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Urological Research

Effects of serum, albumin and immunoglobulins on urease-induced crystallization in urine

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Summary. The effects of serum, albumin and gammaglobulins on urease-induced crystallization have been studied in synthetic and in human urine. Serum and the studied proteins increased urease enzymatic activity in synthetic urine. In human urine only serum had this effect. In synthetic urine, the proteins and serum markedly decreased the precipitation attached to glass surfaces, while the intraluminal precipitation was increased. In human urine, similar but weaker effects on the precipitation were found for serum and albumin. These findings suggest that the proteins studied, in the concentrations in which they are present in human urine, have profound effects on urease-induced crystallization and may be physiological crystallization inhibitors.

Key words: Urease-induced crystallization – Serum – Albumin – Gammaglobulin – Magnesium ammonium phosphate

Infection-induced stones consisting of magnesium ammonium phosphate (MAP) and carbonate apatite constitute 20–30% of all surgically removed upper urinary tract stones in our region [10]. The formation of infection-induced stones is mostly, but not always, linked to an infection with urea-splitting microorganisms [10]. The ammonia produced by the urea breakdown leads to an increase in urinary pH. At alkaline pH, the formation products for MAP and calcium phosphate (CaP) may be exceeded and stone formation take place [5]. The result of this process can vary from large staghorn stones to calyceal and pelvic stones and MAP crystalluria only [10, 14]. This wide variation may naturally be due to differences in the severity of the infections, and it has previously been claimed that the magnitude of the urease-induced pH increase is the major factor in the urease-induced crystallization [16, 19]. More recent studies, however, have shown that the urease-induced crystallization is also influenced by other factors, such as urine composition, and varies markedly between urine from different individuals [8, 11].

Calcium oxalate and calcium phosphate crystallization in urine is said to be influenced by inhibitors affecting both crystal growth and aggregation [6, 18, 20]. Apart from small molecules (Mg^{2+}, Zn^{2+}, citrate and pyrophosphate), large molecules, such as glycosaminoglycans and other protein complexes, have also been shown to affect calcium oxalate crystallization [2, 6]. Some investigators have also found serum to be a potent inhibitor of calcium oxalate crystallization [3, 12]. Of the serum proteins, albumin is known to be a constituent of the stone matrix [15] and it may thereby be involved in the stone formation. It is furthermore known that infected stones are particularly rich in organic material [14]. At least in serum, albumin has a special affinity to calcium ions and is actually their main carrier [1]. It thus seemed especially interesting to investigate the influence of albumin and other serum proteins on urease-induced crystallization.

Methods

Urines

Synthetic urine with the composition described by Griffith et al. [5] and a pH of 5.70 was used. This synthetic urine contains no proteins or other macromolecules. Voided human urine was collected and pooled from persons without a history of stone disease and with negative urine cultures. After collection this urine was immediately chilled to +4 °C, centrifuged (10 min, 3,000 rpm) and sterilized by filtration through a 0.22-μm Millipore filter. The pH and the ammonium ion concentration were measured. The albumin content of the pooled urine was 13.7 $mg \cdot l^{-1}$ (Albumin RIA 100, Commercial Kit, Pharmacia, Uppsala, Sweden) and the total protein concentration was 112 $mg \cdot l^{-1}$ (according to Lowry).

Additives

Serum was obtained from the same persons as those from whom urine was collected. Its content of albumin was 48 $g \cdot l^{-1}$, of IgG 11.2 $g \cdot l^{-1}$, of IgA 2.0 $g \cdot l^{-1}$, and IgM 0.9 $g \cdot l^{-1}$. Human albumin

