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The effects of fractioned human urine on urease-induced crystallisation in vitro*

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Summary. Previous studies have shown human urine to have an inhibitory action on urease-induced crystallisation. Centrifugation and 0.45 μm filtration of the urine did not reduce this activity. This eliminates larger urine particles as being the cause of the inhibitory activity. Both the retenate and the filtrate after ultrafiltration of urine with a 100.000 mol weight cut-off influenced the urease-induced crystallisation of magnesium ammonium phosphate and calcium phosphate. The results indicate that the inhibitory action is exerted by more than one urinary component.

Key words: Urease-induced crystallisation – Fractioned urine – Magnesium ammonium phosphate – Calcium phosphate

Introduction

Colonisation of the urinary tract by urease-producing microorganisms may result in the formation of concrements composed of magnesium ammonium phosphate (struvite) and/or calcium phosphate (carbonate apatite).

For both struvite and calcium phosphate, the increase in urinary pH which is caused by the urea-splitting activity of urease is considered to be the principal faktor determining the degree of saturation [16]. In accordance with this, is has been denied that urine possesses an inhibitory effect on struvite crystallisation [4]. Several substances, both small molecules and ions (Mg^{++}, citrate and pyrophosphate) as well as macromolecules, have, however, recently been shown to have an inhibitory effect on calcium phosphate crystallisation [17]. Recent studies have also shown that factors other than the level of the urease-induced pH increase influence the precipitation of both struvite and calcium phosphate in synthetic as well as in whole human urine [8, 10]. Analysis of the results suggests that it is the crystal aggregation which is influenced. It is thus possible that urine contains inhibitors of crystal aggregation active against this type of crystallisation.

To define the nature of this inhibitory activity better, characterisation of the inhibitor(s) with regard to molecular size has been performed.

Material and methods

Urine specimens were collected as fasting morning urine from 5 healthy adults with no history of stone disease and with negative urinary cultures. The urine was stored al +4°C and used within 4 hours of collection. The urine specimens were used unprocessed or after one, two or all of the following steps of preparation:

- Centrifugation at +4°C at 3,500 r.p.m. for 30 min. The supernatant was collected. The retenate was diluted to original volume with synthetic urine.
- In certain samples, the supernatant was filtered first through Millipore 1.2 and then Millipore 0.45 μm pore size filters.
- Certain of the Millipore-filtered urine samples were filtered in 400 ml portions through a 100.000 mol weight cut-off filter (Millipore Pellicone cassette system with a polysulfone filter) until the retenate volume was reduced to 100 ml. The first filtrate was collected. The retenate was restored to its original volume (400 ml) with distilled sterile water and refiltered through the 100.000 filter. This washing procedure was performed twice to remove electrolytes and substances smaller than the cut-off of the filter from the retenate. The first filtrate and the washed retenate were stored at +4°C and used within 24 h.

The synthetic urine (pH 5.7) used was composed of 11 solutes according to Griffith et al. [9]. The solutes, with concentrations within brackets ($g \cdot l^{-1}$), were as follows: $CaCl_2 \cdot 2H_2O$ (0.65), $MgCl_2 \cdot 6H_2O$ (0.65), NaCl (4.6) Na_2SO_4 (2.3), Na_3 citrate $\cdot 2H_2O$ (0.65), Na_2 oxalate (0.02), KH_2PO_4 (2.8), KCl (1.6), NH_4Cl (1.0), urea (25.0) and creatinine (1.1). The synthetic urine was sterilised by Millipore filtration (0.22 μm) and stored in sealed glass bottles at +4°C until used.

Urease-induced crystallisation

The urease-induced crystallisation in the synthetic urine was studied as encrustation on solid glass rods after innoculation with crystalline Jackbean urease (E.C. 3.5.1.5, 1 unit = 1 mg $NH_3 \cdot 5\,min^{-1}$ at pH 7 and 30°C) according to a previously described experimental model

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Table 1. The effects of unprocessed, centrifuged, centrifuged and filtered (1.2 and 0.45 μm) human urine and resuspended retenate after centrifugation on urease-induced encrustations on glass rods immersed in synthetic urine. Per cent reduction from controls within brackets (results given as mean of two experiments)

