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Regular paper

Various intensity of *Proteus mirabilis*-induced crystallization resulting from the changes in the mineral composition of urine

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Infectious urolithiasis is a result of recurrent and chronic urinary tract infections caused by urease-positive bacteria, especially *Proteus mirabilis*. The main role in the development of this kind of stones is played by bacterial factors such as urease and extracellular polysaccharides, but urinary tract environment also contributes to this process. We used an *in vitro* model to establish how the changes in the basic minerals concentrations affect the intensity of crystallization which occurs in urine. In each experiment crystallization was induced by an addition of *P. mirabilis* to artificial urine with a precisely defined chemical composition. Crystallization intensity was determined using the spectrophotometric microdilution method and the chemical composition of formed crystals was established by atomic absorption spectroscopy and colorimetric methods. Increasing the concentration of all crystals forming ions such as Mg^{2+}, Ca^{2+} and phosphate strongly intensified the process of crystallization, whereas reducing the amount of these components below the proper physiological concentration did not affect its intensity. The inhibitory influence of citrate on calcium and magnesium phosphate crystallization and competitive actions of calcium and oxalate ions on struvite crystals formation were not confirmed. In the case of infectious stones the chemical composition of urine plays an important role, which creates a necessity to support the treatment by developing a model of proper diet.

Key words: *Proteus mirabilis*, crystallization, urinary stones, mineral composition

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INTRODUCTION

Urinary stones are formed mainly due to metabolic disorders and improper diet, but also because of incorrect concentration of crystallization inhibitors and promoters in urine, obesity or bacterial infection (Knoll, 2010; Knoll *et al*., 2011). Different chemical composition of the stones is related to the cause of their formation. Most urinary calculi are composed of calcium oxalate and calcium phosphate. A characteristic feature of infection-induced stones is the presence of mineral components: struvite (ammonium magnesium phosphate hexahydrate, $NH_4MgPO_4 \times 6H_2O$) and apatite (most commonly, carbonate apatite, $Ca_{10}[PO_4]_6CO_3$) (Bichler *et al*., 2002). Infectious stones account for 10% of all urinary stones and pose a serious health problem because of their rapid growth, persistence and high rate of recurrence (up to 50%). If not adequately treated they can develop into staghorn calculi, which can destroy the kidney and result in life-threatening sepsis. The process of kidney stones formation consists of three main stages: supersaturation of urine salts, formation of crystals and their retention in the urinary tract (Basavaraj *et al*., 2007). Each stage in the process of infectious stones formation is a result of the presence of bacteria in the urinary tract. Supersaturation and crystallization of mineral salts are connected with the urease activity. Enzymatic hydrolysis of urea by this enzyme causes the release of ammonia and increases the pH. It leads to a decrease in the solubility of urinary components such as magnesium and calcium phosphate and results in their precipitation as struvite and carbonate apatite crystals (Prywer & Torzewska, 2009). Aggregation and retention of crystals in the urinary tract are dependent on the presence of bacterial polysaccharides (lipopolysaccharide; LPS, capsular polysaccharide; CPS) and inflammatory reaction products. However, the development of this type of kidney stones is also affected by factors normally present in the human body. Urine contains components acting as inhibitors or promoters (their activity depends on kind of the crystals) of struvite and apatite crystallization, including minerals such as magnesium, phosphate, citrate or macromolecular substances: Tamm Horsfall protein, glycosaminoglycans, nephrocalcin (Parmar, 2004). Most urinary stones are not homogenous in terms of their chemical composition since they are a mixture of compounds deposited due to metabolic disorders and infections. Diverse composition of the stones may result from the fact that urolithiasis promotes the development of infections, and bacteria easily colonize porous stone surfaces (Tavichakorntrakool *et al*., 2012). The formation of struvite stones is most frequently caused by bacteria of the genus *Proteus* (about 70% of cases) and rarely by other urease-positive bacteria such as *Providencia, Klebsiella, Morganella* and *Staphylococcus* (Rodman, 1999; Kramer *et al*., 2000). The treatment of patients with infectious urolithiasis involves removal of stones and antibiotic therapy to eliminate bacteria from both the stone and the urinary tract. Antibiotics or chemotherapeutics (such as fluoroquinolones) are administered for up to 3 months in order to prevent infection and recurrence of stones (Bichler *et al*., 2002; Zanetti *et al*., 2008). It is also suggested to use urease inhibitors (e.g. acetohydroxamic acid), acidification of urine through the administration of such compounds as ammonium sulphate, and mod-