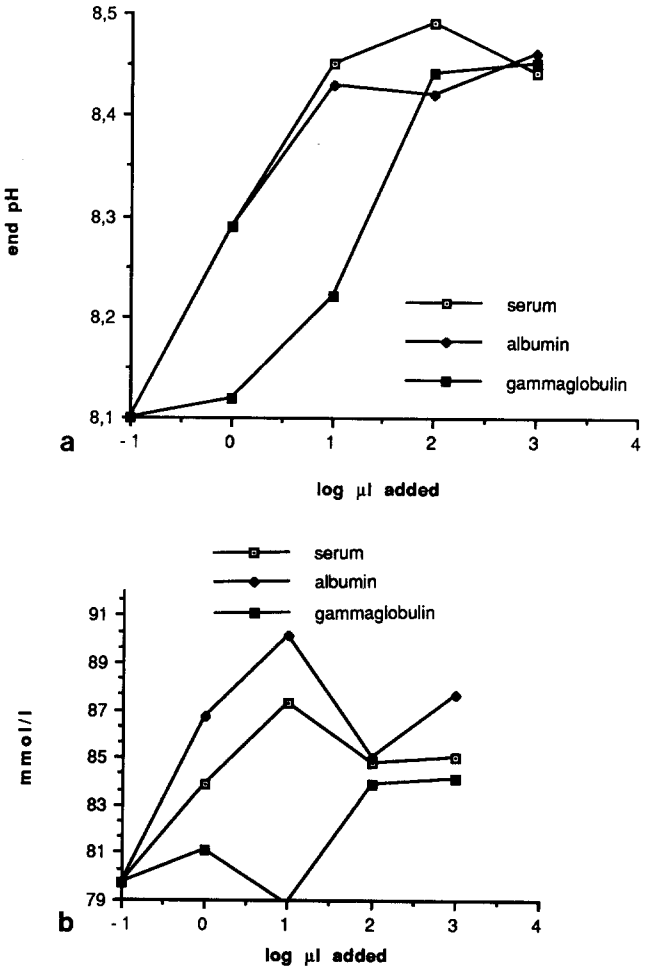

Fig. 1a, b. Effects of serum, albumin and gammaglobulin on a end pH, b ammonium ion concentration in synthetic urine after 4 h urease incubation

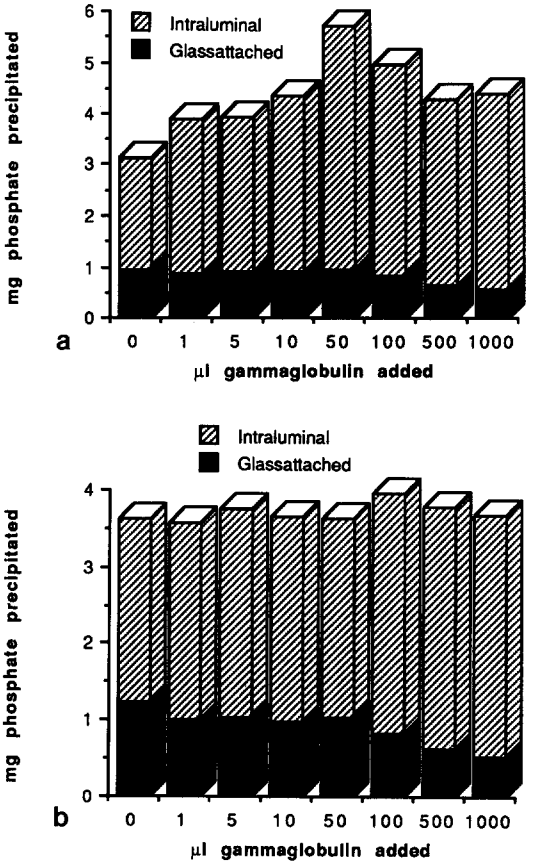

Fig. 2a, b. The urease-induced precipitation in synthetic urine after addition of serum: a magnesium-bound phosphate; b calcium-bound phosphate

(Kabi, Stockholm, Sweden) was dissolved in distilled water to a concentration of $60\,g \cdot l^{-1}$. Gammaglobulin (Kabi) was dissolved in distilled water to a concentration of $55\,g \cdot l^{-1}$ ($50\,g \cdot l^{-1}$ of IgG and $5\,g \cdot l^{-1}$ of IgM).

