Urol Res (1989) 17:177–180

Urological Research
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The influence of zinc and citrate on urease-induced urine crystallisation

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Accepted: September 10, 1988

Summary. Zinc reduced and citrate promoted the urease-induced pH increase in synthetic urine. Secondary to this, the precipitation of magnesium ammonium phosphate and calcium phosphate was influenced. Independent of this pH-related effect, zinc also increased the precipitation of magnesium ammonium phosphate and decreased the calcium phosphate precipitation. These observations were not totally reproducable in human urine.

Key words: Urease-induced crystallisation – Citrate – Zinc

Introduction

Colonisation of the urinary tract by microorganisms producing urease can result in the formation of magnesium ammonium phosphate and calcium phosphate (carbonate apatite) concrements. The formation products of the two salts are exceeded at pHs of about 6.7 and 7.2 respectively, with optimal crystallisation in the pH interval between 7.5 and 8.0 [5, 13]. In-vitro experiments indicated that the crystallisation is also influenced by unidentified components of human urine [3]. Studies in whole human urine have shown a large variation in the degree of crystallisation after urease incubation between urine from different individuals [6]. This effect is not related to the pH or the phosphate content of the urine specimen. These findings indicate that the urease-induced crystallisation also in human urine is not only related to the urease-induced pH-increase. The concentration of citrate in urine is known to vary markedly between individuals [11] and can be anticipated to influence the process of urease-induced crystallisation in several ways. Like other divalent metal ions, zinc has been reported to inhibit urease enzymatic activity [7]. It has also been shown that zinc inhibits calcium phosphate crystallisation [8]. This study was performed to investigate the influence of citrate and zinc on the urease-induced crystallisation in synthetic and human urine.

Materials and methods

Urine specimens were collected without any preservatives as fasting morning urine from 5 healthy adults with no history of stone disease and with negative urinary cultures. The urine portions from all individuals were pooled and stored at +4°C and used in the crystallisation studies within 4 h of collection. The zinc concentration of the pooled urine was measured by atomic absorption spectrometry and the citrate concentration by an enzymatic method [16]. The synthetic urine (pH 5.5–5.7) was composed of 11 solutes, according to Griffith et al. [4]. The solutes, with concentrations in 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), $Na_2 \cdot$ oxalate (0.02), KH_2PO_4 (2.8), KCl (1.6), NH_4Cl (1.0), Urea (25.0), $Na_3 \cdot$ citrate (0.65) and creatinine (1.1).

The urease-induced crystallisation was studied as encrustation of magnesium ammonium phosphate and calcium phosphate on solid glass rods immersed in synthetic or human urine inoculated with urease using an experimental model previously described [3]. 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, Sigma Chemical Company, USA) was used. The incubations were performed in glass vessels, each with four glass rods and filled with 250 ml of synthetic or human urine, at 37°C for 4 h. The pH was determined at the start and at the end of the incubation. The amount of magnesium and phosphate precipitated on the rods was analysed according to Savory et al. and Zilversmith respectively [14, 17]. For each vessel, the mean encrustation per rod was calculated. Assuming that all of the magnesium precipitated was present as magnesium ammonium phosphate, it was possible to calculate the proportion of the precipitated phosphate present as magnesium ammonium phosphate (magnesium-bound phosphate) and calcium phosphate (non magnesium-bound phosphate). The reliability of these calculations has been confirmed in previously performed studies [5]. In certain experiments, the ammonia and ammonium ion concentrations were measured as a sum using a slightly modified commercial kit (No 640-A, Sigma Chemical Company, USA). The measurements were performed at the start and end of the incubation. The increase in concentration reflects urease enzymatic activity.

The effects of zinc in synthetic urine were studied by adding zinc chloride (93.81 mg zinc chloride dissolved in 50 ml of distilled water) to the synthetic urine, giving final concentrations of 0.9, 1.8, 3.6 and 7.2 $mg \cdot l^{-1}$. Two vessels with each zinc concentration were included in each experiment, as well as vessels with no zinc added (controls). Six separate experiments were performed with from 2.5 to 4.0 units of urease per vessel.