Additions to synthetic urine	Encrustations ($mg \cdot rod^{-1}$)		
	Phosphate	Struvite phosphate	Calcium phosphate
Experiment 1			
None (controls)	1.52	0.25	1.27
Unprocessed urine, 5 ml	1.09 (72%)	0.20	0.90
Centrifuged urine			
5 ml	1.16 (76%)	0.23	0.93
15 ml	0.90 (59%)	0.16	0.74
20 ml	0.80 (53%)	0.14	0.66
Experiment 2			
None (controls)	1.70	0.28	1.41
Unprocessed urine, 5 ml	1.28 (75%)	0.24	1.07
Centrifuged and filtered urine:			
5 ml	1.20 (71%)	0.21	0.98
15 ml	1.05 (62%)	0.18	0.85
20 ml	0.96 (56%)	0.16	0.79
Experiment 3			
None (controls)	1.58	0.25	1.33
Unprocessed urine 5 ml	1.20 (76%)	0.21	0.99
Retenate after centrifugtion Equal to:			
5 ml	1.40 (89%)	0.24	1.16
15 ml	1.32 (83%)	0.21	1.11
20 ml human urine	1.50 (95%)	0.23	1.27

(8). Nine vessels (4 glass rods in each vessel) with 250 ml of synthetic urine were incubated simultaneously in each experiment in the same isothermic water-bath at 37°C for 4 h. At the start and end of the incubation, pH was determined and after the incubation the amount of magnesium and phosphate precipitated on the rods was analysed according to the methods described by Savory et al. [18] and Zilversmith [21]. For each reactor, the mean encrustation per rod was calculated. All precipitated magnesium was assumed to be a constituent of struvite. With knowledge of the amount of magnesium, it was thus possible to calculate the proportion of the precipitated phosphate present as struvite and "other phosphate" (calcium phosphate).

The effects of *centrifugation and 1.2 and 0.45* μm *filtration* were studied in three experiments (each performed in duplicate). Each experiment included one vessel filled with synthetic urine only (control) and two vessels with 5 ml of unprocessed human urine added to the synthetic urine. To the remaining six vessels, 5, 15 or 20 ml of human urine (each volume to two of the vessels) was added. In experiment 1 centrifuged urine was added, in experiment 2 centrifuged and 1.2–0.45 μm filtered urine and in experiment 3 the resuspended centrifugation retenate.

To study the effects of *ultrafiltration* (with 100.000 mol weight cut-off), 5 ml of the filtrate and 5 ml of the retenate were each added to two vessels with synthetic urine. One vessels with synthetic urine only (control) and two vessels with 5 ml unprocessed urine were also included in this set of experiments. To study the effects of the dilution medium (the urine retenate was dissolved in distilled water), 5 ml of distilled water was added to another vessel.

Table 2. The effect of urine fractioned on an ultrafilter (nominal molecular weight limit of 100.000) on urease-induced encrustations on glass rods immersed in synthetic urine. Per cent reduction from controls within brackets (mean of four experiments)

Additions to the synthetic urine	Encrustations ($mg \cdot rod^{-1}$)		
	Phosphate	Struvite phoshate	Calcium phosphate
None (controls)	1.73	0.46	1.29
Unfractionated urine, 5 ml	1.18 (68%)	0.32 (69%)	0.86 (67%)
Filtrate, 5 ml	1.43 (82%)	0.30 (65%)	1.13 (88%)
Retenate, 5 ml	1.04 (60%)	0.26 (56%)	0.78 (60%)
Water, 5 ml	1.63 (95%)	0.43 (94%)	1.20 (93%)

Results

In the studies concerning the effects of centrifugation and filtration through 1.2–0.45 μm filters of human urine, the mean end pH was 7.87 ± 0.06. The addition of 5 ml of unprocessed urine reduced the amount of phosphate precipitated on the rods to between 72 and 76% (Table 1). This reduction affected both calcium and struvite phosphate. Neither centrifugation nor 1.2–0.45 μm filtration influenced this inhibitory activity. The phosphate precipitation was thus reduced to 76% when 5 ml of centrifuged urine was added and to 71% when 5 ml of centrifuged and filtered urine was added. The addition of more human urine (15 and 20 ml) gave a volume-related but non-linear reduction (Table 1). That the centrifugation process did not influence the inhibitory activity of the urine was corroborated by the fact that addition of the resuspended centrifugation retenate did not influence the precipitation of phosphate (Table 1).

In the experiments where the effects of ultrafiltration with 100.000 mol weight cut-off were studied, the end pH ranged between 7.74 and 8.25. Five millilitres of the filtrate equalled 6.6 ml of unprocessed urine and 5 ml of retenate 20 ml of unprocessed urine. The retenate gave a pronounced reduction of both struvite and calcium phosphate crystallisation on the rods (Table 2). The filtrate had a more pronounced effect on struvite precipitation than on calcium phosphate precipitation (Table 2). The addition of sterile water instead of human urine did not influence the precipitation (Table 2).