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Abbreviations: LPS, lipopolysaccharide; CPS, capsular polysaccharide; TSB, tryptic soy broth; CFU, colony forming units

ification of diet (Bichler *et al.*, 2002; Sayer *et al.,* 2010). Despite numerous studies, both *in vitro* and *in vivo*, on the crystallization caused by the bacteria, it is still unclear whether controlling the concentrations of the ions which build the crystals is important in the suppression of the crystallization.

The aim of this study was to analyse crystallization induced by ureolytic *P. mirabilis* in environmental conditions where the changes are similar to the alterations in urine caused by metabolic disorders or improper diet.

MATERIAL AND METHODS

Bacterial strains. *Proteus mirabilis* strains designated as K0, K5, K8 and K608 were used. These strains had been previously isolated from human kidney stones and deposited in microorganisms collection of the Department of Immunobiology of Bacteria, University of Lodz. Before each assay, bacteria were cultured on tryptic soy broth (TSB, BTL) for 24 h at 37ºC and then suspended in synthetic urine to a concentration of 5×10^5 CFU/ml (colony forming units per milliter). The number of bacteria per ml was determined spectrophotometrically at 550 nm (Ultrospec2000, Pharmacia Biotech, Vienna Austria) using a standard curve of directly proportional relationship between the absorbance (A 550) and the number of bacteria (CFU/ml) in the suspension.

Synthetic urine. Synthetic urine was prepared according to a recipe previously described by Griffith *et al.* (1976) and consisted of the following components (g/l): $CaCl_2 \times 2H_2O$, 0.651; $MgCl_2 \times 6H_2O$, 0.651; NaCl, 4.6; Na_2SO_4, 2.3; sodium citrate, 0.65; sodium oxalate, 0.02; KH_2PO_4, 2.8; KCl, 1.6; NH_4Cl, 1.0; urea, 25.0; creatine, 1.1 and tryptic soy broth, 10.0 (Sigma, St.Louis, MO, USA). Prior to the experiment, pH was adjusted to 5.8 and urine was sterilized by passing through a 0.2 μm pore-size filter. Synthetic urine should be used within 7 days. The content of the mineral components used corresponded to the mean concentration found during a 24-hour period in regular human urine. To imitate changes in the composition of urine associated with metabolic disorders or improper diet, synthetic urine was modified to include elevated levels of one of the components in the following concentrations: calcium (0.7 g/l), magnesium (0.6 g/l), phosphate (15.5 g/l), citrate (4.0 g/l) or oxalate (0.06 g/l).

Crystallization experiments

Determination of crystallization intensity induced by P. mirabilis in varying concentrations of mineral salts. In this experiment, each studied mineral component was serially diluted in 100 μl synthetic urine that did not contain this component in flat bottom 96-well plates. The final concentrations of ingredients ranged: for calcium between 2.896–0.005 g/l, magnesium — 1.178–0.002 g/l, phosphate — 30.96–0.06 g/l, citrate — 8.125–0.016 g/l and oxalate — 0.512–0.001 g/l. To the wells with a prepared solution of urine was added 5 μl of bacterial suspension with a concentration 5×10^5 CFU/ml. As controls were used 100 μl normal synthetic urine (background control), 100 μl tested *P. mirabilis* strains in normal synthetic urine (extent of crystallization in normal urine) and 100 μl bacterial suspension of urease-negative mutant of *P. mirabilis* O28 (previously described by Torzewska *et al.* (2003)) in the modified composition of urine (control of bacterial growth without crystallization). All wells were covered with mineral oil to prevent the release of ammonia into the environment. After incubation for 5 h and 24 h at 37ºC with gentle agitation the absorbance of suspensions was measured at λ630 and, taking into account the absorbance of the controls, the intensity of crystallization was determined.

