The role of glycoproteins in calcium oxalate crystal development

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OBJECTIVE

To assess the effects of a glycoprotein (mucine) on calcium oxalate crystal development in different conditions and situations, to clarify some of its possible effects.

MATERIALS AND METHODS

Crystallization was assessed using a batch system in presence of mucine suspensions, by kinetic-turbidimetric measurements, and using a flow system in the presence of retained agglomerates of mucine, evaluating the precipitated calcium oxalate.

RESULTS

In batch conditions low mucine concentrations (<15 mg/L) inhibited calcium oxalate nucleation and higher concentrations (<250 mg/L) inhibited calcium phosphate nucleation, whereas at high concentrations there was also promotion. The presence of an aggregate of mucine in the flow system provoked calcium oxalate monohydrate crystallization at 0.691 µg/h per mg of mucine. In flow conditions pyrophosphate at 11.5 µmol/L caused a decrease of 84% in the calcium oxalate crystallized on mucine, 1.32 mmol/L of citrate a decrease of a 83%, 20 mg/L of pentosan polysulphate a decrease by 80%, and 7.58 µmol/L of phytate totally prevented the crystallization of calcium oxalate on mucine.

CONCLUSION

All substances inhibiting calcium oxalate crystallization with the capacity to interact with calcium ions also have crystallization promoting properties when they are at sufficiently high concentrations, because of their capacity to form agglomerates or the insolubility of their calcium salts.

KEYWORDS
glycoproteins, heterogeneous nucleation, crystallization inhibitors, calcium oxalate

INTRODUCTION

The time required to generate a crystal mainly depends on its nature, the supersaturation of the solution (crystallization driving force, a thermodynamic factor), the presence of pre-formed solid particles (the so-called heterogeneous nucleants, a kinetic factor) and the presence of crystallization inhibitors (kinetic variable). The latter are substances that because of their structure interact with the nucleus or the crystal faces, provoking important disturbances in their formation and/or development, thus preventing crystallization. Consequently, crystallization depends on the balance between thermodynamic and kinetic factors. All human urine is supersaturated for calcium oxalate, the supersaturation degree being higher for hypercalciuric/hyperoxaluric individuals. Depending on the pH, urine is also supersaturated for other substances, e.g. calcium phosphates or uric acid. Human urine also contains a variety of heterogeneous nucleants, e.g. protein agglomerates or cellular debris; moreover, the nucleant capacity of the altered epithelium must also be considered. This means that in urine several products can crystallize, depending on time. Fortunately, most urine does not form crystals during the residence time in the upper urinary tract, because of the kinetic retardation by some urinary constituents which may considerably decelerate the crystallization process, and consequently the importance of crystallization inhibitors in preventing calculi formation is unquestionable. Nevertheless, despite the obvious inhibition and the importance of its benefits, this remains a poorly studied and characterized biological process, and there are relatively few reports each year (<100 during 2001), and few inhibitory molecules are of immediate clinical use. The first biological crystallization inhibitor to be recognized and investigated was pyrophosphate, in the 1960s. Thereafter, various substances in urine were described as crystallization inhibitor molecules, some of them of low molecular weight, e.g. magnesium and citrate, and others with high molecular weight like glycosaminoglycans and diverse glycoproteins [1–7]. In the last decade there was a tendency to consider protein inhibitors of stone formation to be of major importance in the natural defence against nephrocalcinosis. Nevertheless, in vitro experiments and clinical data seem to be inconclusive on the inhibitory capacity of these macromolecular substances, some authors defending their inhibitory capacity [8–13], others showing crystallization promoter properties [14–19]. Indeed, there is no unequivocal evidence that any single macromolecular substance or group of them are directly involved in preventing stone formation as crystallization inhibitors. Moreover, their action as anti-adherent substances (lubricants) preventing the adherence of solid particles on the uroepithelium also seems important [20–22], but this is a different action that must not be mistaken for authentic behaviour as a crystallization inhibitor. However, it is obvious that the retention time of a particle in the urinary tract is also a very important kinetic factor that can contribute to stone development and formation. Consequently, the anti-adherent role of glycoproteins expediting crystal elimination must also be considered.

In the present study we assessed the effects of a glycoprotein (mucine) in calcium oxalate
crystal development, to evaluate some of its possible effects.

