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DETERMINANTS OF WEDDELLITE FORMATION: CHONDROITIN SULFATES AND CITRATE DETERMINE WEDDELLITE FORMATION *IN VITRO*

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Abstract

In synthetic urine (SU), addition of oxalate tends to form monohydrates of calcium oxalate. However, addition of oxalate to natural urine preferably forms calcium oxalate dihydrate (COD). Urine apparently contains a determinant for COD formation. To identify the determinant, the effects of pH, temperature, oxalate, calcium, urate, citrate, magnesium, sulfate and chondroitin sulfates (CS) on calcium oxalate crystal formation were studied. Lower temperatures, higher oxalate concentrations and higher pH favored COD formation in a SU. Mixed CS in the presence of citrate were the most decisive determinant of COD formation. Substitution of CS for agar and gelatin produced similar results, indicating that the colloidal effect of the macromolecules determines COD formation. Identification of the determinants led to a simple, reproducible method of COD formation in SU without natural urine. Addition of strontium to SU resulted in dodecahedral bipyramids. Interpenetration twinning of bipyramids occur within seconds of the crystal formation.

Key Words: Weddellite, chondroitin sulfates, citrate, urate, pH, temperature, oxalate, dodecahedra, strontium, interpenetration twinning, brushite.

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Introduction

Despite its lesser thermodynamic stability, calcium oxalate dihydrate (COD) is more prevalent than calcium oxalate monohydrate (COM) in crystalluria and is nearly as common as COM in urinary stones. The mechanism of COD formation in urine is poorly understood. It has been noted that COM and calcium oxalate trihydrate (COT) are preferably formed in synthetic solutions, and the formation of COD in its pure form has been difficult to accomplish. For this reason, COM or non-specified calcium oxalate has been frequently used for the studies of calcium oxalate nucleation and growth *in vitro*; kinetic studies for COD have been scanty.

A very high Ca/oxalate ratio (Tomazic and Nancollas, 1980), the use of a continuous crystallizer (Miller *et al.*, 1977), pyrophosphate (Gardner, 1978), lower temperatures (Kuwahara *et al.*, 1982), higher pH (Martin *et al.*, 1984), the presence of citrate (Oka *et al.*, 1987) or phosphocitrate (Sallis *et al.*, 1995), certain ion compositions (Ackerman *et al.*, 1989), slow diffusion of calcium and oxalate through gel (Franchini-Angela and Aquilano, 1979) or through mucin (Akbarieh and Tawashi, 1991), and the colloidal effect of phosphate (Grases *et al.*, 1990) have been implicated as potential factors that determine COD formation *in vitro*. However, attempts to form COD in its pure form *in vitro* frequently yielded inconsistent results (Werness *et al.*, 1979; Rodgers and Wandt, 1991).

It has been noted that addition of oxalate to filtered urine (FU) (Philipsborn, 1952) or the mixture of FU with synthetic solutions (Werness *et al.*, 1979) causes precipitation of COD. Evidently, urine contains a certain component that determines the formation of COD. Human urine contains up to 150 different compounds (Mroczek *et al.*, 1971), which have hampered identification of the determinant(s) of COD formation. Furthermore, filtration of urine is a tedious task, and it is difficult to obtain FU in large quantities. In an attempt to identify the determinant, various factors, as indicated in the literature, were investigated for their effects on COD formation. Of the components tested, the presence of

Table 1. Synthetic urine.

Amount (g/l)	Solutes
3.33	Na_2SO_4
3.18	NH_4Cl
8.31	KCl
2.00	$MgSO_4 \cdot 7H_2O$
0.58	Na_2HPO_4
4.12	$NaH_2PO_4 \cdot H_2O$
9.25	NaCl
0.80	$Na_3C_6H_5O_7 \cdot 2H_2O$
1.00	$CaCl_2 \cdot H_2O$

both mixed chondroitin sulfates (CS) and citrate in a synthetic urine (SU) was found to have the primary role in COD formation. Identification of the determinants led to a simple, reproducible method of COD formation in quantities in SU without addition of natural urine.

Materials and Methods

Mixing of solutions

Analytical grade chemicals (Sigma Chemical Co., St. Louis, MO) and acid washed glassware were used throughout the study. Deionized and distilled water that was filtered through 0.2 μm pore filters was used. Since it has been said to produce COD only in a continuous crystallizer, a modified Isaacson's SU (Isaacson, 1968; Miller *et al.*, 1977) was chosen for the study (Table 1). When not otherwise specified, crystals were precipitated by pouring 100 ml of SU, with or without FU, at pH 5.5, into a beaker containing 4 ml of 0.05 M ammonium oxalate stock solution (final concentration of 4.8 mM after mixing) and rapidly stirred with a magnetic stirrer for 15 minutes. NaOH and HCl were used to adjust pH. Harvested crystals were briefly washed in H_2O and isolated by centrifugation. Selected samples were harvested within a few seconds of the mixing. Sodium urate was dissolved by boiling immediately prior to the mixing. Freshly prepared SU was used. Overnight storage of the solution containing urate in a refrigerator frequently caused precipitation and tended to produce fewer bipyramids.

In an attempt to trace crystal growth by scanning electron microscopy (SEM) and electron probe microanalysis (EPM), strontium (Sr) was incorporated into COD crystals by replacing 10% of $CaCl_2$ in SU with equimolar $SrCl_2$ prior to forming COD. To determine a possible consumption of the determinant of COD formation, the experiment was repeated with a conditioned SU, with or without 10% FU, in which COD crystals had previously been precipitated. To study

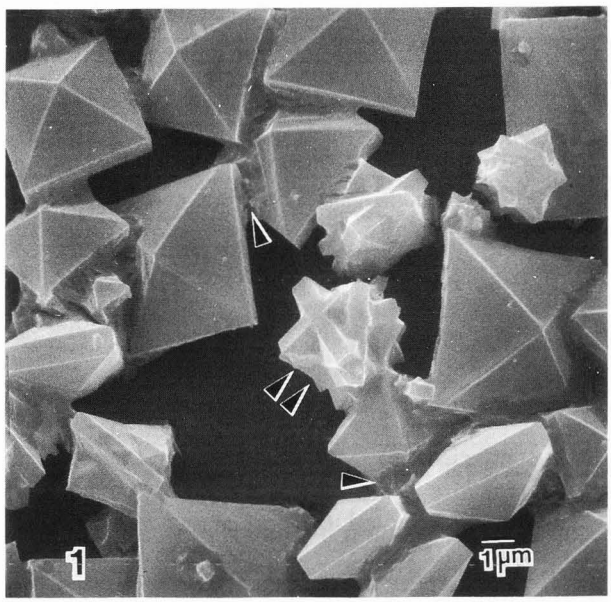

Figure 1. Dodecahedral bipyramid COD formed in 10% FU + SU (pH 5.6, 37°C) in which 10% of $CaCl_2$ was replaced with $SrCl_2$. Crystals were incubated in SU with 10% FU for 3 days. Sintering of bipyramids is evident (arrow). Interpenetrating twins are present (double arrow).

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crystal growth, bipyramid crystals, 10 mg each, were incubated in 10 ml of SU or SU with 10% FU in screw-capped plastic tubes and tumbled on a rotary drum at 37°C for up to a week. Samples were analyzed by SEM and EPM.

Determinants of COD formation

For identification of urine components that determine COD formation, gradient concentrations of sodium urate (Na-Ur) were first added to SU. To SU, with 28 mg/dl of Na-Ur, the concentration that yielded the largest number of bipyramids, mixed CS, chondroitin-4 sulfate (CS-4) and chondroitin-6 sulfate (CS-6) were added. Using SU containing 28 mg/dl Na-Ur and 10 mg/dl mixed CS, the effects of pH (5.5 - 6.0), temperature (18°C to 40°C), and 50-200 mM oxalate concentration in the stock solution (4.76-19 mM after mixing) were tested. In order to identify the potential role of other solutes in SU in COD formation, all the components except $CaCl_2$, NaCl, Na-Ur and mixed CS were deleted from SU. The deleted solutes were replaced consecutively one by one and mixed with oxalate.

