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# CALCIUM OXALATE CRYSTAL GROWTH IN THE PRESENCE OF MUCIN

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## Abstract

Using interfacially-controlled crystallization and gel diffusion crystallization methods, calcium oxalate monohydrate (COM), dihydrate (COD) and trihydrate (COT) crystals were grown by the slow diffusion of reacting ions in the presence of mucin. It was demonstrated that mucin in the growth media dramatically affected the size, habit, surface structure, thermodynamic stability and phase transition kinetics of hydrated calcium oxalate crystals. The results obtained revealed that mucin as a glycoprotein model controlled the growth of COT and COD single crystals as well as cluster formation. Growth inhibition of specific crystal faces and phase transition retardation occurred in its presence. The data confirmed that glycoproteins are more than just adhesive materials, enhancing crystal aggregation in stone formation.

Key Words: Crystal growth, hydrated calcium oxalate crystals, stone formation, mucin, urinary glycoproteins, growth in gel.

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## Introduction

Calcium oxalate crystalluria is a common feature in recurrent renal stone-formers and non-stone-formers. In recent years, there has been much discussion on the influence of different factors, such as ionic and non-ionic inhibitors (stabilizers) and macromolecules, on calcium oxalate stone formation in normal subjects and stone-formers (Berg et al.,1982; Tawashi, 1983; Azoury et al., 1986; Robertson et al.,1986; Scurr and Robertson, 1986; Khan et al., 1988; Kohri et al., 1988; Lanzalaco et al., 1988; Morse and Resnick, 1988; Grases et al., 1988, 1989). An understanding of surface reactions in the mineral phase in urine environments is therefore a prerequisite to solving the problem of urinary calcification.

The relationship between the surface geometry of calcium oxalate crystals and their aggregation and cohesion in stone formation is still unclear (Martin et al., 1984; Marickar and Koshy, 1987). Recent work from our laboratory investigated the dissolution kinetics and surface geometry of COD single crystals in normal and stone-formers' urine (Akbarieh et al., 1987; Akbarieh and Tawashi, 1989). The data obtained suggested that the geometric structure of the surface is likely to be a potential factor in crystal aggregation during stone formation. The phase transition of calcium oxalate trihydrate (COT) to calcium oxalate dihydrate (COD) and calcium oxalate monohydrate (COM), and COD to COM single crystals in a urine environment was also studied (Akbarieh and Tawashi, 1990). These findings indicated that the phase transition of calcium oxalate on the surface of single crystals is a powerful technique for understanding the exact role of urinary macromolecules in stone formation.

Acidic glycoproteins have been detected in urine as well as in the core of renal stones (Grant et al., 1973; Samuelle, 1979; Nakagawa et al., 1987; Sirivongs et al., 1989). The effect of urinary macromolecules such as Tamm-Horsfall mucoprotein (THP) and nephrocalcin on calcium oxalate crystal growth and agglomeration has been studied extensively (Nakagawa et al., 1978; Kitamura and Pak, 1982; Scurr and Robertson, 1986; Wiggins, 1987; Yoshioka et al., 1989; Hess et al., 1989). It is still not clear, however, whether THP is a bystander

or an active participant in stone formation (Kumar and Muchmore, 1990). Although many investigators have reported the inhibition of crystal and/or stone growth by acidic glycoproteins (Nakagawa et al., 1978; Gjaldbaek and Robertson, 1980; Kitamura and Pak, 1982; Scurr et al., 1983; Gambaro et al., 1984), others have demonstrated the promotion of these processes in the presence of THP (Hallson and Rose, 1979; Rose and Sulaiman, 1982). Recently, Worcester et al. (1988) showed that "normal" THP has no effect on calcium oxalate crystal growth. Changes in the molecular configuration of the protein molecule therefore represent another question which needs to be answered. Nakagawa et al. (1985, 1987) observed that nephrocalcin in subjects with recurrent calcium oxalate nephrolithiasis is structurally different from its counterpart in normal individuals. Molecular THP abnormalities in calcium oxalate nephrolithiasis have recently been reported. In fact, Hess et al. (1991) revealed that "abnormal" THP aggregates calcium oxalate crystals.

