series 5 were generally better defined and had "cleaner" faces than those of the AU. We agree that this empirical observation is not apparent from the micrographs. Although the incorporation of fluoride into the COT lattice is a possibility, we do not have any direct evidence in support of this. Nevertheless in a previous study (*J. Urol.*, 138:644-647, 1987), fluoride was found to be present in calcium oxalate mono- and dihydrate calculi.

W.G. Robertson: In the light of recent data that Tamm-Horsfall mucoprotein may interfere with the action of many of the so-called inhibitors of crystallization of calcium oxalate, what effect would you predict that this molecule would have in your system?
Authors: We would tentatively predict that THP might act as a promoter of crystal formation and aggregation as it does in whole urine (*J. Urol.*, 127: 177-179, 1982; *Urol. Res.*, 12: 217-221, 1984). If it does not act in this way, it will indicate that the role of THM in real urine is dependent on the presence of components other than those included in the artificial urine.

W.G. Robertson: From the data in the table, the initial concentrations of most ions are already normal-to-high in terms of the urinary range. Do you not feel that concentrating the system three-fold is excessive since many background factors, such as osmolarity, ionic strength and non-precipitating ion concentrations become quite unrealistic in terms of their ranges even at those sections of the urinary tract where the tubular fluid is at its most concentrated?
Authors: Yes, we agree that concentrating the system three times is excessive. However, we have commented in the text that non physiological conditions were employed in the hope of highlighting trends which might otherwise be masked or too small to be observed. Formulation of a crystallization model might very well be facilitated by investigating systems which initially are extreme or excessive.

B. Tomazic: The formation of whitlockite in series 1 is very interesting and the explanation of its formation and stabilization is plausible. What is the actual evidence of its presence? If available, the SEM and XRD comparison between whitlockite and apatite formed in series 1 would be very appropriate.
Authors: Whitlockite was identified on the basis of its X-ray powder diffraction patterns which matched the standard diffraction patterns as published by Sutor and Scheidt (*Br. J. Urol.*, 40: 22-28, 1968). No SEM evidence of its presence was found.

B. Tomazic: The finding that fluoride-spiking of AU results in formation of smaller COT and COD crystals than in control UA was interpreted as a consequence of the growth retardation process. The fact is that two processes simultaneously take place, calcium oxalate and APA formation. The APA formation is catalyzed by the presence of fluoride; therefore the effective driving forces for calcium oxalate decreases. Therefore the formation of COT crystals of superior quality is not surprising. Based on these comments, is it reasonable to make the direct comparison of growth retardants in series 4 and 5, since more than likely, different mechanisms are involved?
Authors: Your point is a good one. Since different mechanisms are involved, it does not seem reasonable to make comparisons. However, the fact of the matter is that the nett effects of the various additions on the same system are different. We regard the comparison on this basis as valid and of interest.

B. Tomazic: What is the chemical composition of APA formed in series 5? Due to high fluoride content, one may expect the co-formation of fluorapatite.
Authors: We do not know the composition of these deposits as we did not analyse them for fluoride. However, it is extremely likely that fluorapatite is present.

Reviewer IV: What percentage of water is removed from urine in the renal tubules?
Authors: With maximal urinary osmolality of 1200 m Osm per kg H_2O and a daily urine volume of 500 ml, the volume of solute-free water that is reabsorbed is 1500 ml/day. This volume is returned to body fluids during this maximal antidiuresis (*Renal and Electrolyte Disorders*, 2nd edition, ed. Schrier, R.W., Little, Brown and Company, Boston, pp 19).

Reviewer IV: Why was series 2 evaporated to a different level?
Authors: As stated in the text, the degree of evaporation in series 3, 4 and 5 was greater than that in series 2 in our attempt to increase the quantity of precipitate formed.

Reviewer IV: Why weren't the results of series 1 the same as the results of series 2?
Authors: Although the urines which were tested in series 1 and series 2 were identical with respect to composition and pH, different methods of treatment were investigated in the two series, *viz.* ageing and evaporation. In series 1 the crystals which formed did so over a relatively long period of time, without the AU being physically disturbed. However, in series 2, crystals were formed relatively quickly, after evaporation of water from the AU. These factors account for the different results in the 2 series.

Reviewer IV: In experiment series 2 which served as the control for the additive experiments detailed in series 3, 4, and 5, the solutions were evaporated to 50% of the original value, whereas in additive experiments themselves, the solutions were evaporated to 33% of the original volume. How can you be sure that the effects observed in series 3, 4, and 5 should correctly be attributed to the various additives used in these experiments?
Authors: The effects observed in each series are attributable to the various additives in that series regardless of the extent to which the solutions were evaporated. Obviously, concentrating the solutions by evaporation plays a role but this was indeed the philosophy of the experiments in the first place.