SEM, EPM and electron diffraction

Crystal suspensions were placed to cover the entire surfaces of carbon planchets or aluminum stubs. As much solvent as possible was removed with filter paper

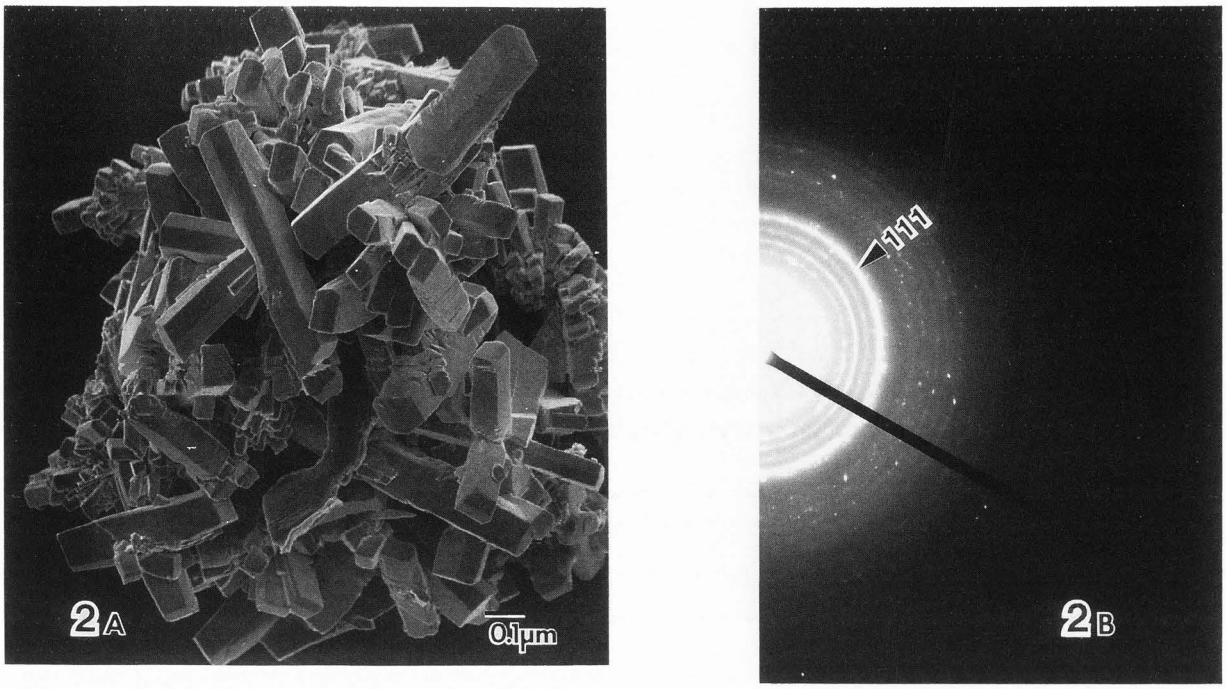

Figure 2. **(A)** Columnar crystal aggregate formed in SU with 2.0 g/l $CaCl_2 \cdot H_2O$ in SU; pH 5.6, 37°C for 24 hours. **(B)** The columns yielded Ca and P by EPM and the ED pattern of brushite. A large amount of apatite was present elsewhere in the same sample.

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avoiding uneven clustering of the crystals, followed by vacuum drying. Dried specimens were coated with carbon or gold in a sputter coater and examined under an ETEC (Hayward, CA) Autoscan scanning microscope equipped with a Kevex (San Carlos, CA) detector and a NS5500 Tracor-Northern (Noran Instruments, Middleton, WI) multichannel analyzer. Micrographs at a standard magnification of 1000x were taken from the center and 4 corners of the stubs. EPM was performed at 20 keV with a specimen tilt of 45°C and an exposure time of 100 seconds; 300 crystals on the micrographs were counted using a grid of 4 cm^2 squares, and the ratio between bipyramids (COD) and other crystals (non-COD) was obtained. For electron diffraction, a drop of crystal suspension was placed on a coated grid, vacuum dried and coated with carbon in an evaporator. Selected area electron diffraction (ED) was performed with a JEOL 100C (JEOL USA, Peabody, MA) electron microscope operated at an accelerating voltage of 80 keV, various camera lengths and a standardized exposure time of 15 seconds. Sputter-coated gold or ThCl was used as the standard at each ED.

Results

As opposed to the claimed result obtained in a continuous crystallizer (Miller *et al.*, 1977), evidently due to the difference in the mode of mixing, the modified Isaacson's SU (Isaacson, 1968) mixed with oxalate in this study yielded scanty bipyramids but mainly plate shaped crystals consistent with COT. When filtered urine (10%) was added to SU and mixed with oxalate, predominantly COD and a few oval shaped COM were formed. When 10% of $CaCl_2 \cdot H_2O$ in SU (with FU) was replaced with $SrCl_2$, dodecahedral bipyramids developed (Fig. 1). However, addition of Sr to SU without FU brought about octahedral bipyramids.

Bipyramids incubated in SU at 37°C underwent their dissolution followed by proliferation of oval COM within a week. When the incubation of COD was repeated with SU in which $CaCl_2 \cdot H_2O$ was increased to 2 g/l, brushite and apatite tended to form in a few days (Fig. 2). Crystal aggregation was minimal in SU. In SU with 10% filtered urine, crystal sintering and aggregation occurred frequently, but no notable increase in the size of crystals was observed (Fig. 1). Incubation of dodecahedra in SU mixed with FU resulted in a complete loss of EPM-detectable Sr in several days, although the crystals retained the dodecahedral habit.

When Na-Ur was added to SU and mixed with oxalate, a small increase in the amount of bipyramid occurred (Fig. 3). The highest COD/non-COD was obtained at 28 mg/dl of Na-Ur. Of the CS concentrations tested, 10 mg/dl of mixed CS in SU (containing 28

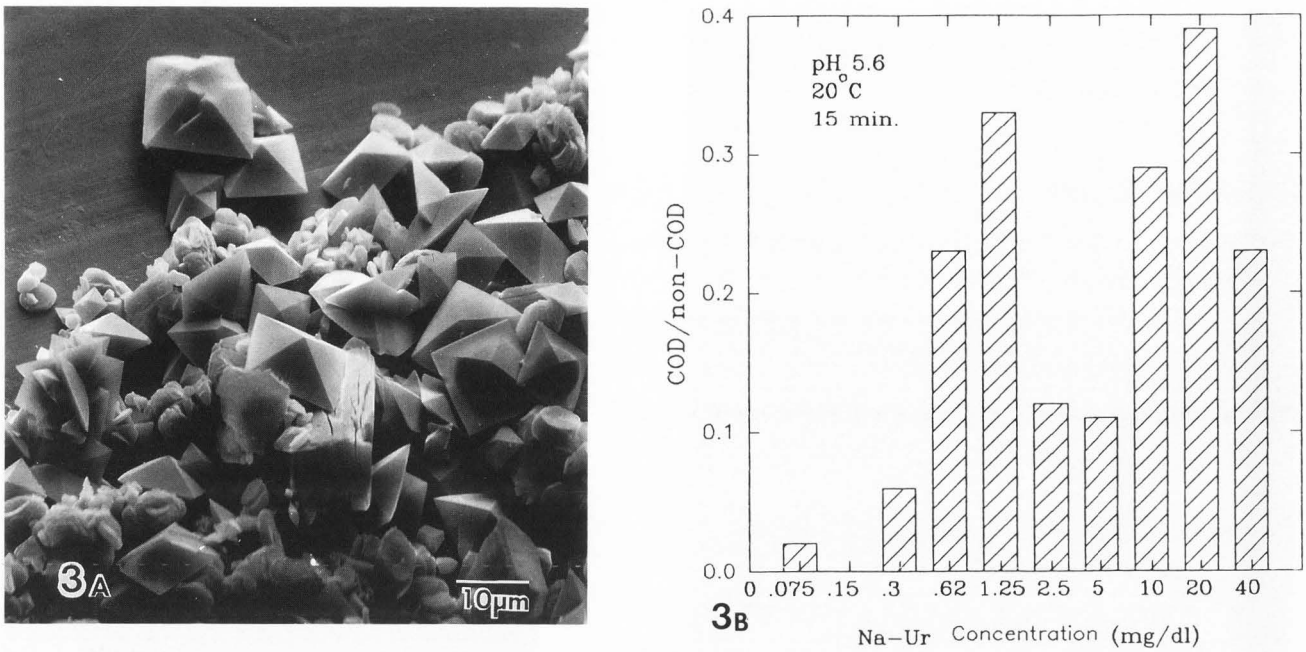


Figure 3. **(A)** Some bipyramids formed in SU with 30 mg/dl of Na-Ur, at pH 5.5 and 37°C. **(B)** The effect of Na-Ur on the COD/non-COD ratio. At approximately 28 mg/dl of Na-Ur in SU, the highest ratio was obtained.