The effects of zinc in human urine were investigated by adding zinc chloride (93.81 mg $ZnCl_2$ dissolved in 50 ml of distilled water) to pooled human urine to give final zinc concentrations of 1.4, 2.3, 4.1 and 7.7 $mg \cdot l^{-1}$. Two vessels with each zinc concentration were

Table 1. The effects of Zn^{++} on the pH increase (end pH) in *synthetic urine* (pH 5.7) incubated with different amounts of urease for 4 h

Zn^{++} concentration ($mg \cdot l^{-1}$)	Units of urease				
	2.5	2.75	3.0	3.25	4.0
0 (controls)	6.44	7.05	7.25	7.45	7.69
0.9	6.44	7.04	7.16	7.32	7.43
1.8	6.43	7.04	7.15	7.32	7.43
3.6	6.35	7.00	7.12	7.22	7.30
7.2	6.32	6.90	6.99	7.05	6.99

Table 2. Effects of Zn^{++} on urease-induced crystallisation of Mg^{++}-bound phosphate (magnesium ammonium phosphate) on glass rods immersed in *synthetic urine* after 4 h incubation with different amounts of Jackbean urease. Results given as mg Mg^{++}-bound phosphate per rod (mean of four rods)

Zn^{++} concentration ($mg \cdot l^{-1}$)	Amount of urease added (units)				
	2.5	2.75	3.0	3.5	4.0
0 (controls)	0.00	0.01	0.01	0.02	0.12
0.9	0.00	0.07	0.13	0.18	0.33
1.8	0.00	0.05	0.11	0.21	0.32
3.6	0.00	0.06	0.12	0.19	0.33
7.2	0.00	0.05	0.06	0.05	0.10

Table 3. Effects of Zn^{++} on urease-induced crystallisation of *non* Mg^{++}-bound phosphate (calcium phosphate) on glass rods immersed in *synthetic urine* after 4 h incubation with different amounts of Jackbean urease. Results are given as mg *non* Mg^{++}-bound phosphate precipitated per rod (mean of four rods)

Zn^{++} concentration ($mg \cdot l^{-1}$)	Amount of urease added (units)				
	2.5	2.75	3.0	3.25	4.0
0 (controls)	0.01	0.71	1.83	1.47	3.43
0.9	0.01	0.42	1.32	1.51	2.08
1.8	0.01	0.32	1.30	1.93	2.41
3.6	0.02	0.66	1.22	1.25	2.05
7.2	0.01	0.34	0.63	0.82	1.58

included in each experiment. To each reaction vessel, 6 units of urease were added.

The effects of *citrate* in synthetic urine was studied by incubating synthetic urine with varying concentrations of citrate (0.6–11.4 $mmol \cdot l^{-1}$) with 4 units of urease per vessel (Table 5). The ion concentration was kept constant, i.e. the same as in the original synthetic urine by increasing or decreasing the sodium chloride concentration relative to the sodium citrate concentration.

The effects of citrate in human urine were investigated by adding sodium citrate to give final citrate concentrations of between 2.0 and 12.8 $mmol \cdot l^{-1}$. In each experiment, 6 units of urease were added. The results are given as mean of two experiments each including one vessel with human urine without any added citrate (controls).

Results

The urease-induced pH increase was reduced by addition of *zinc* to the *synthetic urine* (Table 1). The reduction was more pronounced at higher urease concentrations. The addition of zinc in concentrations of between 0.9 and 3.6 $mg \cdot l^{-1}$ markedly increased the precipitation of magnesium ammonium phosphate compared to in the controls (Table 2). With urease inoculates of 2.75 and 3.0 units of urease, the end pH was virtually the same at zinc concentrations from 0 to 3.6 $mg \cdot l^{-1}$. Consequently, the more pronounced precipitation associated with the addition of zinc in concentrations between 0.9 and 3.6 $mg \cdot l^{-1}$ appears not to be linked to the effect zinc has on the pH-increase. At a zinc concentration of 7.2 $mg \cdot l^{-1}$ the precipitation was as low as in the controls. This, however, is largely a pH effect as at this zinc concentration urease enzymatic activity was strongly impaired and the pH never exceeded 7.05.