Analyses of *P. mirabilis*-induced crystallization occurring in urine with selected concentrations of minerals. For detailed analysis of infection-induced crystallization an experiment was carried out in a volume of 20 ml of urine with a defined chemical composition — normal or modified. In this assay the following concentrations of the tested components were chosen: calcium — 0.7 g/l, magnesium — 0.6 g/l, phosphate — 15.5 g/l, citrate — 4.0 g/l or oxalate — 0.06 g/l. Urine was infected with *P. mirabilis* strains, each to a concentration of 5×10^5 CFU/ml and incubated with gentle shaking (60 rpm) for 5 h or 24 h at 37ºC. At these time points urine pH, bacterial viability and intensity of crystallization were checked. Bacterial viability was determined as a number of CFU/ml (colony forming units per milliliter). The level of crystallization was determined by chemical analysis and direct phase-contrast microscopy. For chemical analyses crystals were washed twice by centrifugation in 0.05 M Tris buffer pH 8.6. The phosphate concentration was determined by the colorimetric method (Ames and Dubin, 1960) and atomic absorption spectroscopy (SpectrAA-300 Varian, Palo Alto, USA) was used to analyse calcium and magnesium concentrations.

Statistics. The U Mann-Whitney test was used to compare the differences in the results obtained in normal and modified urine. The results were considered to be statistically significant at $p \leq 0.05$.

RESULTS

Crystallization intensity induced by *Proteus mirabilis* in varying concentrations of mineral salts

Due to the fact that when crystallization occurs the turbidity of urine increases, a method based on the absorbance measurement was chosen to analyze the impact of urine composition on *P. mirabilis*-induced crystallization. It allowed examining the influence of such compounds as: Ca^{2+}, Mg^{2+}, PO_4^{3-}, citrate and oxalate in a wide range of concentrations both below and above their normal levels in urine. There was no significant differentiation in the degree of crystallization among the strains, so the results presented in Fig. 1 are the average values obtained for all strains tested. In the case of Ca^{2+}only was the course of curves the same for 5 h and 24 h incubation (Fig. 1). Ca^{2+} ions content lower than in normal urine did not change the degree of turbidity or affect the crystallization process. However, when the level of this cation reached 0.362 g/l, crystallization intensity increased in a direct proportion to its concentration. In the case of Mg^{2+}, which is the main component of struvite crystals, an increase in crystallization intensity was seen above 0.294 g/l, and only after 5 h of incubation compared to the control. Changes in the degree of urine turbidity due to the increasing concentrations of phosphate were similar regardless of the incubation time. Crystallization was more intense above the concentration of 3.87 g/l, which was seen especially after 24 h of incubation. In the case of citrate other dependencies could be observed. Citrate is a known inhibitor of crystallization but in this experiment the inhibitory effect was visible only after 5 h of incubation at a concentration above 2.03 g/l. At other concentrations and longer duration of the experiment citrate had no inhibitory effect or even intensified the crystallization process.