mg/dl Na-Ur) yielded the highest COD/non-COD (Fig. 4). Mixed CS gave a higher COD/non-COD than CS-4, CS-6 or a mixture of CS-4 and CS-6 (Fig. 5). In addition to discrete bipyramids, numerous interpenetrating twins of bipyramids were observed within a few seconds of the mixing (Figs. 1A and 4A). Both urate and CS in SU displayed saturable effects on bipyramid formation (Fig. 3B and 4B). Concentrations of Na-Ur higher than 30 mg/dl frequently resulted in uric acid hydrate crystals. Mixed CS greater than 15 mg/dl caused mainly oval shaped COM. Increases in calcium concentration in SU had little effect on COD formation.

The temperature of SU containing urate and mixed CS showed a considerable effect on the COD/non-COD ratio. The lower the temperature, the higher the ratio was (Fig. 6). At 37°C, scanty bipyramids but a large amount of thin plate shaped crystals consistent with COT were formed. At 18°C, mainly bipyramids and a small amount of oval COM formed. A significant effect on the ratio was elicited by pH of the solution, as well. The higher the pH, the more bipyramids formed (Fig. 7). Although higher concentrations of oxalate yielded a higher COD/non-COD ratio, bipyramids thus formed were variable in size with numerous microcrystals. Oxalate concentrations also showed a saturable effect on bipyramid formation (Fig. 8). When SU was mixed with the stock solution that exceeded 200 mM in oxalate concentration, nearly pure COM was produced. When conditioned SU, in which COD had been previously precipitated and calcium was readjusted to 1 mM, was mixed with oxalate, pure COM of oval plate shape was formed (Fig. 9).

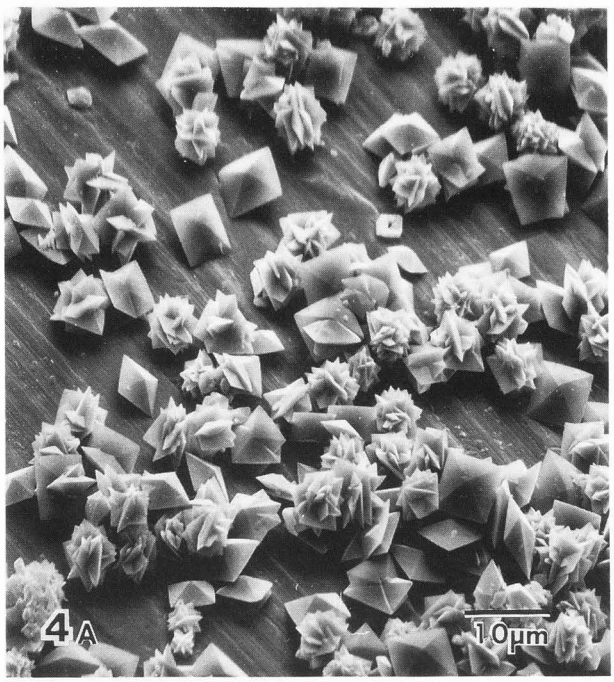
When solutes other than $CaCl_2$, NaCl, Na-Ur or mixed CS were deleted from SU and mixed with oxalate, no bipyramids but plate shaped crystals consistent with COT were formed. Omission of the components of SU, i.e., CS, urate and other solutes, appeared to favor COT formation. When deleted solutes were consecutively replaced, phosphate failed to produce any bipyramids. However, addition of citrate caused a dramatic increase in COD/non-COD. Magnesium and sulfate had no notable effect on the ratio. Addition of KCl appeared to further increase the ratio (Fig. 10).

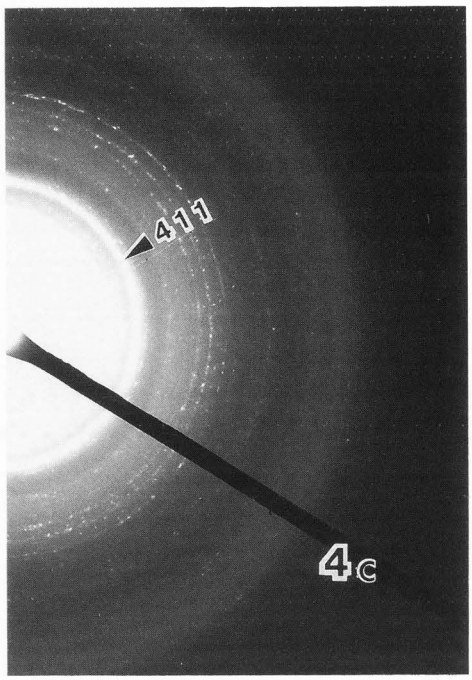
Taking into account all the factors, namely addition of SU containing 28 mg/dl Na-Ur, 10 mg/dl mixed CS at pH 6 and 20°C and mixing with 0.05 M ammonium oxalate with rapid stirring, morphologically pure bipyramids of fairly uniform size were consistently obtained (Fig. 11).

When mixed CS were substituted for 10 mg/dl of agar, gelatin, Bacto-peptone (Difco, Detroit, MI) and sucrose, very high COD/non-COD resulted. Agar and gelatin were as effective as mixed CS in bipyramid formation (Fig. 12).

Discussion

The prevalence of COD in crystalluria has mainly been attributed to the selective inhibitory effect of impurities in urine for COM (Martin *et al.*, 1984; Tomazic and Nancollas, 1979). Although a variety of





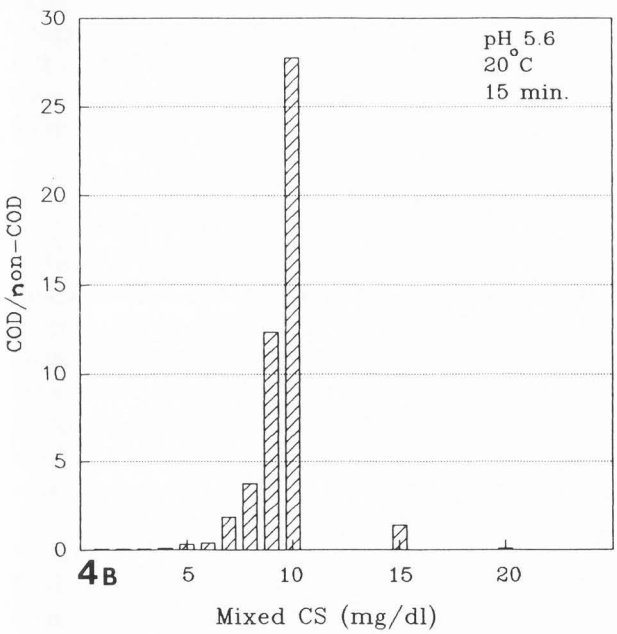


Figure 4. **(A)** SU with 28 mg/dl of Na-Ur and 10 mg/dl mixed CS, produced nearly pure bipyramids. **(B)** The effect of CS is saturable. The highest COD/non-COD ratio was obtained with 10 mg/dl mixed CS. **(C)** The ED pattern of COD.

