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# How variations in the composition of urine influence urease-induced crystallization

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**Summary.** To Study how the composition of urine influences urease-induced crystallization, human urine samples were incubated with urease and the subsequent precipitation measured. Beside the pH increase, the urinary content of magnesium and calcium had profound effects on the precipitation of magnesium ammonium phosphate and calcium phosphate, respectively. Urine phosphate, ammonium and osmolarity had no direct effects on the precipitation. Among the urine components with potential inhibitory properties, only albumin was found to be correlated with such an effect. This inhibitory activity was especially influential in urines with high calcium and magnesium levels. These findings suggest that the composition of urine could also influence the formation of stones consisting of magnesium ammonium phosphate and calcium phosphate.

**Key words:** Urease-induced crystallization – Urine composition – Urinary pH – Urinary calcium – Urinary magnesium – Urinary albumin

The association between urea-splitting microorganisms and urinary tract stones consisting of magnesium ammonium phosphate (MAP) and carbonate apatite is well-established [2, 8]. Urease splits urea to ammonia with a subsequent pH increase and, secondary to that, precipitation of MAP and calcium phosphate (CaP). Some authors have claimed that the pH increase is the major determining factor for MAP and CaP crystallization in urine [14, 18]. In synthetic urine, crystallization reaches a maximum between pH 7.5 and 8.0 for CaP and between pH 8.0 and 8.5 for MAP [5]. Urine from different individuals varies markedly in crystal-forming tendency when incubated with urease, however, despite similar end pH [6]. A large interindividual variation is also noted clinically. An infection with urea-splitting microorganisms can thus rapidly lead to the formation of large staghorn stones in some patients, while in others no stones at all are formed [21].

It has been observed that patients with infection-induced stones frequently have hypercalciuria [16, 17, 21]. Urinary magnesium, on the other hand, has not been found to differ between MAP stone formers and idiopathic calcium stone formers [9]. How urine composition affects urease-induced crystallization in the individual urine has so far not been investigated.

Crystallization inhibitors are thought to play an important role in idiopathic calcium stone formation [13]. It has recently been shown that human urine has inhibitory effects on urease-induced crystallization of MAP and calcium phosphate in synthetic urine [1]. Citrate, zinc and albumin have also been shown to influence this crystallization [4, 10]. The role of these inhibitors is not fully evaluated but they could be one explanation for the observed interindividual variation in MAP stone formation after infection with urease-producing microorganisms.

This investigation addressed the issue how urease-induced crystallization is influenced by variations in urine composition in urines from different individuals.

## Materials and methods

Morning urine was collected on 8 different days from seven healthy persons with no history of stone disease or urinary tract infection. The pH level was measured immediately on collection. Samples were taken for analysis of different parameters in urine (Table 1) and for ordinary bacterial culture. Urine buffer capacity was determined by titrating a 10-ml urine portion from pH 5.7 to 8.0 with 0.1 M NaOH. The remaining urine was centrifuged for 10 min at 3,000 rpm, sterilized by filtration (Millipore 0.22 μm) and stored at +4 °C. The urine specimens were processed with 7 weeks of the day they were collected. Previous studies have shown that this urine preparation and storing do not influence the urease-induced crystallization [7]. Immediately before incubation, the pH of all samples was adjusted to 5.7 with 0.1 M HCl or 0.1 M NaOH, and new cultures were performed. All urine samples were incubated at 37 °C under sterile conditions with urease (jack bean urease E.C. 3.5.1.5. Sigma, St. Louis, Mo., USA) for 4 h in glass tubes (15 ml) with one glass rod immersed in the solution. The amount of urease added was calculated empirically from the buffer capacity in order to reach a

**Table 1.** Parameters in urine from seven healthy adults and method of analysis

| Parameter | Analytical method |
|---|---|
| Calcium | Atomic absorption spectrometry [12] |
| Magnesium | Atomic absorption spectrometry [12] |
| Phosphate | Trichloracetic precipitation [14] |
| Ammonia and ammonium ion | Sigma, commercial kit [22] |
| Citrate | Enzymatic method [16] |
| Albumin | Radioimmunoassay (Albumin RIA 100, Commercial Kit, Pharmacia, Uppsala, Sweden) |
| Glycosaminoglycans | Precipitation with Alcian blue [17] |
| Total protein | Lowry method [20] |
| Osmolarity | Freeze-point method |

final pH between 7.5 and 8.5. The final pH and ammonium ion concentration were measured. The precipitation on the glass rods was dissolved in concentrated nitric acid and analysed for phosphate and magnesium. The slurry of salt precipitated in the solution (= intraluminal precipitation) was separated from the solution by Millipore filtration (0.22 μm), and its content of phosphate and magnesium was measured. All precipitated magnesium was assumed to be a constituent of MAP. With knowledge of the amount of magnesium, it was thus possible with stoichiometic methods to calculate the amount of magnesium-bound phosphate and calcium-bound phosphate precipitated. These fractions are referred to hereinafter as MAP and CaP. Previous studies have verified the reliability of these calculations [1, 5].