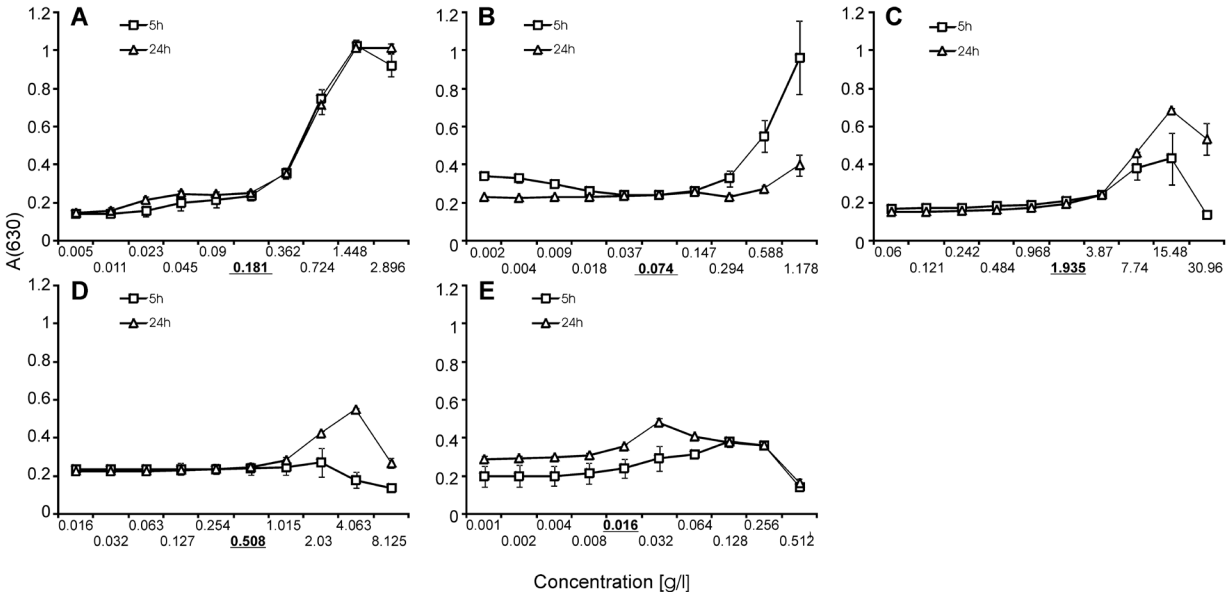

Figure 1. Crystallization intensity induced by *P. mirabilis* strains in urine with various concentrations of:
(**A**) calcium, (**B**) magnesium, (**C**) phosphate, (**D**) citrate, (**E**) oxalate. Absorbance values presented are the mean with standard deviation for all tested strains in 5 independent experiments. The value underlined corresponds to the average concentration found during a 24-hour period in normal human urine.

Table 1. The pH level and the number of bacteria after incubation at 37°C in the urine of varying chemical composition.
In the urine with a modified composition the compounds were used in the following concentration [g/l]: calcium — 0.7, magnesium — 0.6, phosphate — 15.5, citrate — 4.0 or oxalate — 0.06.

Urine composition	Bacterial growth (CFU/ml x 10^8)		pH	
	5 h	24 h	5 h	24 h
Normal urine	1.67 ± 0.41	ng	8.96 ± 0.09	9.68 ± 0.04
Urine with excess:				
calcium	1.26 ± 0.66 ‡	ng	8.07 ± 0.07 *	9.65 ± 0.06 ‡
magnesium	1.41 ± 0.12 ‡	ng	8.87 ± 0.13 *	9.64 ± 0.06 ‡
phosphates	1.14 ± 0.59 ‡	ng	8.33 ± 0.31 *	9.64 ± 0.03 ‡
citrate	1.38 ± 0.43 ‡	ng	8.72 ± 0.24 *	9.40 ± 0.10 †
oxalate	1.54 ± 0.15 ‡	ng	8.97 ± 0.09 ‡	9.23 ± 0.25 †

Results are presented as mean with standard deviation of 8 independently performed experiments. ng — no growth of the bacteria, *p<0.005, †p<0.05, ‡p>0.05 compared with control (normal urine)

The presence of oxalates, even in high concentrations, did not have a marked influence on the turbidity of urine because of the crystallization caused by bacteria. This stage of the research allowed selecting concentrations of the tested compounds which have a strong impact on *P. mirabilis*- induced crystallization for further and more detailed research.