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factors have been said to promote COD formation *in vitro*, the purity of COD produced has seldom been addressed, and the definitive determinant(s) of COD formation in urine has not been established. Findings in

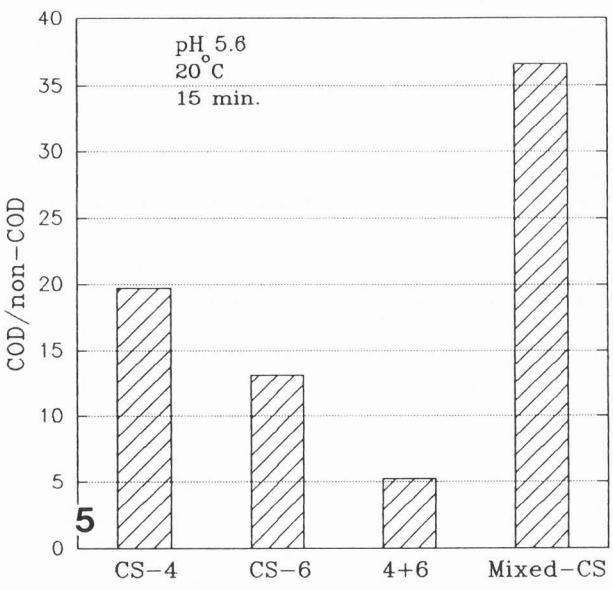

Figure 5. Mixed CS produced a higher COD/non-COD ratio than CS-4, CS-6 or their mixture.

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this study indicate that the synergistic action of CS and citrate play the primary role in COD formation. The mechanism of the synergism, however, remains to be determined.

Of the factors that determine COD formation, pH has been studied in the greatest detail (Martin *et al.*, 1984). At pH 6.5, only COD formed in human urine. Addition of pyrophosphate, citrate, yeast RNA or heparin (Martin, 1995) and creatinine (Rodgers and Wandt,

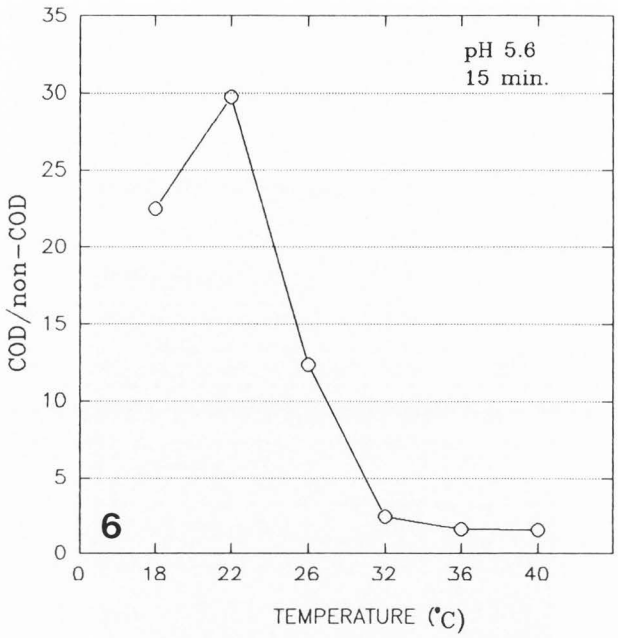

Figure 6. The effect of temperature on COD formation in SU with 28 mg/dl Na-Ur and 10 mg/dl CS. Higher COD/non-COD ratios were obtained at lower temperatures.

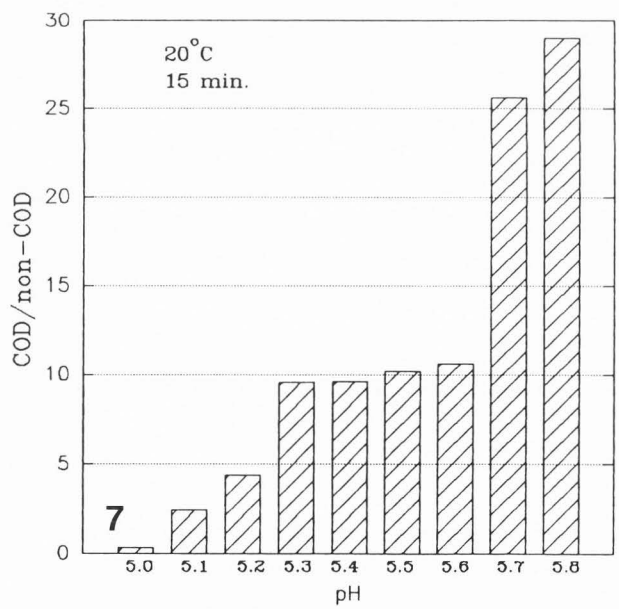

Figure 7. The effect of pH on COD formation. The higher the pH, the greater COD/non-COD ratio.

1991) to a simple supersaturated solution of calcium oxalate increased the yield of COD in a dose dependent manner. In comparing COM and COD stone formers, COD stones were more common among younger male with higher urine calcium and pH (Pierratos *et al.*,

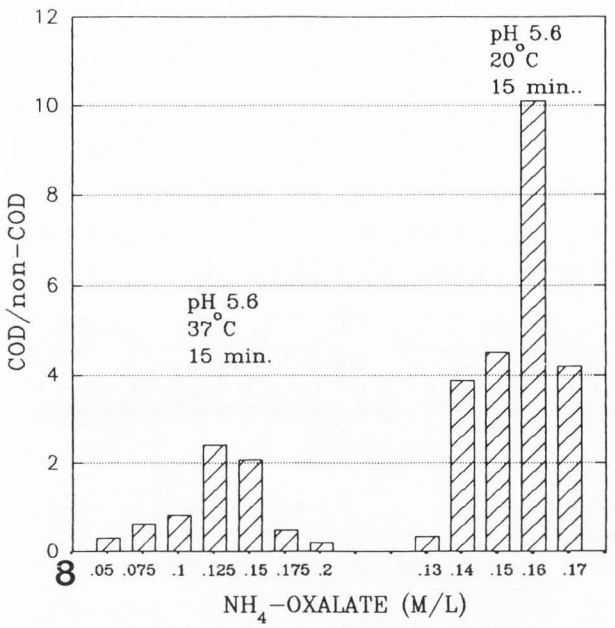

Figure 8. A composite graph of the effect of oxalate concentration (in the stock solution of ammonium oxalate) on COD formation. The left half of the graph shows the results at 37°C, the right half, at 20°C. The effect of oxalate concentration is saturable. Oxalate concentration exceeding 200 mM in the stock solution of ammonium oxalate resulted in COM only.

1994). In addition, pH has been shown to have an additive effect on the potency of inhibitory action of calcium oxalate crystal growth by citrate (Curreri *et al.*, 1981), by pyrophosphate (Tiselius, 1981) and by multidentate phosphate (Meyer *et al.*, 1977). The effect of lower temperature favoring COD formation has been known for some time (Kuwahara *et al.*, 1982) and calls for a caution in identification of COD in urine stored at a lower temperature.

Interpenetration twinning is common in urinary stones (Kim, 1981). The deviation of the major axis of the crystal growth is believed to cause the twinning (Bloss, 1971). The twinning apparently takes place in the very early stage of COD crystal growth. As opposed to large-sized bipyramid crystals frequently encountered in urinary stones, COD in this study are relatively small and uniform in size. Grases *et al.* (1990) described synthesis of large COD crystals by using the solution in which COM had previously been precipitated. Conversely, when oxalate was added to the conditioned SU in which COD has previously been precipitated, pure COM resulted in this study. These observations suggest that determinants of COD and COM formation are distinctive and are consumed during crystal