Overall, the precipitation, deposition and growth of calcium oxalate crystals are influenced by the presence of natural polymers, such as THP, nephrocalcin, RNA, lipids, heparin and chondroitin sulphate (Martin et al., 1984; Khan and Hackett, 1984; Iwata et al., 1985; Khan et al., 1988; Lanzalaco et al., 1988). The effect of these polymers on calcium oxalate crystallization is a function of the concentration of anionic polyelectrolytes (Manne et al., 1990). Given these facts, the purpose of this work was to determine the influence of another natural mucoprotein, mucin as an acidic glycoprotein model, on the growth of calcium oxalate single crystals and to identify possible structural and chemical changes at the interface.

## Materials and Methods

To study the effects of mucin on hydrated calcium oxalate crystal growth, we used aqueous and gel growth media, two different techniques already well developed in our laboratory.

### Interfacial crystallization

Different hydrated calcium oxalate crystals were grown under slow liberation of reacting ions. Growth was based primarily on the slow hydrolysis of diethyloxalate (Sigma Chemical Co., MO, USA) in the presence of freshly double distilled water containing calcium chloride (Fisher Scientific, Canada) at 4°C. In this work, 0.1 M and 1 M calcium chloride solutions were used to grow COT and COD crystals, respectively. Slow diffusion of oxalate at the interface, which separates the two insoluble phases, controlled the reaction and growth of calcium oxalate crystals. The chemical reaction took place in calcium chloride solution. The harvested crystals were washed with chilled absolute ethanol (0°C) and preserved in a dry vacuum state for further study and analysis. The technique of growth and identification has been reported in detail elsewhere (Lachance and Tawashi, 1987). Lyophilized type I bovine submaxillary gland mucin (Sigma) was added to the calcium chloride solution at a concentration of 50mg/L. In all cases, a control experiment was performed without mucin.

### Gel diffusion crystallization

COD and COM crystals were grown in a gel medium with or without mucin. The growth medium used in this study was prepared with calfskin purified gelatin (Lot #13413, Eastman Kodak Co., Rochester, NY, USA). A 5% aqueous gelatin solution was constituted by gentle heating (below 50°C) for one hour. The solution was buffered to pH 6.2 with tromethamine. When the effect of the glycoprotein was investigated, 50 ppm of mucin was added to the aqueous solution prior to gelling. The solution was poured into a cooled (4°C) Pyrex Culture Petri dish (100x22 mm) in which the bottoms of two large test tubes were suspended (Fig. 1a). The system was allowed to gel for 24 hours at 4°C. The suspended test tubes were then carefully removed one by one from the gel by gently heating them. To do so, 10 ml of hot water was poured into each test tube prior to its removal. This is a crucial step because the depression made must have a smooth surface and should not be scratched. With this technique, a gel growth media and its two chambers could easily be observed under a stereomicroscope (Fig. 1b). One chamber was filled with 5 ml of a 0.5 M calcium chloride solution and the second was loaded with a 0.5 M oxalic acid solution. Both solutions were cooled to 4°C before use. The system thus prepared was kept in