Discussion

Centrifugation and filtration with 1.2 and 0.45 μm filters did not influence the inhibitory effect of human urine on urease-induced precipitation of struvite or calcium phospate. Both the retenate and the filtrate after ultrafiltration with 100.000 mol weight cut-off inhibited the urease-induced precipitation of calcium and struvite phosphate. It thus appears that both urine macromolecules and components with a mol weight less than 100.000 affect the urease-induced crystallisation. That 5 ml of the retenate had a more pronounced effect than 5 ml of the filtrate is

probably caused by the fact that the retenate, due to experimental conditions, represented a larger volume of urine than the filtrate. The effect of the filtrate on calcium phosphate was, however, so small that it appears that larger molecules were the major inhibitors for this salt. The precipitation of struvite was on the other hand affected by the large and small molecular weight fractions in a similar way.

To be able to filter urine through ultrafilters without clogging the filter pores, it is necessary to remove larger urinary components (small crystals, desquamated epithelial cells and microorganisms). This was achieved in this as well as in other experimental studies by centrifugation supplemented by filtration with coarser filters [1]. Our results show that these preparative procedures did not reduce the inhibitory effect of human urine on urease-induced crystallisation in synthetic urine. They also indicate that the larger urinary components which had been removed dit not play any role in the inhibitory effect human urine has on this type of crystallisation. This is further reinforced by the fact that these substances (as centrifugation retenate) did not have any effect on the urease-induced crystallisation in synthetic urine.

At a pH above 7, the initial event in the process of calcium phosphate crystallisation is the precipitation of amorphous calcium phosphate. By a process of phase transformation, this non-crystalline precipitate is gradually transformed to stable crystalline phosphates, hydroxyapatite and octacalcium phosphate [3, 14]. Heterogeneous nucleation of calcium phosphate is influenced by inhibitors present in urine [15]. The phase transformation is also claimed to be sensitive to inhibitors present in urine as glycosaminoglycans (GAGs) and pyrophosphate [5, 14]. The preceding event in the formation of a concrement is crystal growth and aggregation. Known inhibitors are pyrophosphate, citrate and magnesium [20]. Recent studies have shown that high molecular weight urinary components also account for an appreciable fraction of the total urinary inhibition, at least concerning calcium oxalate crystallisation [1, 13]. Urine contains a large number of organic macromolecules (glycoproteins, glycopeptides and heteropolysaccharides) as well as numerous electrolytes [2]. The glycoproteins and glycopeptides range in mol weight from a few hundred up to several million (Tamm-Horsfall's mucoprotein). Almost all of the heteropolysaccharides are glycosaminoglycans (GAGS) with molecular weights ranging from 2.000 to 50.000. Studies concerning the role of the macromolecules in urinary crystallisation have mainly focused on calcium oxalate, where the effects of different urinary fractions have been studied [1, 6, 11, 12]. The results are far from uniform, however, and both high and low mol weight fractions have been claimed to possess the major inhibitory activity. The statement that the net inhibitory activity in whole urine is a function of a number of substances is probably close to reality although confusion exists regarding their relative importance and the way in which they interact [7, 19].

The process of struvite crystallisation has been less intensively investigated and it is not known whether it is preceded by an amorphous phase. It has previously been claimed that the urease-induced crystallisation is not influenced by the presence of inhibitors. The dominating factor determining the degree of saturation and the subsequent crystallisation has instead been claimed to be the dissociation of HPO_4^{--} to the less soluble PO_4^{---} in an alkaline milieu [16]. Using the same experimental model as in the present study, we recently showed that other factor(s) than the level of the pH-increase also influenced the precipitation of struvite and calcium phosphate both in synthetic and in whole urine [8, 10].

The results obtained in the present study show that the urease-induced crystallisation of struvite and calcium phosphate were influenced by fractionated urine in different ways. For calcium phosphate, macromolecules appeared to have the greatest influence. Studying calcium oxalate crystal aggregation, Koide et al. [12] also found that high mol weight urinary compounds accounted for the inhibitory activity, excluding the GAGs as being the most important inhibitor. The process is complicated by the fact at least the low molecular weight inhibitors magnesium, pyrophosphate and citrate have a strongly pH-dependent activity [20]. Struvite was influenced by both macromolecules and smaller urine components in a way which suggests that interacting urinary components are involved in the process.

To conclude, this study suggests that the inhibitory effect on urease-induced crystallisation is caused by different, probably interacting, compounds from small molecules up to macromolecules, in agreement with what appears to be the case for other types of concrements [1, 19]. It is becoming more and more evident that the urease-induced crystallisation in urine and the subsequent concrement formation is a very complex process.

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