The correlation between the four fractions of precipitation (MAP, intraluminal and on rods, as well as CaP, intraluminal and on rods) and the measured urine parameters was studied using multiple regression analysis (Table 2). In this analysis, the final pH was incorporated in the regression analyses as a pH function. This pH function was calculated from the mean precipitation in every 0.2 pH interval (Fig. 1). In this way, one pH function was constructed for each of the four fractions of precipitation.

It might be expected not only that substances in urine influence the precipitation in a way that would satisfy a linear function, but also that two or more urine components might potentiate each other's effects. In order to detect such potentiating effects, products of the different ions and of potential inhibitors and these ions were also included in the multiple regression analyses. In previouis work (unpublished data), albumin and citrate were found not to influence the precipitation in a linear way but followed the logarithm for

**Table 2.** Variables included in multiple regression analysis of urease-induced precipitation in urine, studied in seven healthy adults

| For MAP[a] | For CaP[b] |
|---|---|
| Calcium | Calcium |
| Magnesium | Magnesium |
| Phosphate | Phosphate |
| Osmolarity | Osmolarity |
| Ammonia | Ammonia |
| Added urease | Added urease |
| Magnesium · phosphate | Calcium · phosphate |
| Magnesium · phosphate · ammonia | Total protein |
| Total protein | GAG |
| GAG | Log citrate |
| Log citrate | Log albumin |
| Log albumin | Calcium · log citrate |
| Magnesium · log citrate | Calcium · log albumin |
| Magnesium · log albumin | pH function |
| pH function | Individual factors |
| Individual factors | |

[a] Magnesium ammonium phosphate; [b] calcium-bound phosphate

u-albumin and u-citrate, respectively. These parameters were therefore incorporated in the multiple regression analyses as log u-albumin and log u-citrate. The precipitations in urine from each individual were also incorporated in the multiple regression analyses as a factor in order to detect any differences in urines from different individuals.

All data were computerized (Medlog Clinical Data Management and Analysis System, IAC, USA), and statistical analyses have been based on Student's *t*-test.

## Results

All urine parameters measured varied markedly both inter- and intraindividually (Table 3).

End pH after 4 h urease incubation ranged between 6.0 and 9.0 in the different samples. In 44 (79%) of the samples, the end pH was in the region 7.5–8.5. A precipitation was recorded even at an end pH of only 6.3 in some samples. Maximum intraluminal and rod-attached precipitation of CaP was noted around pH 8.0 and of MAP around pH 8.5 (Fig. 1). The degree of precipi-

**Table 3.** The inter- and intraindividual variations in urine from seven individuals from whom urine was collected and analysed on 8 different days (SD = standard deviation of mean)

| Parameter | Mean | Range | Mean intra-individual variation (SD) | Inter-individual variation (SD) |
|---|---|---|---|---|
| Calcium (mmol · $l^{-1}$) | 3.37 | 0.79–11.00 | 1.454 | 2.084 |
| Magnesium (mmol · $l^{-1}$) | 3.91 | 0.41–11.1 | 1.72 | 2.50 |
| Phosphate (mmol · $l^{-1}$) | 24.1 | 6.3–50.0 | 8.2 | 9.3 |
| Osmolarity (mosmol · $l^{-1}$) | 559 | 207–1060 | 141 | 183 |
| Albumin (mg · $l^{-1}$) | 6.87 | 0.92–36.58 | 3.65 | 6.84 |
| Citrate (mmol · $l^{-1}$) | 1.79 | 0.39–5.37 | 0.73 | 0.98 |
| Glycosaminoglycans (mg · $l^{-1}$) | 5.7 | 1.5–14.3 | 2.5 | 2.8 |
| Total protein (mg · $l^{-1}$) | 27.6 | 8.8–58.9 | 11.4 | 14.7 |
| Buffering capacity (ml 0.1 M NaOH) | 3.84 | 1.45–7.20 | 1.24 | 1.74 |