MATERIALS AND METHODS

Synthetic urine supersaturated for calcium oxalate (Ca\(^{2+}\), 2.5–3.5 mmol/L, oxalate 0.455 mmol/L) was prepared by mixing equal volumes of solutions A and B, of the compositions shown in Table 1. The range of calcium was selected to avoid conditions that represent hypercalciuria. Conditions of mild hyperoxaluria were chosen to dispose to slight lithogenic urinary conditions. Solutions were stored for ≤1 week at 4 °C. Chemicals of reagent-grade purity were dissolved in deionized and re-distilled water. All solutions were filtered through a 0.45 μm pore filter before being used.

To study calcium oxalate crystal formation in the presence of mucine, kinetic turbidimetric measurements were made using a photometer equipped with a measuring cell and fibre-optic light guide, with an attached 2 × 10 mm reflector in the light path, and using monochromatic light (550 nm). Crystallization was carried out in a thermostatically controlled (37 °C) and magnetically stirred cylindrical glass flask (height 12.5 cm, diameter 9 cm); 250 mL of solution A were added to the flask, the measuring cell immersed in the solution and the magnetic stirrer activated. Then 250 mL of solution B and different amounts of a mucine-water suspension were added to the flask with no oxalate was subtracted from the former values.

To assess crystallization in the presence of mucine in flowing conditions, a cuvette of 1 × 1 × 1 cm was placed in a temperature-controlled chamber (Fig. 3), and 15 mg of mucine placed at the bottom of the flask. Synthetic urine (with a calcium concentration of 3.5 mmol/L at pH 5.5) was introduced by a multichannel peristaltic pump at 1.5 L/day 3 mm from the bottom of the flask and overflowing constantly. The system was operated continuously for 48 h, after which the substrate was removed from the system, rinsed with distilled water and dried at room temperature in a desiccator. The amount of calcium oxalate crystallized on the substrate was evaluated by dissolving the precipitate in 0.1 mol/L HCl and determining calcium by atomic emission spectroscopy. To avoid ‘blank’ errors the calcium content obtained from a mucine substrate treated in identical conditions but using synthetic urine without calcium was subtracted from the results.

The effects of citrate (sodium salt) at 0.529–5.29 mmol/L, phytate (sodium salt, Sigma Chemical Co., St Louis, MO) at 1.52–7.58 mmol/L, pyrophosphate (sodium salt at 5.74–40.2 μmol/L) and sodium pentosan polysulphate (Sigma) at 10–40 mg/L were assayed by adding different amounts of them to synthetic urine in the presence of agglomerates of mucine in flowing conditions. Each experiment was repeated three times to check the reproducibility of the results.

Because of the high concentrations of citrate, and considering its complexing capacity for calcium ions, in experiments in which the action of citrate ions was evaluated calcium was also added to obtain the same calcium oxalate supersaturation present with no citrate. A decrease in supersaturation implies

![Image](https://example.com/image.png)

**TABLE 1** Composition of synthetic urine, values in mmol/L.

<table>
<thead>
<tr>
<th>Solution A</th>
<th>Solution B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(_2)SO(_4)-10H(_2)O</td>
<td>Na(_2)HPO(_4)-2H(_2)O</td>
<td>19.34</td>
</tr>
<tr>
<td>MgSO(_4)-7H(_2)O</td>
<td>Na(_2)HPO(_4)-12H(_2)O</td>
<td>5.93</td>
</tr>
<tr>
<td>NH(_4)Cl</td>
<td>NaCl</td>
<td>86.73</td>
</tr>
<tr>
<td>KO</td>
<td>Na(_2)C(_9)O(_4)</td>
<td>162.60</td>
</tr>
</tbody>
</table>

Different volumes of a 1 mol/L calcium solution (prepared by dissolving calcium carbonate with hydrochloric acid) were added to solution A to obtain a final calcium concentration in the range of 2.5–3.5 mmol/L.
a decrease in the crystallization rate that could not be assigned to inhibitory effects. The amount of added calcium ions was potentiometrically calculated using a selective calcium electrode and a potentiometer. The activity of free calcium ions was the same with or with no citrate, and consequently when citrate was added an appropriate amount of calcium to fulfil these conditions was added, i.e. an increase of 0.15 mmol/L in calcium concentration was required for each 0.53 mmol/L of citrate. Because low concentrations of phytate and pyrophosphate were used the decrease in the free calcium concentration was practically negligible, confirmed by potentiometry, and consequently it was not necessary to add calcium.

Solid samples from the different experiments were assessed by X-ray diffraction techniques and by scanning electron microscopy, showing the formation of calcium oxalate monohydrate (COM) crystals when the pH was adjusted to 5.5 and calcium phosphate (brushite) at pH 7.0.