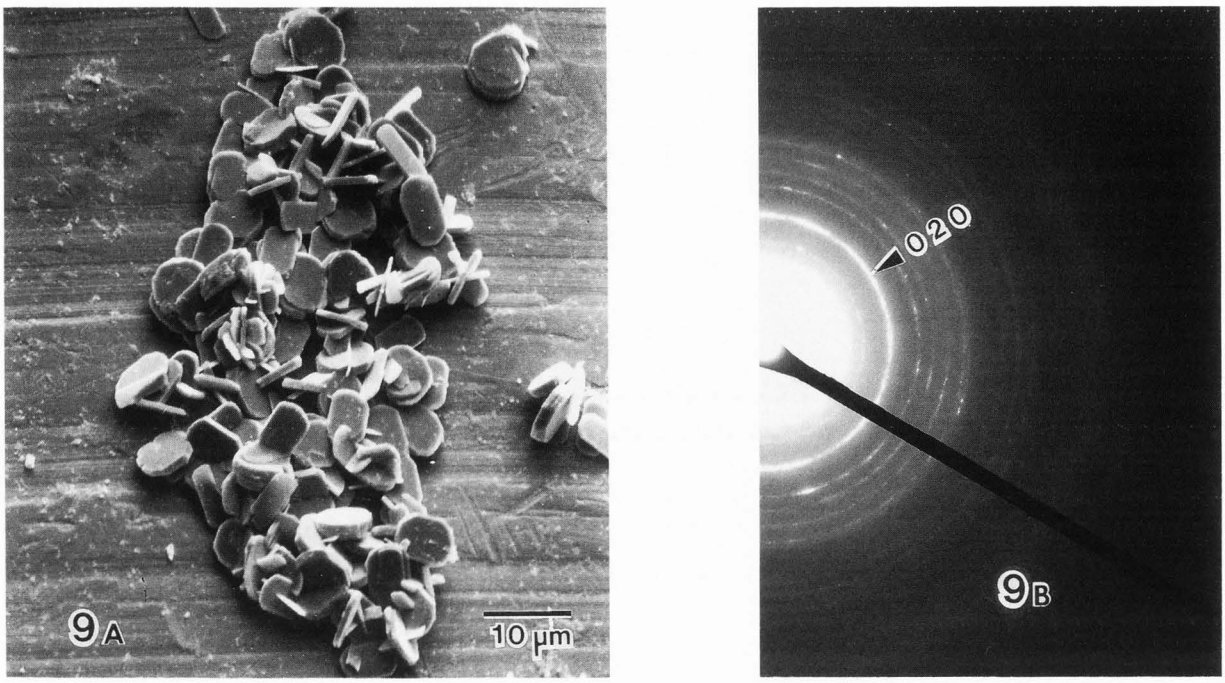

Figure 9. (**A**) Pure oval plate shaped COM formed in a conditioned SU, in which COD had previously been precipitated. Calcium was readjusted to 1 mM and mixed with oxalate. The finding indicates that the determinant of COD was consumed during previous COD formation. (**B**) The ED pattern of COM.

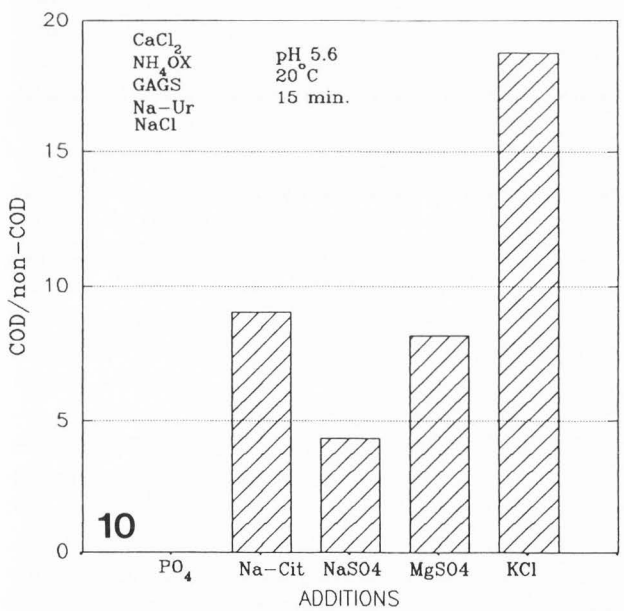

Figure 10. Solutes were deleted from SU, leaving only $CaCl_2$, NaCl, mixed CS and Na-Ur, and mixed with oxalate. No bipyramids were formed. Deleted solutes were added consecutively to see their effect on COD formation. Replacement of phosphate and sulfate had no effect on COD formation. Replacement of citrate resulted in a dramatic increase in the COD/non-COD ratio. Addition of KCl appeared to enhance further bipyramid formation. OX: oxalate; Cit: citrate.

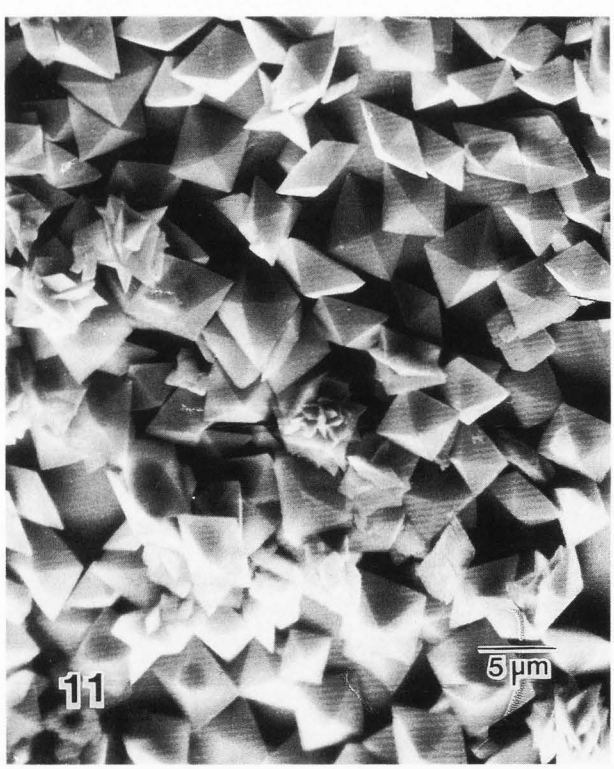

Figure 11. Pure bipyramids formed in SU with 28 mg/dl Na-Ur, 10 mg/dl CS, at pH 5.8 and 20°C.

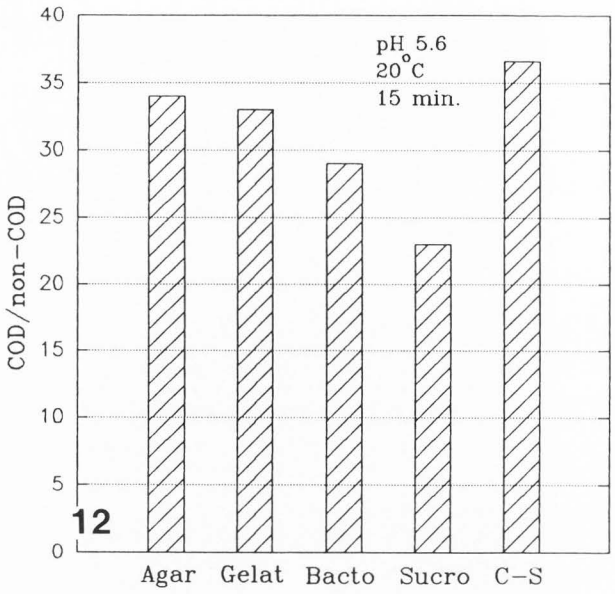

Figure 12. Substitution of CS for agar, gelatin (gelat), Bacto-peptone (Bacto) and sucrose (sucro) produced very high COD/non-COD ratios, indicating the effect of CS on COD formation is non-specific.

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formation. Dodecahedra formation by addition of Sr to SU indicates that certain impurities in urine are likely to be responsible for their occurrence in urinary stones. The loss of Sr from bipyramids in SU demonstrates that aberrantly fit Sr during COD growth is unstable and a dynamic exchange of ions occurs between the crystal and the solution to correct the instability.

The role of urate in calcium oxalate stone formation has been controversial (Ettinger, 1989; Ryall *et al.*, 1991). Dissolved urate has been shown to promote crystallization of COM. The effect of urate appears not to be dependent on heterogeneous nucleation (Grover *et al.*, 1993). Tamm-Horsfall protein (THP) inhibits calcium oxalate crystal aggregation caused by urate (Grover *et al.*, 1994). Addition of urate to certain urine ultrafiltrates yielded COD crystal (Grover *et al.*, 1992). The effect of urate on COD formation in this study raises a possibility that the promotive effect of urate in oxalate stone formation (Coe, 1978) is mediated in part through its effect on COD formation.