Effect of chosen minerals concentrations on *P. mirabilis*-induced crystallization

In the next stage of the research, the impact of selected concentrations of urine mineral compounds on the *P. mirabilis*-induced crystallization was analyzed after incubation for 5 and 24 hours. Analysis of crystallization included a qualitative and quantitative chemical analysis of crystals and estimation of crystals size and morphology.

Viability of bacteria and change in the urine pH

Modifications of the urine composition did not affect the viability of bacteria (Table 1), at 5 h the number of bacteria expressed as CFU/ml was comparable to the control (normal urine) but after 24 h of incubation, because of the high pH for each urine sample, there was no bacterial growth observed. In both periods of incubation the pH of urine was measured. After 5 h, in all urine samples the pH values varied and ranged from 8.07 to 8.97. In the presence of all tested components, except oxalate, pH was lower compared to the control (p<0.05), the lowest value was noted in the urine with an increased amount of calcium. With time, the differences in pH became smaller and after 24 h of incubation the pH levels were between 9.23–9.68 and only in the urine with an excess of citrate and oxalate, the pH was lower than the control (p<0.05).

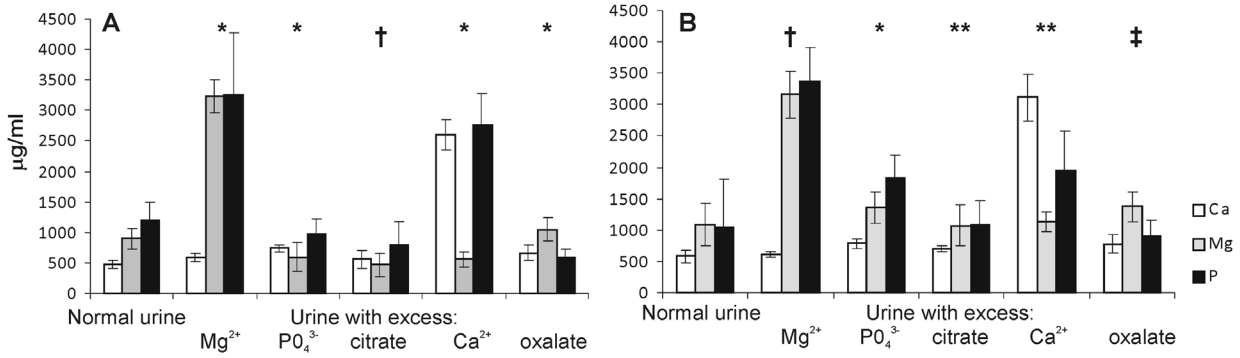

Figure 2. Amounts of calcium and magnesium phosphate precipitated for 5 h (A) and 24 h (B) after the addition of the bacteria in urine with different composition.
The concentration of each ion is presented as the mean with standard deviation for all tested strains in 8 independently performed experiments. *$p \leq 0.05$ for all ions, **$p > 0.05$ for Mg ions, †$p > 0.05$ for Ca ions, ‡$p > 0.05$ for phosphate ions.

Crystallization intensity

A total amount of crystallized salt was established by determining the concentration of Ca^{2+}, Mg^{2+} and phosphate ions. As shown in Fig. 2, the most intense crystallization occurred in the urine with increased concentrations of Mg^{2+} and Ca^{2+} ($p \leq 0.0005$). In these cases more crystals of magnesium phosphate or calcium phosphate were formed, depending on the amount of which cation was higher. Other modifications of urine also influenced the degree of crystallization but no longer to such an extent. The level of crystallization of both apatite and struvite was apparently higher while increasing the concentration of phosphate after 5 and 24 h incubation ($p \leq 0.02$ and $p \leq 0.043$ for all ions tested, compared to the control). Increased concentrations of sodium citrate did not affect the level of crystallization in the experimental model as compared with normal urine. In the case of an excessive amount of oxalate in urine, a higher level of Ca^{2+}, Mg^{2+} and phosphate ions was noted as compared to the control, but this difference was statistically insignificant for phosphate after 24 h of incubation (Fig. 2B).