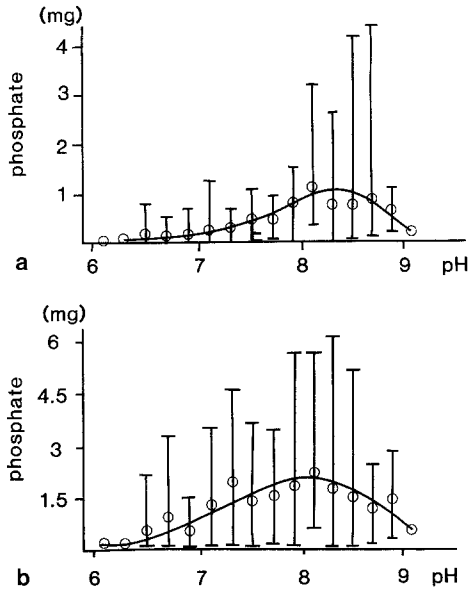


**Fig., 1a, b.** The precipitation obtained after a 4-h urease incubation in various human urines related to end pH after incubation. The *bars* represent maximal and minimal precipitation and the *circles*, the mean precipitation obtained in every 0.2-pH interval. **a** Magnesium-bound phosphate; **b** calcium-bound phosphate

**Table 4.** Differences in multiple regression coefficients for the difference fractions of precipitation when different variables were included in the analyses.

| Variables | MAP | | CaP | |
|---|---|---|---|---|
| | Rod | Intra-luminal | Rod | Intra-luminal |
| All variables | 0.90 | 0.88 | 0.93 | 0.91 |
| Mg | 0.65 | 0.65 | | |
| Mg + Mg · log alb | 0.78 | 0.70 | | |
| Mg + Mg · log alb + pH | 0.78 | 0.80 | | |
| Mg + pH | 0.67 | 0.80 | | |
| Ca | | | 0.72 | 0.74 |
| Ca + Ca · log alb | | | 0.82 | 0.75 |
| Ca + Ca · log alb + pH | | | 0.83 | 0.84 |
| Ca + pH | | | 0.74 | 0.84 |

tation varied widely between different samples at each pH interval, however (Fig. 1a).

Multiple regression analysis resulted in very high regression coefficients, around 0.9, for all four fractions of precipitation and the measured urine parameters (Table 4). However, looking at the individual variables in each regression function, only a few parameters influenced the process in a highly significant way while the others had only a weak and nonsignificant effect. Accordingly, the regression functions could be simplified to comprise only two variables for each fraction and still maintain high regression coefficient around 0.80. For MAP precipitation on rods, u-Mg (positively correlated) and the product of u-Mg and log albumin (negatively correlated) were the only two variables with statistically significant effects (Table 5). For intraluminal MAP precipitation, the only significant variables were u-Mg and pH (positively correlated).

For precipitation of CaP on rods, u-Ca (positively correlated) and the product u-Ca · log albumin (negatively correlated) were significantly correlated. For the intraluminal precipitation of CaP, were the only two variables with significant effect u-Ca and pH.

Still when the individual factors were taken into account was the effect of the significant variables (pH, U-Ca, u-Mg and u-albumin) virtually the same, i.e. around 0.8.

The buffering capacity in urine correlated to u-phosphate with a correlation coefficient of 0.70. If total urinary protein was included, a multiple regression coefficient of 0.91 was achieved.

**Table 5.** Multiple regression functions obtained for different fractions of precipitation after urease inoculation of different human urines

| Fraction | Variable | Coefficient | Standard deviation | *t*-Statistic | *P*-value |
|---|---|---|---|---|---|
| MAP on rods | Intercept | −0.0037 | 0.0050 | −0.74 | 0.4611 |
| | Magnesium | 0.58 | 0.065 | 8.83 | < 0.0001 |
| | Magnesium · log albumin | −0.11 | 0.022 | −5.12 | < 0.0001 |
| MAP intraluminal | Intercept | −0.44 | 0.088 | −5.04 | < 0.0001 |
| | Magnesium | 2.67 | 0.29 | 9.33 | < 0.0001 |
| | pH | 1.32 | 0.24 | 5.45 | < 0.0001 |
| CaP on rods | Intercept | −0.076 | 0.031 | −2.41 | 0.0196 |
| | Calcium | 0.123 | 0.0135 | 9.063 | < 0.0001 |
| | Ca · log albumin | −0.021 | 0.0043 | −5.03 | < 0.0001 |
| CAP intraluminal | Intercept | −4.24 | 0.737 | −5.76 | < 0.0001 |
| | Ca | 0.634 | 0.057 | 11.18 | < 0.0001 |
| | pH | 1.96 | 0.320 | 6.14 | < 0.0001 |

## Discussion

The pH dependence CaP and MAP precipitation was confirmed (Fig. 1). However, within each pH interval, there was a large variation in precipitation between different urines. Urines from certain individuals showed no or minimal precipitation when incubated with urease, despite an end pH between 7.5 and 8.5, which is the range within which maximal precipitation takes place. In urines from other individuals a marked precipitation of both MaP and CaP was noted at pH levels as low as 6.3. This must indicate that factors other than pH influenced the precipitation of MAP and CaP. Previous studies have shown that the intraluminal fraction consists mostly of amorphous material present as a slurry in the tubes [1, 5]. This fraction mainly reflects the initial phases of the urease-induced precipitation, i.e. nucleation and growth. In this experiment, it constituted about 90% of the total precipitation. The material attached to the glass rods can, according to previous studies, be assumed to be more strongly affected by later phases in the process of crystallization, especially aggregation [1].