Magnesium has been shown to inhibit both nucleation and growth of calcium oxalate crystals (Li *et al.*, 1985). Magnesium or trace metal may stabilize COD (Hesse *et al.*, 1976). Oka *et al.* (1987) observed a higher inhibitory activity of magnesium on COM than COD crystal formation. The presence of magnesium in SU did not appear to have a significant effect on COD formation in this study.

The inhibitory effect of citrate on calcium oxalate is known. Hypocitruria is common among idiopathic calcium oxalate stone formers. Potassium citrate administration has been effective in the prevention of calcium oxalate stone recurrences (Pak, 1991). Citrate has an effect on supersaturation with respect to calcium oxalate and phosphate, and inhibits the growth of these crystals by reducing the ion activity products. Citrate also inhibits aggregation of these crystals (Tiselius *et al.*, 1993). Citrate and THP have been shown to have a synergistic effect on agglomeration of COM (Erwin *et al.*, 1994).

The propensity to form COD in solutions containing citrate is also known. The effect is attributed to its selective inhibition for COM formation. Citrate and pyrophosphate accounted for 45% of the formation of COD in undiluted urine (Martin *et al.*, 1984). However, citrate had an effect on COD formation only when CS or urate coexisted in this study.

The role of urinary macromolecules in calcium oxalate stone formation has been extensively studied but remains controversial. The discrepancy apparently stems from the variation in experimental conditions, especially of the composition of solutions used in the studies. In view of the conflicting results in the literature, Rodgers (1995) proposed that the ultimate test for the role of macromolecules in crystallization should be performed in natural urine. Urinary macromolecules may act either as inhibitors of growth or promoters of nucleation. Macromolecules affect the overall order of the growth mechanism of calcium oxalate. In a constant kinetic study, the presence of isolated stone matrix macromolecules caused the effective order to be approximately 6.6, indicating a higher order polynuclear growth mechanism (Nancollas *et al.*, 1991). The higher COD/non-COD ratio obtained with mixed CS than with CS-4 or CS-6 in SU indicates that a certain other components in mixed CS is involved in COD formation. It is interesting that heparan sulfate was incorporated in preference to CS into calcium oxalate crystals formed in human urine (Suzuki *et al.*, 1994).

Of the macromolecules in urine, glycosaminoglycans (GAGs) have been of particular interest. Most studies have indicated that GAGs inhibit calcium oxalate crystal nucleation, growth and aggregation (Hesse *et al.*, 1991; Robertson *et al.*, 1976). Although an interaction with the crystal surface and adsorption are the suspected cause of the inhibition, the mechanism of GAGs' inhibitory actions appears to be not yet clear. Chondroitin sulfates, human serum albumin and THP had no effect on the size and the rate of calcium oxalate crystallization in human urine (Ryall *et al.*, 1991).

The majority of studies on urinary macromolecules, including GAGs, has been aimed at their roles in promotion or inhibition of oxalate crystal growth and aggregation. The possible role of macromolecules in determin-

ing different hydrates of calcium oxalate has hardly been touched upon. Drach *et al.* (1982) demonstrated in a continuous crystallizer that high molecular weight macromolecules of less than 50 kDa present in urine enhance the nucleation rate of COD but inhibit its linear growth rate. Occasional descriptions of negative effect of CS in COD formation (Martin *et al.*, 1984; Rodgers and Wandt, 1991) are attributable to the synergistic role of CS and citrate and the saturable effect of CS; both CS and citrate are required to yield COD in SU. The similar effect by foreign macromolecules, i.e., agar and gelatin, however, indicates that the role of CS in COD formation is non-specific and is mediated probably through the colloidal effect. Since urine contains a variety of colloidal materials, it is likely that they play a collective role in COD formation *in vivo*.

Boevé *et al.* (1994) observed a difference in the zeta potential distribution of THP derived from normal and stone formers' urine. The more potent capacity to shift the zeta potential of COM surface by THP from normal urine may account for its greater inhibition of COM agglomeration. The measurement of the potential for GAGs in relation to COD appears to be not yet available. Apparently, citrate and CS affect the molecular arrangements during crystal growth. Both citrate and CS have been shown to be incorporated into non-specified calcium oxalate and COM (Suzuki *et al.*, 1994; Wierzbicki *et al.*, 1995).

The transformation of COD to COM, as observed in this study, has been shown to occur through dissolution and re-nucleation (Tomazic and Nancollas, 1980). The transformation does not appear to occur by heterogeneous nucleation (Deganello, 1986; Grover *et al.*, 1993). The transformation may account for the higher incidence of COD in crystalluria and a lower incidence of COD in stones.

Summary

The synergistic effect of CS and citrate is the primary determinant of COD formation in a SU. The presence of urate and potassium, higher pH, lower temperatures and higher concentrations of oxalate favor COD formation, as well. The effect of CS appears to be non-specific; foreign macromolecules, i.e., agar and gelatin, are as effective as CS in the formation of COD. **The effect of these macromolecules in COD formation is attributable to their colloid nature.** Interpenetration twinning occurs in the very early stage of COD formation. Addition of Sr to SU resulted in dodecahedral bipyramids, indicating certain impurities in urine are responsible for dodecahedral formation in urinary stones.

Identification of the determinants led to a simple and reproducible method of COD formation in quantities without addition of natural urine.

References

Ackerman D, Brown P, Khan SR (1989) Preparation and application of calcium oxalate dihydrate crystal seeds. Urol Res **17**: 143-151.

Akbarieh M, Tawashi R (1991) Calcium oxalate crystal growth in the presence of mucin. Scanning Microsc **5**: 1019-1027.

Bloss FD (1971) Crystallography and Crystal Chemistry. Holt Reinhart and Winston, Inc., New York. pp. 324-338.

Boevé ER, Cao LC, de Bruijn WC, Robertson WG, Romjin JC, Schröder FH (1994) Zeta potential distribution on calcium oxalate crystal and Tamm-Horsfall protein surface analyzed with Doppler electrophoretic light scattering. J Urol **152**: 531-546.

Coe FL (1978) Hyperuricosuric calcium oxalate nephrolithiasis. Kidney Int **13**: 418-426.

Curreri PA, Onoda G, Finlayson B (1981) A comparative appraisal of adsorption of citrate on whewellite seed crystals. J Crystal Growth **53**: 209-214.

Deganello S (1986) Phase transitions of calcium oxalate trihydrate and epitaxy in the weddellite-whewellite system. Scanning Electron Microsc **1986**; IV: 1721-1728.

Drach GW, Kraljevich Z, Randolph AD (1982) Effects of high molecular weight urinary macromolecules on crystallization of calcium oxalate dihydrate. J Urol **127**: 805-810.

Erwin DT, Kok DJ, Alam J, Vaughn J, Coker O, Carriere BT, Lindberg J, Husserl FE, Puselier H Jr, Cole FE (1994) Calcium oxalate stone agglomeration reflects stone forming activity: Citrate inhibition depends on macromolecules larger than 30 kilodalton. Am J Kidney Dis **24**: 893-900.

Ettinger B (1989) Does hyperuricosuria play a role in calcium oxalate lithiasis? J Urol **141**: 738-741.

Franchini-Angela M, Aquilano D (1979) Growth morphology of weddellite $CaC_2O_4{\cdot}2H_2O$. J Cryst Growth **47**: 719-726.

Gardner GL (1978) Effects of pyrophosphate and phosphonate anions on the crystal growth kinetics of calcium oxalate hydrates. J Phys Chem **82**: 864-870.

Grases F, Millan A, Conte A (1990) Production of calcium oxalate monohydrate, dihydrate or trihydrate. A comparative study. Urol Res **18**: 17-20.

Grover PK, Ryall RL, Marshall VR (1992) Calcium oxalate crystallization in urine: Role of urate and glycosaminoglycans. Kidney Int **41**: 149-154.