Crystals size and morphology

In addition, for each of the urine solutions the morphology and size of the crystals formed were determined, which gives additional information about the crystallization process. Figure 3 shows representative

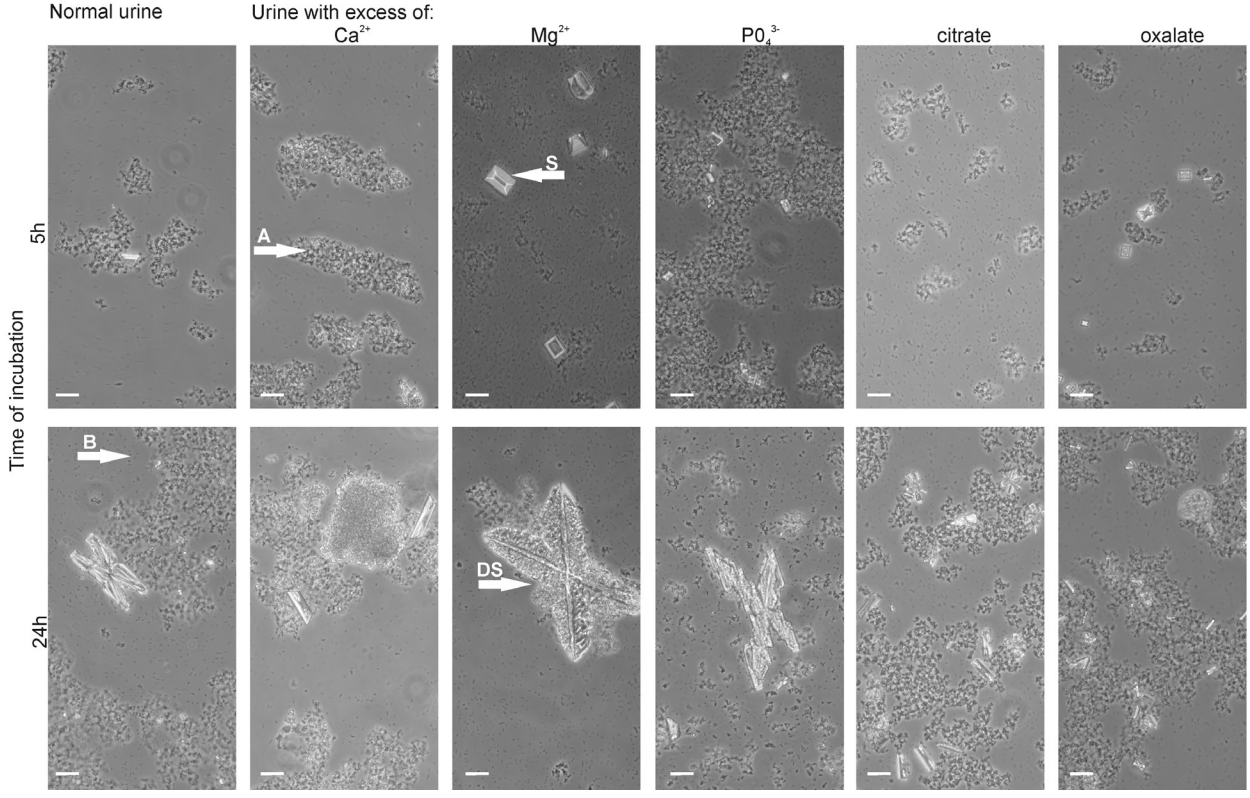

Figure 3. The size and morphology of struvite/apatite crystals formed in urine with a modified composition after short and long time of incubation in the presence of *P. mirabilis* K0.
The bar represents 20 µm. S, struvite crystals; DS, dendrites of struvite; A, apatite crystals; B, bacteria.