Urinary calcium was found to have a profound effect on the intraluminal precipitation of CaP. This precipitation only correlated to pH and to u-Ca; no other ions, including phosphate, or the studied potential inhibitors significantly influenced it. In the same way, u-Mg and pH were found to influence the intraluminal precipitation of MAP strongly, while u-phosphate and amount of urease added, which reflect the amount of ammonia produced, had no significant effects. Despite the observation that u-phosphate had no influence on the precipitation of either MAP or CaP, it can, by being the major urine buffer, protect against the urease-induced pH increases and thereby reduce the precipitation of both MAP and CaP.

Urinary calcium and magnesium varied markedly between different individuals and also showed wide day-to-day variations. Urines with no precipitation despite optimal pH always had low concentrations of u-Ca and u-Mg. This means that, if crystallization of MAP and CaP is to take place, not only must the pH be elevated but the urinary content of calcium and magnesium must also exceed critical levels. This may be one explanation of why certain patients do not develop urinary tract stones despite long-standing infection with potent urea splitters such as *Proteus mirabilis*.

Precipitation on glass rods seemed to be less pH-dependent. In the multiple regression analyses for CaP and MAP on glass rods, only u-Ca and u-Mg, respectively, were found to have promoting effects. Of the substances studied, only u-albumin had a negative influence on this precipitation. However, if the products of log albumin and u-Ca and u-Mg, respectively, were used, even higher regression coefficients were obtained. The protective effect of albumin was thus especially effective at high u-Ca and u-Mg levels. In previous studies albumin, citrate and zinc [4, 10] have shown to influence precipitation of MAP in synthetic urine. Zinc was not measured in this study. Citrate had a weak but not statistically significant inhibitory effect on MAP crystallization. For calcium phosphate crystallization, citrate, albumin, GAGs and magnesium have documented inhibitory properties [3, 10]. For urease-induced precipitation, this study could only confirm the inhibitory activity of u-albumin.

In previous studies, the activity products of MAP and CaP have been estimated [14, 18]. Risk indices have been constructed using the concentrations of the ions included in the salt and urinary pH. The results of this study suggest that the situation is more complicated.

First, pH seemed to have a less critical effect than previously thought. For rod encrustation no pH dependence was registered, while the intraluminal precipitation was more affected by the end pH. This could be interpreted as indicating that a pH increase was necessary to initiate the nucleation but the following phases of the crystallization process necessary for stone formation, i.e. phase transformation, crystal aggregation and crystal growth, were less pH-dependent.

Second of the ions included in the salts, i.e. $Mg^{2+}$, $PO_4^{3-}$, $NH_4^+$ and $Ca^{2+}$, only variations in the concentrations of u-Mg and u-Ca had any effects on the precipitation. That u-Ca is of importance for infected stone formation is supported by previous clinical studies where MAP stone formers have been shown often to have hypercalciuria [16, 17]. Ammonium ions and phosphate always seemed to be present in urine in such concentrations that the normal variations in their concentrations had only minor effects on the precipitation. This study was performed on urines from persons with no history of stone disease, however. Whether this is also the case for stone formers remains to be investigated. The precipitation varied rather widely in urines from different subjects. The precipitation was thus always low in urine from certain individuals but high in urine from others. These differences were not statistically significant if individual factors were taken into account in the multiple regression analyses. This implies that the crystallization at least in urine from the individuals studied was more related to be composition of urine sample than to the individual.

Third, u-albumin had a strong negative correlation with crystallization of both MAP and CaP. This "protective" effect of high albumin concentrations was most pronounced in urines with high calcium and magnesium levels. The observation that albumin affected only the glass-attached crystallization suggests that an inhibition of crystal growth and/or crystal aggregation is the major effect and that the nucleation rate is less strongly affected.

To sum up, this study showed that urease-induced crystallization of MAP was affected by urinary pH and the urine content of magnesium and albumin. For the urease-induced crystallization of CaP, u-calcium, u-albumin and urinary pH were determining factors. The end pH was dependent on the phosphate content, however, since it contributes to the buffering capacity in urine. Other urine parameters seemed to have only a slight influcence on the process.

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