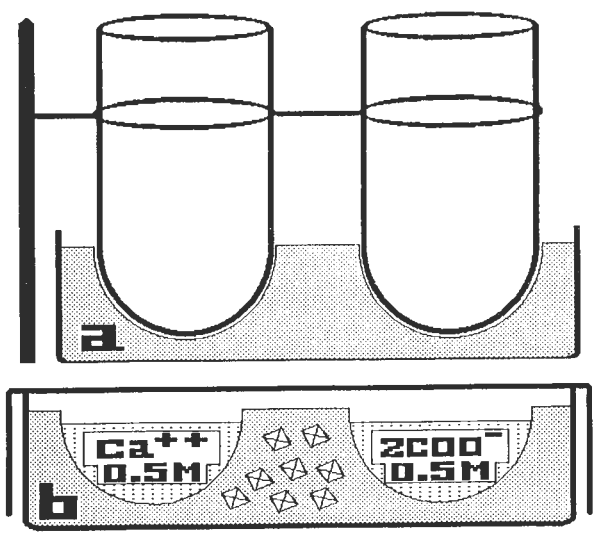


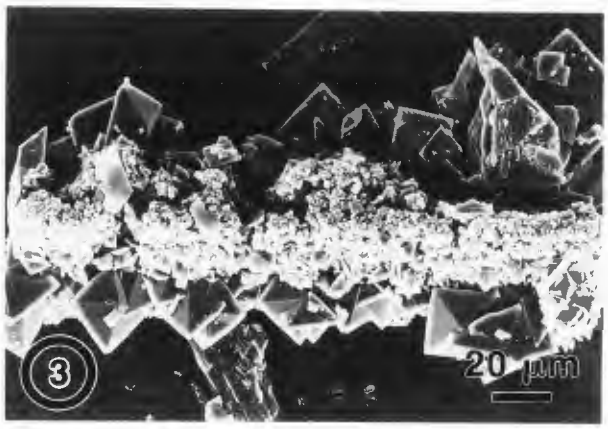

**Figure 1.** Schematic presentation of gel growth medium preparation. a: the test tubes were suspended before pouring the gel. b: after removal of the test tubes, solutions were poured into the two chambers and covered to limit evaporation.

a controlled-temperature incubator at 4°C for the duration of the experiment. The crystals formed in the calcium chamber were washed with chilled absolute ethanol (0°C) and preserved in a dry vacuum state for further study and analysis.

Crystals obtained by these methods were characterized by polarizing microscopy, scanning electron microscopy (SEM) X-ray surface analysis and X-ray powder diffraction. For SEM X-ray surface analysis, uncoated crystals were examined with a SEM energy dispersive microanalyzer. In this study, we used a JSM 840 scanning electron microscope (JEOL, Tokyo, Japan) equipped with a LINK-10000 energy dispersive X-ray spectrometer in a windowless mode to compute the calcium/oxygen ratio in the crystals. Surface analysis was performed at 5kV for a minimum of 50 readings on each crystal. Details of this technique have been reported previously (Akbarieh and Tawashi, 1990). Using a Philips PW-1130 diffractometer for X-ray powder diffraction analysis, calcium oxalate crystals were examined for their degree of hydration according to a method described earlier (Lepage and Tawashi, 1982).

## Results and Discussion

Two different hydrated calcium oxalate crystals, namely COT and COD, were grown separately under the slow liberation of reacting ions by changing the concentration of calcium in the aqueous medium (0.1 M for COT and 1 M for COD crystals). The interfacial crystallization method generated COT single crystals up to 2 mm in diameter (Fig. 2) when mucin was present in the calcium chloride solution. To obtain crystals of this size, four weeks of incubation were needed. COT crystals did not exceed 250 $\mu$m in the controls as demonstrated earlier (Lachance and Tawashi, 1987). Maximal crystal size in the controls was reached after three weeks. Further incubation of the controls led to the transformation of COT to less hydrated forms, mainly COD (Fig. 3). In fact, X-ray powder diffraction analysis of the system after four weeks of incubation did not reveal any COT

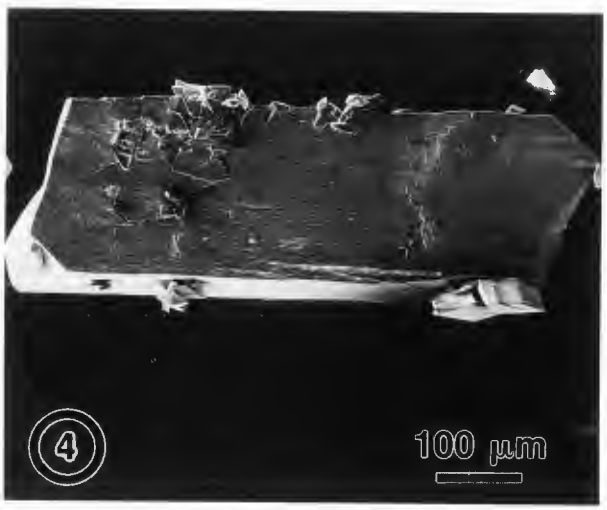




**Figure 2.** COT single crystal grown after four weeks of incubation using an interfacial crystallization medium in the presence of mucin.