Experimental model

Twenty-four glass tubes equipped with one central glass rod were filled under sterile conditions with human or synthetic urine. Between 1 and 1,000 µl serum or albumin or gammaglobulin solution was added, resulting a final volume of 15 ml in each tube. The concentrations achieved ranged for serum from 0.007% to 6.7%, for albumin from $4\,mg \cdot l^{-1}$ to $4\,g \cdot l^{-1}$ and for gammaglobulin from $3.7\,mg \cdot l^{-1}$ to $3.7\,g \cdot l^{-1}$. When necessary, pH was adjusted to 5.70 in all tubes with 0.1 M HCl. The tubes were then simultaneously incubated with jack bean urease (E.C. 3.5.1.5.; $7\,units \cdot mg^{-1}$; unit = $1\,mg\,NH_3 \cdot 5\,min^{-1}$ at pH 7.0 and 30 °C, Sigma, St. Louis, USA) for 4 h at 37C without stirring. Of these 24 tubes, 3 were controls with urine only, serum was added to 7 tubes, albumin was added to 7 tubes and gammaglobulin was added to the last 7 tubes.

Analyses

Before and after urease incubation, pH and ammonium ion concentration were measured. The precipitation in the solution (intraluminal precipitation) was collected by filtration through a 0.22-µm Millipore filter and dissolved in nitric acid. The precipitation on the glass rod and inner surface of the tube was washed twice with cooled distilled water in order to wash away urine and remaining traces of the intraluminal percipitation. The rods and the tubes were then dried and the precipitation dissovled in nitric acid. The magnesium and phosphate content of the precipitates was measured according to the methods described by Savory et al. [17] and Zilversmith and Davis [21]. The pH measurements were performed with a surface electrode connected to a precision pH meter (Model 701 A, electrode 91-35, Orion Research). The ammonia-ammonium ion concentration was measured as a sum, using a slightly modified commercial kit (Sigma no. 640 A). All precipitated magnesium was assumed to be a constituent of MAP. With knowledge of the magnesium concentration, stoichiometric calculations were performed to determine the proportions of precipitated phosphate present as magnesium-bound and as calcium-bound phosphate. Previous studies have shown these fractions to consist of magnesium ammonium phosphate (MAP) and calcium phosphate (CaP) [7]. These fractions are referred to hereinafter as MAP and CaP.

All experiments were performed in triplicate. Results are given as means of the three experiments in each case.

Results

In synthetic urine, the urease-induced ammonium ion production and the subsequent pH increase were augmented by the addition of serum, albumin and gammaglo-

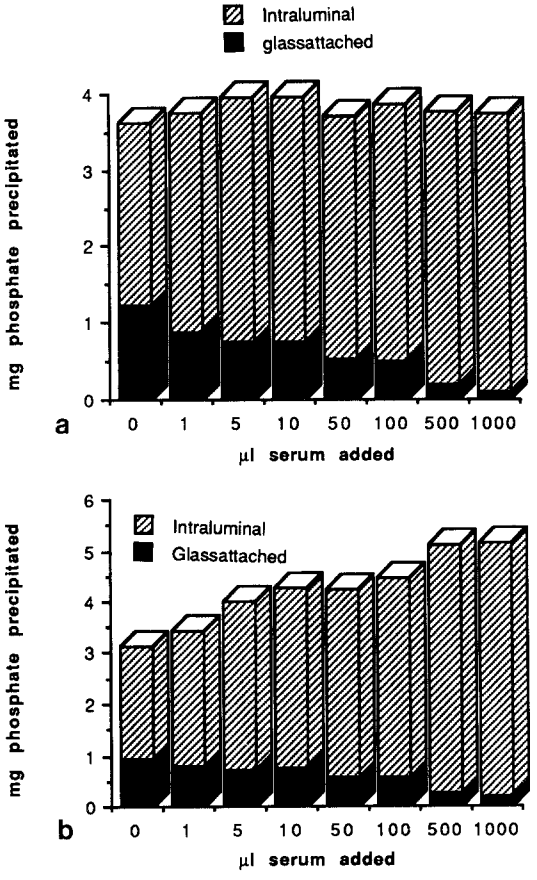

Fig. 3a, b. The urease-induced percipitation in synthetic after addition of albumin. **a** magnesium-bound phosphate; **b** calcium-bound phosphate