**RESULTS**

The effects of suspended mucine on calcium oxalate crystal formation in synthetic urine in batch conditions are shown in Fig. 4. When the pH was adjusted to 5.5, at low concentrations (<15 mg/L) mucine strongly inhibited calcium oxalate crystal nucleation, whereas at high concentrations (>75 mg/L) it also acted as a promoter. When the pH was adjusted to 7.0 and calcium phosphate (brushite) was formed, mucine showed very similar behaviour, i.e. at low concentration (<250 mg/L) mucine inhibited calcium phosphate crystal nucleation, whereas at high concentrations (>500 mg/L) it promoted nucleation. The presence of an aggregate of mucine retained in a flow system provoked COM crystallization at 0.691 μg/h per mg mucine. The COM crystals formed in such conditions are shown in the Fig. 5.

In flowing conditions the effects of pyrophosphate on COM heterogeneous nucleation on mucine are shown in Fig. 6a. Pyrophosphate at 11.5 μmol/L caused a decrease of 84% in the calcium oxalate crystallized on mucine. Nevertheless, there was no further decrease when the pyrophosphate level was increased. The citrate effects are shown in Fig. 6b; 1.32 mmol/L citrate caused a decrease of 83% in the calcium oxalate crystallized on mucine, and there was no further decrease at greater citrate concentration. Pentosan polysulphate (a semisynthetic glycosaminoglycan) also inhibited the heterogeneous nucleation of calcium oxalate on mucine, and 20 mg/L decreased by 80% the calcium oxalate.
crystallized on mucine (Fig. 6c), but, as before further increase did not prevent crystal formation. The effects of phytate are shown in Fig. 6d; at concentrations of 1.52 μmol/L, the amount of calcium oxalate crystalized on mucine was reduced by 71% and at 7.58 μmol/L the crystallization of calcium oxalate on mucine was totally prevented.

**DISCUSSION**

From the present results mucine (a glycoprotein) is an inhibitor of calcium oxalate and calcium phosphate crystallization, but the inhibition cannot prevent crystallization when prolonged and at higher mucine concentrations, attaining maximum inhibition at relatively low mucine values. Because brushite precipitates at pH 7.0 and the aim of the study was to assess calcium oxalate formation, no further measurements were made at this pH. However, agglomerates of mucine were clearly heterogeneous nucleants of calcium oxalate crystals, as shown in Fig. 5. All these apparently contradictory results can be explained and understood given the general mechanisms of the inhibition of calcium oxalate crystallization and the nature of glycoproteins. Thus, crystallization is inhibited by the interaction between the inhibitor, mucine and crystals, which must be attributed to the affinity between the glycoproteins and calcium ions. Through this interaction there are disturbances in the nucleation, growth and aggregation of calcium oxalate crystals, and consequently crystallization is inhibited [8–13]. Moreover, by this affinity the glycoproteins present in urine can coat calcium oxalate crystals and thereby block their adhesion to renal tubular cells, preventing crystal retention in the kidney [20–22]. However, also through this capacity to interact with calcium ions, if these macromolecules form retained solid particles, then they could also act as active heterogeneous nucleants, enhancing the formation of calcium oxalate crystals [14–19]. Indeed, all inhibitors of calcium oxalate crystallization with a capacity to interact with calcium ions can show this behaviour when they are present at high enough concentrations, because of their ability to form agglomerates or the insolubility of their calcium salts (Grases F, unpublished data). Therefore the retention of agglomerates of glycoproteins can effectively induce calcium oxalate crystal development, and consequently any renal cavities of very low urodynamic efficiency could be an important risk factor for stone formation at high urinary levels of glycoprotein. In such cases inhibitors of the heterogeneous nucleation of calcium oxalate on mucine could prevent or minimize calcium oxalate crystal development. Thus citrate, pyrophosphate and pentosan polysulphate (a semisynthetic glycosaminoglycan) effectively reduced calcium oxalate crystal nucleation on mucine, whereas phytate totally blocked crystal development. Importantly, the effective inhibitory concentrations of these substances were of the same order as concentrations normally found in urine. Finally, in previous studies, pentosan polysulphate did not inhibit crystallization when a salt like calcium fluoride acted as a heterogeneous nucleant of calcium oxalate (Grases F, unpublished data), whereas in the present study it was an inhibitor, attributed to the more favourable chemical interactions between pentosan polysulphate and glycoprotein.

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**CONFLICT OF INTEREST**

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Abbreviations: COM, calcium oxalate monohydrate.