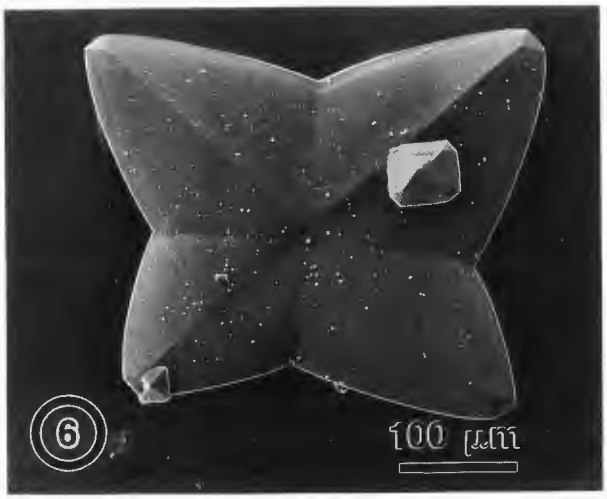
**Figure 3.** Rapid phase transformation of COT single crystal to other hydrated forms, mainly COD, after four weeks of incubation in an interfacial crystallization medium without mucin.

**Figure 4.** Slow phase transformation of COT single crystal to COD and COM after four weeks of incubation in an interfacial crystallization medium in the presence of mucin.

crystals. However, in the presence of mucin, phase transition started only after four weeks and took two more weeks to complete COT→COD transformation (Fig. 4). The SEM energy dispersive X-ray spectrometer in a windowless mode was used to determine the degree of hydration on different areas of the same crystal (Fig. 5). The data obtained from the computation of Ca/Ox ratios were similar to those reported in a previous paper (Akbarieh and Tawashi, 1990).

With COD crystals grown by interfacial crystallization, two major differences were noted in the presence of mucin. Crystal growth in the controls continued for up to two weeks, and COD transformation to COM occurred thereafter. The size of the largest crystal produced was about 350 $\mu$m with a typical tetragonal bipyramidal morphology (see Akbarieh et al., 1987; Akbarieh and Tawashi, 1989, 1990). However, when the calcium chloride solution contained mucin, COD crystal transformation did not occur before four weeks and the final crystals grew up to 1 mm. More strikingly, the crystal habit was different (Fig. 6). In fact, {110} planes (according to Franchini-Angela and Aquilano, 1979) were recognizable on all COD prepared by this method in the presence of mucin. A longer incubation period (two more weeks) was needed for phase transformation of COD single crystals and the formation of COM clusters (Fig. 7). X-ray powder diffraction confirmed that these crystals were exclusively COM. It is noteworthy that this type of cluster was previously observed in a study of crystalluria (Werness et al., 1981).

In this investigation, we used gelatin gel to control the slow diffusion of ions. It has been reported that gelatin gel is a favourable growth medium for calcium oxalate nucleation (Bisaillon and Tawashi, 1976). Good results have been obtained with calcite growth in gelatin gel under variable conditions (Wolter and Tawashi, 1977). In these two investigations, the U-shaped tube system used required several washings to discard gelatin from the crystals for size analysis. This new experimental design offered the possibility of observing and sizing the crystals as well as determining their growth rate under the microscope.