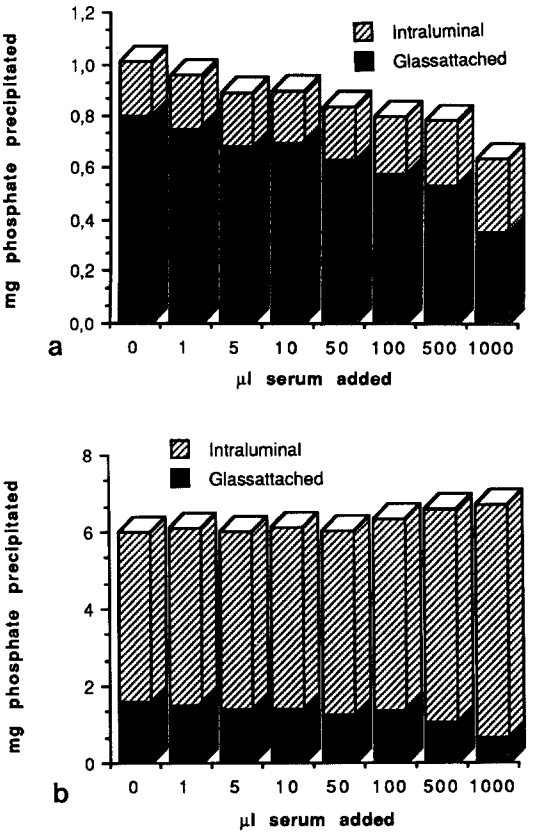

Fig. 4a, b. The urease-incuded precipitation in synthetic urine after addition of gammaglobulin: **a** magnesium-bound phosphate; **b** calcium-bound phosphate

bulin in very low concentrations, for serum 0.007%, for albumin $4\,mg \cdot l^{-1}$ and for gammaglobulin $3.7\,mg \cdot l^{-1}$ (Fig. 1). This effect was concentration-related up to additions of 0.03% serum and $20\,mg \cdot l^{-1}$ albumin and $37\,mg \cdot l^{-1}$ gammaglobulin. Larger additions did not increase the pH further. In human urine, only the addition of larger amounts of serum (more than 0.67%) had effects on the urease-induced pH increase. When 6.7% serum was added, pH increased to 8.27, as against 8.11 in controls. Albumin and gammaglobulins had no effect on the urease-induced ammonia production or the pH increase in human urine.

In synthetic urine, addition of serum, albumin or gammaglobulin markedly reduced the glass-attached precipitation (Figs. 2–4). This reduction was concentration-related and included both MAP and CaP. It was most pronounced for serum. When 6.7% was added, the CaP precipitation was reduced to 9% and the MAP precipitation to 12% (Fig. 2). A similar but less pronounced effect on the precipitation was noted also for albumin and gammaglobulin (Figs. 3, 4).

Together with a reduced glass surface-attached precipitation, an increased intraluminal precipitation was registered (Figs. 2–4). The total precipitation (glass surface-attached + intraluminal) of MAP increased with increasing additions of serum, albumin or gammaglobulin (Figs. 2a, 3a, 4a). For CaP, the intraluminal increase and glass surface decrease balanced each other, resulting in an unchanged total precipitation (Figs. 2b, 3b, 4b).

In human urine, serum and albumin had the same but weaker effects on the precipitation as in synthetic urine (Fig. 5). The intraluminal precipitation of MAP was not as much augmented as in synthetic urine, however, so that the total precipitation of MAP showed a decrease, in contrast to the increase registered in synthetic urine. Nonetheless, intraluminal precipitation of CaP showed a pattern similar to that in synthetic urine. The addition of gammaglobulin did not influence the precipitation in human urine.