morphology and size of crystals formed under all conditions tested. In normal urine infected with *P. mirabilis* after 5 h of incubation struvite crystals with a characteristic shape and numerous amorphous carbonate apatite crystals were visible. With time (after 24 h) struvite crystals became larger and formed dendrites. Comparing all tested crystallization conditions, it can be seen that the largest crystals of struvite were formed in the urine with an increased concentration of magnesium. It should also be pointed out that under these conditions the fewest crystals of apatite were detected. Conversely, if the urine contained an excess of calcium, a large number of apatite and no struvite crystals after 5 h of incubation were noticed. After 24 h both apatite and struvite crystals were visible. The excess of phosphate did not significantly affect the crystallization of analysed minerals. The crystals size and morphology were comparable with those found in normal urine. Significant differences were seen in the case of the urine with an excess of oxalate and citrate. The process of struvite crystallization was slower and the crystals formed had an altered morphology, they were smaller and longer compared to the crystals formed in the urine with normal levels of these compounds. In these cases in both 5 and 24 h of incubation apatite crystals were also visible, but in a smaller number than in normal urine.

DISCUSSION

The treatment scheme of infectious urolithiasis includes the removal of deposits, as well as long-term antibiotic therapy supplemented with the administration of bacterial urease and crystallization inhibitors. Recently, increased attention has been paid to the need for the modification of diet in patients with infectious urolithiasis. This applies particularly to patients with coexisting constant (metabolic disorders) or temporary (e.g. improper diet) changes in the chemical composition of urine. To evaluate the usefulness of the diet in the treatment of infectious urolithiasis in the present study it was determined how the concentrations of the mineral component of urine affect the rate and intensity of struvite and apatite crystallization induced by bacteria of the genus *Proteus*. The results showed that a concentrations higher than normal physiological levels of each mineral building struvite and apatite crystals leads to an increase in crystallization intensity. This effect was particularly evident in the urine with higher concentration of calcium and magnesium. In these cases total amount of crystallization products increased almost three times (Fig. 2). So far there is little detailed information on the relationship between the chemical composition of urine and intensity of crystallization caused by bacteria. Previously, Hugosson and coworkers (1990) examined how the composition of urine influenced urease-induced crystallization but this study was performed without bacteria, and crystallization was induced by adding jack bean urease to human urine. The authors determined correlations between the concentrations of calcium, magnesium, phosphate, ammonium, citrate, albumin, glycosaminoglycans and crystallization struvite and apatite in urine. Among all the compounds analyzed only the concentrations of calcium and magnesium had a positive effect on the formation of struvite and apatite crystals, while the concentration of albumin was found to have negative correlations with crystallization. Therefore, it seems important to control and/or reduce urinary phosphate, magnesium and calcium levels. Takeuchi and coworkers (1991) showed that marked reduction in the amount of urinary magnesium and/or phosphate may prevent struvite stone formation in rats with urinary tract infections. However, as shown in our studies (Fig. 1), diminishing the quantity of all substrates for struvite crystallization below the normal level does not cause a reduction in the degree of crystallization. Therefore, lowering the content of these salts in the diet might have no influence on the course of infectious urinary calculi formation but could affect the proper functioning of the body. Evaluating the presence and concentrations of crystallization inhibitors in urine seems to be an interesting solution. A compound naturally present in urine which is cited most often as a crystallization inhibitor is a citrate. Its inhibitory influence on crystallization and formation of stones containing calcium phosphate, calcium oxalate and also struvite is well documented (Grases *et al.,* 1989; Achilles *et al*., 1990; Wang *et al*., 1993). Having analyzed urease-induced crystallization in the presence of citrate, Wang and coworkers (1993) showed that this compound affected the nucleation, growth as well as aggregation of struvite crystals. However, as suggested by the authors of these studies, an inhibitory activity can be seen only above the concentration found in normal urine (3 mM/24 h) and may be limited because of the ability of bacteria to utilize citrate as a source of carbon and energy. This could explain the absence of the citrate effect on the *P. mirabilis*-induced crystallization process observed in our study. In the present work, only at a concentration above 2 g/l citrate weakly decreased crystallization intensity as evidenced by the morphology of the crystals and the results of the spectrophotometric method. In this study, the impact of elevated levels of oxalate on the crystallization of struvite and apatite was also examined. At elevated pH, like in the course of *P. mirabilis* infection, oxalate is formed from ascorbate present in the urine (Hokama *et al*., 2000). Therefore, it is possible, to have a situation where calcium oxalate and struvite with apatite crystallized at the same time. In these conditions calcium cations can compete with oxalate and phosphate groups in the crystallization. It has not been confirmed in our study that the increased concentration of oxalate did not affect the crystallization of calcium and magnesium phosphate (Figs. 2, 3). An important role played by the amount of calcium and magnesium in bacteria-induced crystallization had been mentioned previously. In the context of the formation of infectious stones not only the concentration of these two cations but also the ratio between them are of great importance. This relationship is crucial because, as demonstrated in previous clinical studies, patients with struvite urolithiasis often have hypercalciuria (Segma *et al*., 1981). Le Corre and coworkers (2005) assessed the impact of Ca^{2+} ions on struvite crystallization measuring the absorbance, as well as the particle size and characterized formed struvite crystals through X-ray diffraction and SEM-EDS analysis. These analyses showed that increasing the calcium concentration (especially when the molar ratio was 1:2 — Mg:Ca) reduces struvite crystals size, inhibits growth and causes the amorphous structure of the crystals, i.e. deprived of their characteristic shape. Calcium interferes with the crystallization of struvite by blocking active growth sites and competing for phosphate to form calcium phosphate. As in the case of urinary calculi associated with metabolic disorders, the formation of infectious urolithiasis is a result of many physical and chemical processes. Environment of urine is just as important as bacterial factors in the development of the urolithiasis development, which emphasizes the importance of proper diet