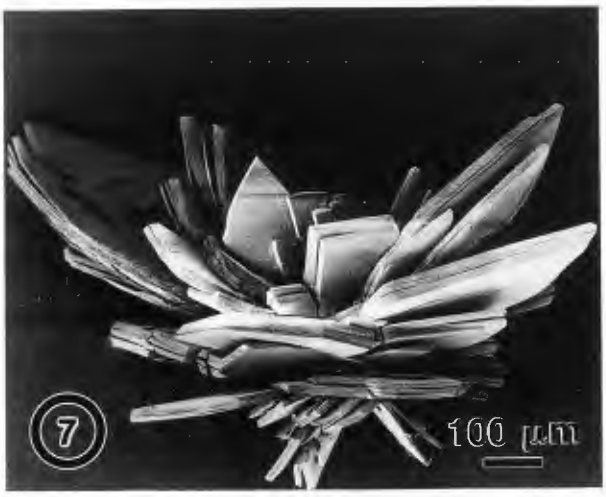




**Figure 5.** Different phases revealed by energy dispersive X-ray microanalysis of the COT crystal presented in Figure 4. Transformation of COT (a) to COD (b) and COM (c) after four weeks of incubation in interfacial crystallization medium in the presence of mucin.

**Figure 6.** COD single crystal grown after four weeks of incubation in interfacial crystallization medium in the presence of mucin.

**Figure 7.** COM cluster produced by COD transformation after six weeks of incubation in interfacial crystallization medium in the presence of mucin.

With gel diffusion crystallization, single tetragonal bipyramidal COD crystals of 5-10 $\mu$m formed inside the gel after two weeks. These crystals could be observed and sized under the stereomicroscope (Fig. 8a). Furthermore, COD crystals in clusters and/or in chains were grown in the calcium chamber (Fig. 9). In the controls, these packs of crystals were free in the calcium chloride solution. However, in the presence of mucin, they were attached to the gel (Fig. 8b). These clusters could be separated from the gel by the very slight force of a needle. Strikingly, we observed growth pits on the attachment site of each crystal of the cluster (Fig. 10). Longer incubation periods initiated phase transition and after the fifth week COM crystals in the calcium chamber were found in the controls (Fig. 11). This crystal habit was studied by other investigators and identified as dumbbells of COM crystals (Khan & Hackett, 1986; Werness et al., 1981). The phase transformation of COD crystals in the presence of mucin led to spherullization after six weeks (Fig. 12). These spherical structures were free in the calcium chloride solution (Fig. 8c) and X-ray powder diffraction analysis revealed that they were exclusively COM. Although these COM crystals did not reach 1 mm in size, their morphology resembled the milk of calcium found as deposits in the lower pole of the kidney (Grynpas et al., 1984).

In this work, we used a lyophilized powder of bovine submaxillary gland mucin, which is a mucoprotein with a high ratio of carbohydrate and sialic acid (Ozeki and Yosizawa, 1971). This choice was made primarily because of the ratio of sialic acid (approximately 5%) which is close to the amount (4%) found in THP (Fletcher et al., 1970). To determine the effect of mucin

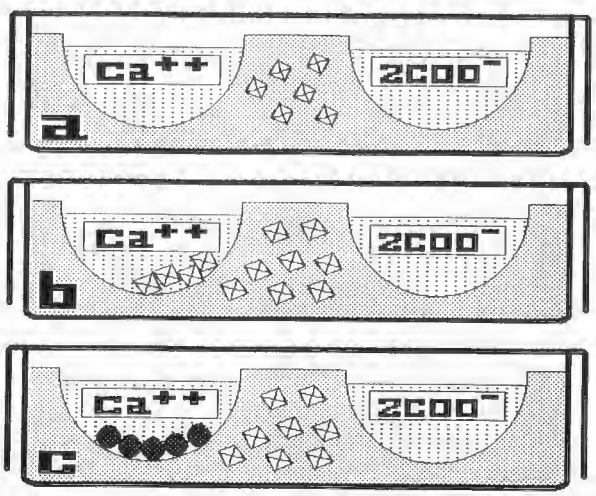
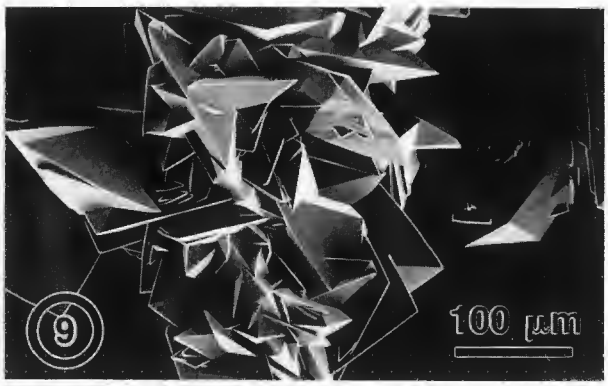