The precipitation of calcium phosphate was reduced by the addition of zinc (Table 3). This appears largely to be a pH-independent process except at a Zn^{++} concentration of 7.2 $mg \cdot l^{-1}$. At this concentration, the pH-increase was markedly reduced and the low precipitation – lower than in controls – is explained by the low pH obtained.

In the pooled *human urine,* which had an initial zinc concentration of 0.5 $mg \cdot l^{-1}$, the addition of zinc influenced the urease-induced pH increase weakly (Table 4). Zinc also had a weak effect on the precipitation of both magnesium ammonium phosphate and calcium phosphate.

The effects of *citrate* on urease-induced precipitation in *synthetic urine* are demonstrated in Table 5. By increasing the citrate concentration, the pH of the synthetic urine (before urease inoculation) was elevated from 5.52 without sodium citrate to 5.86 when the citrate concentration was 11.4 $mmol \cdot l^{-1}$. Urease enzymatic activity, measured as NH_3-NH_4^+ increase, was positively correlated to the citrate concentration (Table 5). Due to these two factors in combination, an increasing citrate concentration resulted in a higher end pH. The encrustation of both magnesium ammonium and calcium phosphate was strongly influenced by the citrate concentration, with a maximum when the citrate concentration was 2.9 $mmol \cdot l^{-1}$ (Table 5). This is largely a pH effect since the urease-induced crystallisation is most pronounced at the pHs obtained at this citrate concentration [5]. Furthermore, at citrate concentrations between 1.2 and 2.3 $mmol \cdot l^{-1}$ the initial pH, the end pH and the encrustation of calcium phosphate were independent of the citrate concentration. These findings do not support the suggestion that citrate influences the precipitation of calcium phosphate except for its pH-effect. In human urine, the addition

Table 4. The effect of Zn^{++} (zinc chloride) on urease-induced pH increase and crystallisation on glass rods immersed in pooled *human urine* after 4 h incubation with 6 units of urease. The Zn^{++} concentration in the human urine before addition of zinc chloride was 0.5 mg·l^{-1} (results given as mean of 4 experiments)

Zn^{++} concentration (mg·l^{-1})	End pH	Encrustation (mg·rod^{-1})	
		Mg-bound phosphate	non Mg-bound phosphate
0.5 (controls)	7.50	0.06	0.22
1.4	7.46	0.07	0.25
2.3	7.50	0.08	0.22
4.1	7.35	0.04	0.12
7.7	7.43	0.05	0.15

of citrate had a very weak effect on the pH of the urine (Table 6). No certain effect on the urease-induced NH_3-NH_4^+ production was observed. Consequently, the end-pH was not influenced by the amount of sodium citrate added. The precipitation of both magnesium ammonium and calcium phosphate was unaffected by the addition of citrate.

Discussion

Zinc inhibited and citrate promoted the urease-induced pH-increase in synthetic urine. Secondary to this, the precipitation of both magnesium ammonium phosphate and calcium phosphte was influenced. Independent of this pH-related effect, zinc also increased the precipitation of magnesium ammonium phosphate and reduced the precipitation of calcium phosphate. Citrate did not appear to influence the precipitation apart from its influence on pH.

In human urine, on the other hand, the addition of zinc had only very weak effects and citrate had no effects on the pH-increase or the precipitation of magnesium ammonium phosphate and calcium phosphate. Certain conclusions can be drawn from these observations. Urease enzymatic activity and the urease-induced crystallisation are not solely dependent on the pH-increase but can be influenced by substances present in human urine like zinc and citrate. The process can be studied in synthetic urine to gain an understanding of the mechanisms involved and of how they are interrelated. One should, however, be cautious

Table 5. The effects of urease inocculation in synthetic urine with different citrate concentrations (results given as mean of two experiments)