Discussion

Serum, albumin and immunoglobulins promoted urease enzymatic activity in synthetic urine. In human urine, serum had a weak effect, while albumin and gammaglobulins had no effect on urease enzymatic activity. In synthetic urine, the proteins increased the total precipitation of MAP but did not influence the total precipitation of CaP. Serum markedly and albumin and gammaglobulin to a degree decrease the glass-attached precipitation of both MAP and CaP.

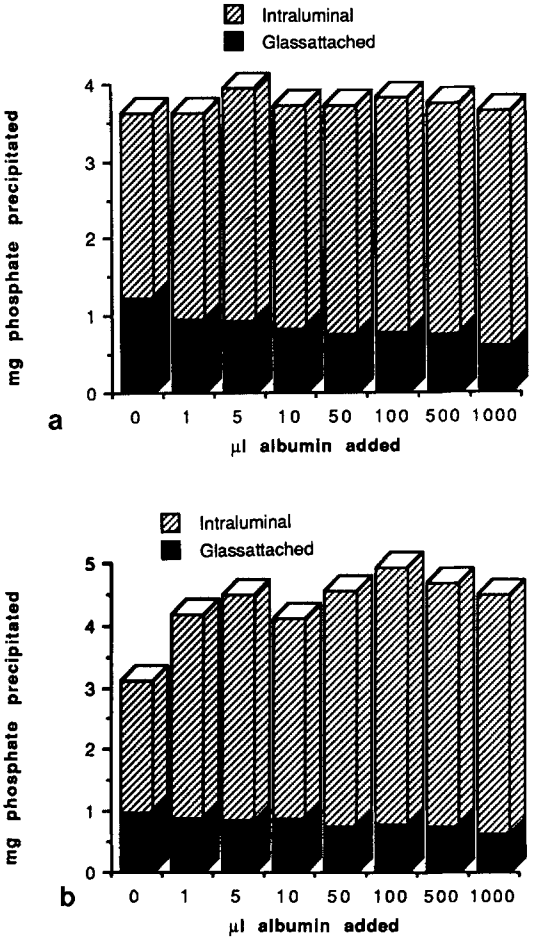

Fig. 5a, b. The urease-induced precipitation in human urine after addition of serum: **a** magnesium-bound phosphate; **b** calcium-bound phosphate

In human urine, serum and albumin influenced the precipitation in a similar way. The changes were less pronounced than those in synthetic urine, however. Calcium oxalate stone formation is influenced by inhibitors thought to play an important role in the stone-forming process [2]. Serum is known to be one of the most potent calcium oxalate crystallization inhibitors [12]. Serum acts in concentration of up to 0.1% as a potent inhibitor of calcium oxalate aggregation when added to whole urine but is only a relatively weak inhibitor of crystal growth [3]. Despite this, little interest has been focused on the role of the major serum proteins, i.e. albumin and immunoglobulins, in crystallization in urine. Prebladder urine has a high inhibitory capacity, and it has been suggested that this inhibition is due to glomeruli-filtered substances [12]. The main urine protein derived from glomerular filtration is albumin. Its urine concentration varies in healthy individuals from 5 to 40 $mg \cdot l^{-1}$ [11]. Other serum proteins present in urine are IgG (0.20–6.50 $mg \cdot l^{-1}$), IgA (0.0–2.25 $mg \cdot l^{-1}$) and IgM (0.0–1.34 $mg \cdot l^{-1}$).