**Figure 8.** Schematic presentation of gel diffusion system. COD single crystals were produced in the gel (a) and at the same time COD clusters were grown in the calcium chamber (b). Phase transition then occurred and produced COM spheres (c).



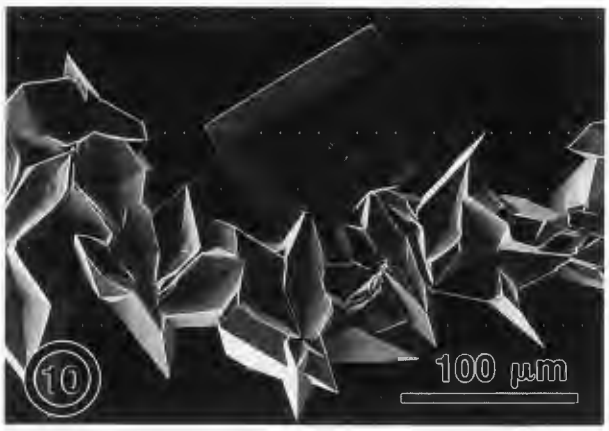





**Figure 9.** COD cluster grown in the calcium chamber of the gel crystallization system after two weeks in the absence of mucin.

**Figure 10.** COD cluster grown in the calcium chamber of the gel crystallization system after two weeks in the presence of mucin.

**Figure 11.** COM crystals produced by phase transformation of COD in the calcium chamber of the gel crystallization system after six weeks in the absence of mucin.


**Figure 12.** Different stages of phase transition of COD in the calcium chamber of the gel crystallization system after six weeks in the presence of mucin. The COD cluster (a) was transformed to COM by spherullization (b) and produced a smooth spherical crystal (c).

on calcium oxalate crystal growth, a 50 mg/L concentration was employed since it is close to the amount of THP in urine. The longer time of crystal growth and the larger size of COT and COD single crystals could be attributed to phase transformation inhibition,and the acidic portion of mucin can be responsible for this result. It has already been shown that nonpolyelectrolytes have no effect on the crystallization of calcium oxalate and, in the presence of these macromolecules, only COM crystals are formed (Manne et al., 1990). Working with supersaturated solutions, Manne et al. produced COT and COD crystals only in the presence of 1-50 ppm of polyelectrolytes, regardless of molecular weight. Some other investigators have found that many polyhydroxycarboxylic acids inhibit COM crystal growth (Grases et al., 1988). The change in COD and COM crystal habit observed in this study could be due to the selective adsorption of the inhibitors. In fact, it has been suggested that adsorption of polyhydroxy-carboxylic acids at active growth sites is the cause of the reduced COM crystal growth rate. The selective adsorption of urine inhibitors was responsible for the decrease in the dissolution rate of calcium oxalate single crystals (Akbarieh and Tawashi, 1989). In this work, 0.1 M and 1 M calcium chloride solutions were used to grow COT and COD crystals, respectively. However, significant variations of the calcium oxalate crystal growth rate were demonstrated at different ionic strengths or pH levels (Kitamura and Pak, 1982; Worcester et al., 1988; Yoshioka et al., 1989). Heijnen and van Duijneveldt (1984) reported that during growth of the tetragonal bipyramidal COD crystal the {110} face could develop, generated by certain changes in charge distribution. Mucin produces this effect, as seen in Figure 6. Work is continuing along these lines to elucidate the influence of pH levels and ionic strength on the exact role of mucin in the structural surface properties of newly-formed oxalate crystals and in crystal aggregation.