in the therapy. This diet should not only involve the reduction of Ca^{2+}, Mg^{2+} and phosphate. Previously, it was shown that the intensity of struvite crystallization is positively correlated with an increase in the pH level (Ariyanto *et al*., 2014). Therefore, it is suggested that in this case a good therapeutic or preventive diet would lower the urine pH. Suller and coworkers (2005) suggested that pH can be elevated by decreasing the calcium, magnesium and phosphate concentrations. In the present study, none of the factors affected significantly the pH of urine and the activity of bacterial urease. The urine pH may be reduced by administering an inhibitor of bacterial urease or by acidification. Recently, it has been found that the phenolic substances of plant origin can act as inhibitors of crystallization and urease activity (Torzewska & Rozalski, 2014). Acidification of urine and the corresponding ionic strength also seem to be significant (Stratful *et al*., 2001). Ionic strength increases electrostatic interactions between the urine ions and reduces their activity, e.g. by affecting the availability of phosphate to form struvite crystals (Desmidt *et al*., 2013). This parameter is responsible for the nucleation and growth of crystals and further for their aggregation, which contributes to the development of urolithiasis.

To sum up, our *in vitro* experiments demonstrate that among all the tested compounds the concentrations of calcium and magnesium in the urine have the strongest influence on the crystallization induced by *P. mirabilis*. A slight increase in their concentrations results in a significant acceleration of the crystallization process. This illustrates the need to control the content of calcium and magnesium in the urine of *P. mirabilis*-infected patients and modify a diet to reduce amount of excreted cations and pH of urine which is responsible for the induction of crystallization.

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