Grover PK, Ryall RL, Marshall VR (1993) Dissolved urate promotes calcium oxalate crystallization: Epitaxy is not the cause. Clin Sci **85**: 303-307.

Grover PK, Marshall VR, Ryall RL (1994) Tamm-Horsfall mucoprotein reduces promotion of calcium oxalate crystal aggregation induced by urate in human urine *in vitro*. Clin Sci **87**: 137-142.

Hesse A, Berg W, Schneider H-J, Hienzsch E (1976) A contribution to the formation mechanism of calcium oxalate urinary calculi. I. Stabilizing urinary constituents in the formation of weddellite. Urol Res **4**: 125-128.

Hesse A, Wuzel H, Vahlensieck W (1991) Significance of glycosaminoglycans for the formation of calcium oxalate stones. Am J Kidney Dis **17**: 414-419.

Isaacson LC (1968) Urinary ionic strength, osmolarity, and specific conductivity. Invest Urol **5**: 406-413.

Kim KM (1981) Calcium oxalate crystal growth in human urinary stones. Scanning Electron Microsc **1981**; III: 147-154.

Kuwahara M, Kageyama S, Kurosu S, Orikasa S (1982) Effects of calcium chelating agents and acid mucopolysaccharides on the growth of calcium oxalate dihydrate crystals. Nippon Hinyogika Gakkai Zashi **73**: 1436-1443.

Li MK, Blacklock NJ, Garside J (1985) Effects of magnesium on calcium oxalate crystallization. J Urol **133**: 123-125.

Martin KL (1995) Scanning electron microscopy and molecular modeling of inhibition of calcium oxalate monohydrate crystal growth by citrate and phosphocitrate. Calcif Tissue Int **56**: 297-304.

Martin X, Smith LH, Werness FG (1984) Calcium oxalate dihydrate formation in urine. Kidney Int **25**: 948-952.

Meyer JL, Lee KE, Bergert JH (1977) The inhibition of calcium oxalate crystal growth by multidentate organic phosphates. Effect of pH. Calcif Tissue Res **23**: 83-86.

Miller JD, Randolph AD, Drach GW (1977) Observations upon calcium oxalate crystallization kinetics in simulated urine. J Urol **117**: 342-345.

Mroczek JE, Butts WC, Rainey WT Jr, Burtis CA (1971) Separation and identification of urinary constituents by use of multiple-analytical techniques. Clin Chem **17**: 72-77.

Nancollas GH, Smesko SN, Campbell AA, Richardson CF, Johnsson M, Iadiccico RA, Binette JP, Binette M (1991) Physical chemical studies of calcium oxalate crystallization. Am J Kidney Dis **17**: 392-395.

Oka T, Yoshika T, Koide T, Takaha M, Sonoda T (1987) Role of magnesium in the growth of calcium oxalate monohydrate and calcium oxalate dihydrate crystals. Urol Int **42**: 89-93.

Pak CYC (1991) Citrate and renal calculi: New insights and future directions. Am J Kidney Dis **17**: 420-425.

Philipsborn H (1952) Uber calciumoxalat in Pflanzenzellen (On calcium oxalate in plant cells). Protoplasma **41**: 415-424.

Pierratos AE, Khalaff H, Cheng PT, Psihramis K, Jewtt MAS (1994) Clinical and biochemical differences in patients with pure calcium oxalate monohydrate and calcium oxalate dihydrate kidney stones. J Urol **151**: 571-574.

Robertson WG, Knowles F, Peacock M (1976) Urinary acid mucopolysaccharide inhibitors of calcium oxalate crystallization. In: Urolithiasis Research. Fleisch H, Robertson WG, Smith LH, Wahlensieck W (eds.). Plenum Press, New York. pp. 331-334.

Rodgers AL (1996) The role of urinary macromolecules in urolithiasis: Review of methodologies and a proposal for a standard reference crystallization system. Scanning Microsc **10**: 535-546.

Rodgers AL, Wandt MAE (1991) Influence of aging, pH and various additives on crystal formation in artificial urine. Scanning Microsc **5**: 697-706.

Ryall RL, Grover PK, Marshall VR (1991) Urate and calcium stones - Picking up a drop of mercury with one's finger? Am J Kidney Dis **17**: 426-430.

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

Sallis JD, Parry NF, Meehan JD, Kamperman H, Anderson ME (1995) Controlling influence of phosphocitrate *in vitro* and *in vivo* of calcium crystal formation and growth. Scanning Microsc **9**: 127-136.

Suzuki K, Mayne K, Doyle IT, Ryall RL (1994) Urinary glycosaminoglycans are selectively included into calcium oxalate crystals from whole human urine. Scanning Microsc **8**: 523-530.

Tiselius H-G (1981) The effect of pH on the urinary inhibition of calcium oxalate crystal growth. Br J Urol **53**: 470-474.

Tiselius H-G, Berg C, Fornander AM, Nilsson M-A (1993) Effects of citrate on the different phases of calcium oxalate crystallization. Scanning Microsc **7**: 381-390.

Tomazic BB, Nancollas GH (1979) The kinetics of dissolution of the calcium-oxalate hydrates. J Crystal Growth **46**: 355-361.

Tomazic BB, Nancollas GH (1980) The kinetics of dissolution of calcium oxalate hydrates. II. The dihydrate. Invest Urol **18**: 97-101.

Werness PG, Duckworth SC, Smith LH (1979) Calcium oxalate dihydrate crystal growth. Invest Urol **17**: 230-233.

Wierzbicki A, Sikes CS, Sallis JD, Madura JD, Stevens ED, Martin KL (1995) Scanning electron

microscopy and molecular modeling of inhibition of calcium oxalate monohydrate crystal growth by citrate and phosphocitrate. Calcif Tissue Int **56**: 297-304.

Discussion with Reviewers

H-G. Tiselius: Obviously, a normal body temperature favors the formation of COM, whereas a pH above 5.6 apparently favors that of COD. In view of the predominance of COD in urine, do you think that temperature has any effect *in vitro*?
Author: Although the lower temperature may help prepare COD seed crystal *in vitro*, it can not be of any clinical significance. So is the case with higher pH. Alkalinuria results in struvite and apatite instead of calcium oxalates.

K. Suzuki: To determine the percentage of COD, the author used micrograph counting. Findings from the scanning electron micrographs provide little information about whole crystal characteristics. There is a simple method using infrared spectroscopy to calculate the percentage of COD. Did the author compare the result of micrograph's method using other methods?
T. Koide: To determine COD crystallines, the author employed imaging counter under SEM. It seems to be exact to use infrared spectrophotometer in determining COD crystallines or COD/COM ratio.
Author: The ratio of intensities of the lines in X-ray diffraction and of the absorbance in infrared spectroscopy have been used to estimate the ratio of crystal components in urinary stones. Although these methods have an advantage of dealing with bulk samples, it is known that the methods overlook minor components of the stones (see Kim *et al.*, 1985). For identification of the minor non-COD components that may contaminate COD preparations, no other method can excel the sensitivity of direct SEM observation of the crystals formed; this is possible because of the well defined, bipyramid habit of COD. In cell morphometry, a sample size of 300 cells is usually considered sufficient. No comparison with other methods for determination of the ratio was made.

K. Suzuki: At present, heparan sulfate (HS) is thought to have a remarkable effect against CaOx crystal formation, growth and aggregation. Why didn't the author use HS in the experiment?
T. Koide: What does "mixed" chondroitin mean? Human urine contains various GAGs; chondroitin sulfate A, B, C; dermatan sulfate, etc. The major role of GAGs on stone formation has focussed on heparan sulfate, recently. Did the author examine the effect of heparan sulfate in the experimental system?
Author: Experiments in this study have been performed prior to the knowledge of heparan sulfate adsorption to calcium oxalates. A great significance has been attached to the adsorption of macromolecules to calcium oxalate crystals. However, the significance of the adsorption requires a careful interpretation. As pointed out, the effect of CS in COD formation is not specific. Sigma used to supply "mixed chondroitin sulfates." The product appears to have been discontinued. It was my understanding that "mixed CS" were a crude extract from shark cartilage that consisted of a mixture of non-specified GAGs.