## Conclusion

Different hydrated calcium oxalate single crystals, namely COM, COD and COT, were grown under slow diffusion of reacting ions, using interfacially-controlled crystallization as well as gel diffusion crystallization in the presence of mucin. The size, habit, structure, thermodynamic stability and phase transition kinetics of hydrated calcium oxalate crystals were dramatically affected by the presence of mucin in the growth media. Our data confirm that glycoproteins are more than an adhesive material in calcium oxalate stone formation.

## Acknowledgments

Thanks are due to the $MC^2$ Laboratory of Ecole Polytechnique (Montreal, Canada) for their help and to the MRC of Canada for supporting this work.

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## Discussion with Reviewers

**W.G. Robertson:** In the absence of additive, it is usually found that a high calcium/oxalate ratio leads to COD and a low calcium/oxalate ratio to COM as the primary crystals formed. Do you have evidence to support this?
**Authors:** We believe that the growth of calcium oxalate dihydrate crystals in the calcium chamber (Fig. 8b) supports previous observations indicating that the high calcium/oxalate ratio primarily leads to formation of COD crystals.

**W.G. Robertson:** Does varying the concentration of mucin affect the formation of crystals, their degree of agglomeration and the transformation to another phase?
**Authors:** With a mucin concentration of 50 ppm, there was significant effect on the formation of crystals, their degree of agglomeration and the transformation. However, at lower concentrations the results were variable. In fact, phase transition was not observed.

**S.R. Khan:** I would like to know whether it is the calcium concentration in the medium or the duration of the experiment that results in the formation of large crystals?
**Authors:** Beside the effect of mucin, the calcium concentration and the slow diffusion of ions controlled by the gel are important factors in the formation of large calcium oxalate crystals.

**S.R. Khan:** Why do the crystals form in calcium chamber and not in oxalate chamber?
**Authors:** When equimolar ratios of calcium and oxalate are employed, the ionic equilibrium will shift in favour of oxalate and the crystals will grow in the calcium chamber. This shift appears to be controlled by the acidic pH of the gel.

**Y. Nakagawa:** The authors harvested crystals, then washed them with absolute ethanol and preserved them in a dry vacuum state. I wonder whether calcium oxalate trihydrate and dihydrate convert to calcium oxalate monohydrate during these processes?
**Authors:** The phase transformation of the hydrated calcium oxalate crystals to a less hydrated form involves dissolution and recrystallization process. This requires the presence of water molecules. During the harvesting and preservation we tried to keep the crystals as dry as possible.

**Y. Nakagawa:** The authors obtained commercially available mucin isolated from submaxillary gland. I wonder how pure this mucin is, since many crystal growth inhibitors might be in this mucin and effect crystal growth?
**Authors:** The mucin obtained from Sigma might contain impurities which act as crystal growth inhibitors. This kind of impurities could affect the growth rate of crystals

(size). We believe that the concentration level of these impurities is not sufficient to affect agglomeration and/or phase transformation.

**H. Iwata:** I am interested in the fact that the phase transition of COD to COM took place in the calcium chamber but it didn't take place inside the gel. How can you explain this?
**Authors:** As a matter of fact, calcium oxalate phase transformation occurred in both calcium chamber and inside the gel. However three weeks after phase transition occurred in calcium chamber, the COD crystals formed inside the gel started phase change.

**H. Iwata:** Low temperature, low oxalate-to-calcium ratio and presence of magnesium ion are factors which facilitate the formation of COD rather than COM. There seems to be a possibility that some of the COM crystals are formed initially (i.e. not transformed from COD) after several weeks, because the prolonged incubation increases the oxalate-to-calcium ratio in the calcium chamber. Can you distinguish the initially formed COM from the transformed one?
**Authors:** The possibility exists. Indeed, there is no available technique, to our knowledge, capable to detect submicron COM crystals initially formed in calcium chamber in this type of experiment.