Citrate (mmol·l^{-1})	Initial pH	NH_3-NH_4^+ increase gN·l^{-1}	End pH	Encrustation (mg·rod^{-1})	
				Mg-bound phosphate	non Mg-bound phosphate
0	5.52	0.47	7.34	0.016	0.395
0.6	5.54	0.50	7.54	0.009	0.630
1.2	5.53	0.54	7.68	0.013	1.159
1.7	5.53	0.55	7.69	0.116	1.139
2.3	5.55	0.56	7.69	0.144	1.241
2.9	5.65	0.56	7.74	0.290	1.428
5.7	5.72	0.57	7.89	0.185	0.811
8.6	5.74	0.66	8.03	0.130	0.492
11.4	5.86	0.67	8.23	0.040	0.201

Table 6. The effects of citrate (sodium citrate) on urease-induced pH increase and crystallisation on glass rods immersed in pooled human urine after 4 h incubation with urease. The citrate concentration of the human urine was 1.40–1.45 mmol·l^{-1} (results given as mean of two experiments)

Citrate concentration (mmol·l^{-1})	Initial pH	NH_3-NH_4^+ increase gN·l^{-1}	End pH	Encrustation (mg·rod^{-1})	
				Mg-bound phosphate	non Mg-bound phosphate
1.40	5.94	0.42	8.11	0.135	0.198
1.96	5.96	0.44	8.10	0.175	0.238
2.52	5.97	0.41	8.14	0.144	0.216
3.08	6.00	0.44	8.06	0.221	0.268
3.64	6.00	0.40	7.93	0.147	0.208

when extrapolating from the results obtained in synthetic urine to the situation in voided urine.

The urinary zinc concentration is approximatively 0.5 mg · l^{-1}. Zinc in a concentration of 2.6 mg · l^{-1} has been found to give a 50% reduction of urease enzymatic activity [7]. Zinc has also been shown to affect the growth of calcium phosphate crystals when present at physiological concentrations [8]. The urinary zinc excretion can be increased to levels (3.5 mg · l^{-1}) which ought to impair urease activity with an oral intake of zinc far below that carrying a risk of toxic effects [15]. The excretion of zinc is also increased by hydrochlorothiazide [12]. Zinc consequently appears to be of potential therapeutic interest in the context.

In synthetic urine, we could verify that zinc reduced urease activity in concentrations which can be achieved in urine by oral zinc administration. This inhibitory effect could not be reproduced in human urine, however. This could be due to complex formation or adsorption of zinc to ions or macromolecules present in human but not in the synthetic urine used. Whatever the explanation, our findings do not support the suggestion that an increase in the urinary zinc excretion could be of therapeutic use in reducing urease-induced concrement formation.

Citrate is a potent chelator of calcium and it also exerts an inhibitory effect on hydroxyapatite crystallisation apart from its Ca^{++}-complex formation. Trace metal citrate complexes have also been shown to influence calcification and crystal growth (9). The urinary citrate excretion varies greatly between individsuals, 2.67 ± 1.79 mmol · l^{-1} per 24 h according to Burr et al. [1] and between 0.38 and 1.96 mmol per 24 h according to Ogawa et al. [11]. An increase of the sodium citrate concentration in the synthetic urine and sodium citrate addition to the human urine increased the pH. Variation of the citrate concentration of the synthetic urine within the limits of the variation in citrate concentration occurring in human urine did not influence the initial pH, however. Within these limits, the NH_3-NH_4^+-increase was positively correlated to the citrate concentration. It thus appears that citrate stimulates urease enzymatic activity. This effect was even more pronounced at higher unphysiological citrate concentrations. It cannot be categorically stated whether this is due to a direct stimulation or whether the citrate-induced pH alteration is kinetically favourable for the enzyme. This stimulatory effect could not be reproduced in human urine.

A high prevalence of hypocitraturia has been found in stone formers with no other abnormalities [10]. The role citrate plays in the formation of different types of stones is, however, far from clear [1, 10]. An interesting fact in the context of infection stone formation is that bacteria use citrate as a source of carbon and urinary citrate can be reduced in infect urines [2]. Our results demonstrate that citrate influences the urease induced crystallisation but whether this has any clinical significance for infection stone formation needs further evaluation.

Acknowledgements. Supported by the Swedish Medical Research Council, Grant No B88-17X-05437-10A, Tore Nilssons Fond, Göteborgs Läkaresällskap and Svenska Läkaresällskapets forskningsfond.

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