T. Koide: The experimental method was a little complicated, and the results obtained from various additives, such as urate, oxalate, foreign macromolecules and the change of pH and temperature easily affected COD crystalline formation in this study. Why could the author emphasize the synergistic role of mixed chondroitin sulfate and citrate without considering the role of other factors?
Author: As pointed out, urine is complex. Many additional urine components are likely to have some effects on COD formation. It will be an endless task to test the effect of every urine component on COD formation. The results in this study, however, indicate that the synergism of CS and citrate is a powerful determinant of COD formation. When either CS or citrate was absent in SU, no COD was formed.

T. Koide: Regarding the macromolecules, the author stated that the effect of those in COD formation was non-specific and attributable to the colloidal nature. However, ultrafiltered urine, in which COD formation is ordinary, contains various macromolecules as the non-colloidal form. Why does the author speculate the mechanism of macromolecules on COD formation as above? If the speculation was exact, why did CS-4 and/or CS-6 not affect the COD crystalline formation?
Author: The effect of colloid on crystal formation has long been known. Alterations of the zeta potential of the colloid molecules are thought to have a role in the modification crystal formation. The colloid effect seems to be the most likely common denominator for GAGs, agar, gelatin and Bacto-peptone. The reason for the varied effects on COD formation by different CS is not clear. Identification of the factors that underlie the difference may hopefully lead to a better understanding of the macromolecular role in COD formation or crystal formation in general. The findings in this study by no means exclude the potential role of non-colloidal components of urine on COD formation.

S.R. Khan: The impression is given that reproducible methods of COD formation are not available. This is

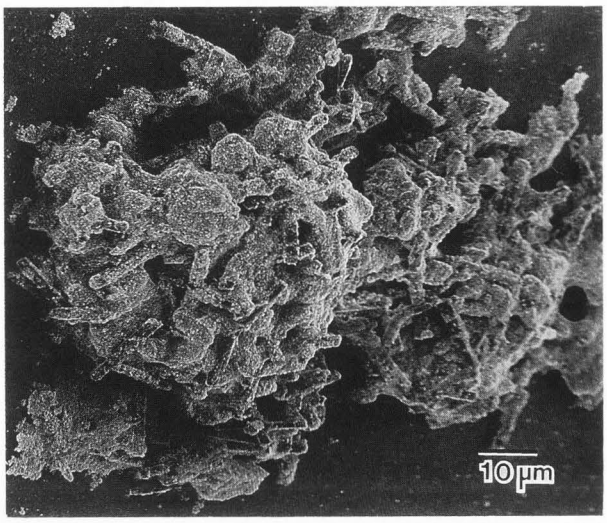

Figure 13. Brushite heavily coated by a layer of apatite crystals.

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not so; many laboratories, including our own, produced almost pure CODs for various uses, including investigation of crystal/renal epithelial cell interaction.

Author: No one other than Martin *et al.* (1984, text ref.) has published detailed quantitative data in regard to the purity of COD produced *in vitro*. The purity of COD is difficult to judge without such quantitative data. It is well known that crystal formation is frequently difficult to reproduce evidently due to the certain, subtle, unnoticed changes in the environment in which crystals form. Consistent reproduction of the same crystal in pure form, e.g., COD, calls for powerful determinant(s) of their formation, i.e., CS and citrate, as shown in this study. For morphological studies, the purity of crystals may not be of the great significance. However, for kinetic studies, the purity, or the knowledge of the degree of purity of crystals, is critical.

S.R. Kahn: It will be helpful to provide some information about the composition of native urine and how it differs from the synthetic urine. How do the *in vitro* conditions compare with the *in vivo* conditions?

Author: SU is culminated through extensive analyses of normal human urine (Isaacson, 1968; text ref.); Burns and Finlayson, 1980). However, the composition of SU is highly selective and ignores most organic components, especially macromolecules that exist in urine. Urine contains 22 mM of uric acid (ca. 40 mg/dl Na-Ur; Grover *et al.*, 1993, text ref.) and 10-20 μM (ca. 2-5 mg/dl) of glycosaminoglycans are excreted through urine a day (Hesse *et al.*, 1991, text ref.). The simple mixing of solutions in a beaker cannot be compared with what happens *in vivo* since the exact mode of COD or any crystal nucleation *in vivo* is not yet understood. Nevertheless, the involvement of CS and citrate in COD formation is significant since urine contains both.

A. Rodgers: How do you know that apatite is present in Figure 2A? I am sure that you would not have been able to detect its electron diffraction pattern, and since it contains Ca and P, as does brushite, it would not be possible to identify by electron microprobe.

Author: Figure 2A is from a highly selected area of the sample in an earlier stage of incubation and does not show any apatite. Apatite was present elsewhere in the same sample. In most cases, brushite was heavily coated by a layer of apatite crystals (Fig. 13). I agree that EPM is a not reliable method of crystal identification, especially those with the same elemental contents, i.e., brushite and apatite.

The major advantage of ED is its ability to diffract a selected area of the silhouette of crystals that are sorted out by transmission electron microscopy (see Kim, 1982). Needle shaped crystals in the samples yielded the pattern of apatite, whereas fragments of the columns yielded the pattern of brushite.

A. Rodgers: The occurrence of brushite and apatite in Figure 2 is intriguing. Could this be attributed to a pH effect? If so, what was pH?

Author: The original pH of the solution was 5.6. Brushite and apatite were formed in SU in which $CaCl_2 \cdot H_2O$ was increased to 2 g/l and incubated at 37°C for more than 24 hours. It is known that brushite forms at a lower pH. This is the reason that brushite is relatively common in urinary stones, whereas it does not usually occur in tissue calcification. Brushite is also known to spontaneously "transform" into apatite (Pak, 1978).

A. Rodgers: Figure 4A is notable for the high degree of crystal aggregation. To what factor(s) can this be attributed?

Author: The study was not designed to investigate crystal aggregation. The aggregation on electron micrographs may in part be artifacts formed during drying. However, addition of FU, chondroitin sulfates and urate to SU appeared to enhance the aggregation.

A. Rodgers: As a result of the experiment involving Sr, you have concluded that certain impurities in urine are responsible for dodecahedra formation in kidney stones. Can you speculate as to what these impurities might be?

Author: Dodecahedral COD has long been observed in urinary stones but the mechanism of their formation has

been unknown. The formation of dodecahedra by addition of Sr to SU mixed with FU certainly indicates that in addition to Sr substitution for Ca, a certain urine component is involved, as well. What causes dodecahedra *in vivo* remains to be determined. Hopefully, the finding in this study will lead to identification of the determinant(s) of dodecahedra in urine.

F. Grases: The incorporation of Sr^{2+} ions to the lattice of COD crystals will provoke disturbances that could favor the formation of intergrown crystals with a complex crystal arrangement (primary agglomeration). Has the author evidence of such phenomena in his experiment?
Author: Interpenetration twinning and agglomeration were not monitored in the study. However, it was of the impression that addition of urate, CS and FU enhanced COD aggregation.

F. Grases: Figure 3 shows a dramatic decrease in the ratio of COD/non-COD crystals when increasing mixed CS concentration over 10 mg/dl. How can this be explained? Must this fact be attributed to the size of colloidal particles?
Author: Practically every factor that caused an increase in the COD/non-COD ratio showed dose dependent responses. I am unable to offer an explanation for the phenomena. The phenomena certainly call for an interpretation by expert physical chemists.

Additional References

Burns JR, Finlayson B (1980) A proposal for a standard reference artificial urine in *in vitro* urolithiasis experiments. Invest Urol **18**: 165-169.

Kim KM (1982) The stones. Scanning Electron Microsc **1982**; IV: 1635-1660.

Kim KM, Alpaugh HB, Johnson FB (1985) X-ray microanalysis of urinary stones, a comparison with other methods. Scanning Electron Microsc **1985**; III: 1239-1246.

Pak CYC (1978) Calcium Urolithiasis, Pathogenesis, Diagnosis and Management. Plenum Medical Book Co., New York. pp. 5-36.