It has recently been shown that human urine has an inhibitory action on urease-induced crystallization [4]. The nature and significance of the inhibitory action human urine has on urease-induced crystallization is still obscure. Citrate and zinc are so far the only known inhibitors of urease-incuded crystallization and they have only a very weak effect in human urine [9]. In this experiment, serum, albumin and gammaglobulin (IgG and IgM) were found to have a strong inhibitory effect on glass-attached crystallization in synthetic urine, while the intraluminal precipitation was unaltered or increased. The glass-attached precipitation probably reflects aggregation and growth of crystalline material, while the intraluminal deposit rather reflects earlier phases like nucleation and growth [7]. Different modes of action could explain the observe inhibition of the glass-attached precipitation; inhibition of the phase transformation from an amorphous to a crystalline phase, or inhibition of crystal aggregation and/or crystal growth. The inhibitory effect serum has on calcium oxalate crystallization has been said to be an inhibition of crystal aggregation [3]. Adhesion to calcium oxalate crystals has previously been described for urinary mucoproteins as well as for albumin and gammaglobulin [13]. This adhesion could then prevent the crystals formed from aggregating by covering their surfaces. This adhesion may not cover the crystals' growth sites, however, and crystal growth is therefore not affected [3]. It thus seems plausible that the inhibitory effect serum, albumin and gammaglobulin also had on urease-incuded crystallization was due to inhibition of the aggregation.

The changes in intraluminal crystallization caused by serum and the proteins studied could not be explained by their effect on the urease-incuded pH increase. The optimal pH for calcium phosphate crystallization is about 7.5 and for MAP 8.0. At higher pH the precipitation tends to decline [11]. In synthetic urine with serum or the protein solutions added, the end pH increased from 8.1 to 8.5. The precipitation would thus decrease with an increase in pH within this pH interval. Instead, the intraluminal precipitation of MAP increased and CaP was unchanged, in contrast to an expected reduction. A reasonable explanation for this observation is that a strong inhibition of crystal agrregation leaves more crystal growth sites "free" and if crystal growth is or only weakly, inhibited, not an increased crystal volume is to be expected, as described by Ryall et al. [3]. Another possible explanation is that the added proteins acted as nuclei for heterogeneous nucleation. In human urine, a decreased intraluminal precipitation of MAP was found instead. This may indicate that human urine contains inhibitors directed against the other steps of crystallization acting against an increase in intraluminal precipitation.

The changes in human urine were, less marked, however, especially when small amounts of serum or albumin were added. That serum and albumin exerted effects only when added in larger amounts (serum $> 0.3\%$ and albumin $> 40\ mg \cdot l^{-1}$) is probably due to the fact that whole human urine already has a strong inhibitory effect on crystallization. Only when larger, unphysiological, amounts of proteins are added will effects be noted.

Albumin and gammaglobulin had definite effects on the crystallization, but these were not so pronounced that they could explain the total effect of serum. Apart from

albumin and immunoglobulins, serum must contain other substances with inhibitory effects on urease-induced crystallization. Albumin and IgG, however, are of special interest, since they are present in human urine.

The promoting effect serum, albumin and gammaglobulins had on urease enzymatic activity is interesting. This effect was most pronounced in synthetic urine and was already apparent at very low protein concentrations, and when proteins were added in concentrations higher than normally seen in urine ($>20\,mg \cdot l^{-1}$ of albumin) no further stimulation was registered. The promoting effect was noted in human urine only at very high concentrations of serum. This can be explained by the fact that human urine contains proteins (in this urine $112\,mg \cdot l^{-1}$) in concentrations which thus appear to stimulate urease enzymatic activity almost maximally. The promoting effect was registered both as an increase in the final pH after 4 h urease inoculation and as an increased ammonium production in the urines where proteins were added. The rise in ammonium concentration was, however, less pronounced than the pH increase. The mechanism behind the promoting effect on urease enzymatic activity is not clear. Albumin also has this effect on other enzymes, however, and it is thought to be mediated via a better presentation of substrate to the enzyme [1]. Zinc has an inhibitory action on urease enzymatic activity [9]. It thus appears that human urine contains both urease inhibitors (zinc) and urease promotors (proteins). Whether this is also true of urease from uropathogenic microoganisms and to what extent this contributes to modulation of the stone-forming process remains to be evaluated. This effect has another important implication, however. In studies of the crystallization process, synthetic urines have often been used. Since the synthetic urines used usually only contain inorganic substances, the effects of proteins and other macromolecules in whole human urine will be missed and the results will not completely reflect the in vivo situation.

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