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Urease-induced crystallizations of calcium phosphate and magnesium ammonium phosphate in synthetic urine and human urine

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Abstract An aggregometer technique was used to study urease-induced crystallizations in synthetic urine and human urine from healthy subjects and patients with chronic spinal cord injuries. The two different phases of crystallization, calcium phosphate and magnesium ammonium phosphate, were easily evaluated with a single assay using this technique. The crystallization of calcium phosphate and magnesium ammonium phosphate varied markedly among the different urine specimens after incubation with urease. The turbidity curves from human urine were divided into four patterns. We assumed that the variations in the patterns of the turbidity curves appeared to be mainly due to differences in the composition of the urine and in the original pH, and that the calcium and magnesium concentrations were very important in the urinary constituents.

Key words Urease-induced crystallization · Magnesium ammonium phosphate · Calcium phosphate Aggregometer

Urease-producing microorganisms can induce the crystallization of calcium phosphate and magnesium ammonium phosphate. Experiments in vitro have also shown that urease alkalinizes synthetic urine, resulting in the crystallization of struvite and calcium phosphate [6,7]. However, it remains to be clarified how the different phases involved in urease-induced crystallization of calcium phosphate and magnesium ammonium phosphate are influenced by variations in the composition of urine. In this study, an aggregometer technique was used to study urease-induced crystallization in synthetic urine, and to obtain information on the processes of calcium phosphate and magnesium ammonium phosphate crystallization. We also examined how urease-induced crystallization was influenced by variations in the composition of urine from healthy subjects and patients with chronic spinal cord injuries.

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Materials and methods

Synthetic urine of the composition described by Griffith et al. [5] containing (g/l) $CaCl_2 \cdot 2H_2O$ (0.65), $MgCl_2 \cdot H_2O$ (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), and an initial pH of 5.7, was used. In the morning, urine was collected without the use of a preservative from 15 healthy subjects (9 men and 6 women) and 28 patients (24 men and 4 women) with chronic spinal cord injuries. These patients were not given any antibiotics during this study, and none of them exhibited renal dysfunction. The healthy subjects were hospital staff members who had no previous history of kidney or urinary stone disease. None of the subjects were given any dietary restrictions. Urine specimens were prepared as follows, as soon as they reached our laboratory. The specimens were centrifuged for 10 min at 1500 rpm and filtered through a 0.45-μm membrane filter. The original pH was measured with a Corning 125 pH meter, and the calcium and magnesium contents were estimated consecutively by atomic absorption spectrometry. Urinary phosphate was measured by the Fiske-Subbarow method [3].

An aggregometer (Hema Tracer 1, Model pat-4M, MC Medical Co., Tokyo, Japan), devised to measure the thrombocyte aggregation capacity by means of optical turbidity at 660 nm, was used to estimate the crystallization of calcium phosphate and magnesium ammonium phosphate. In our system, both synthetic urine and human urine, in 200-μl portions, was stirred constantly at 37°C. Then, 10 μl urease solution (1000 μmol U/ml) was added and incubated for 30 min at 37°C. Jack bean urease was purchased from Sigma (St. Louis, MO, USA). The optical densities at 660 nm were simultaneously recorded as turbidity curves on a chart during incubation. Measurements were taken of 0% and 100% turbidity from distilled water and 100 m*M* copper sulfate, respectively. In each human urine sample, 0% turbidity was adjusted with the filtered urine itself.

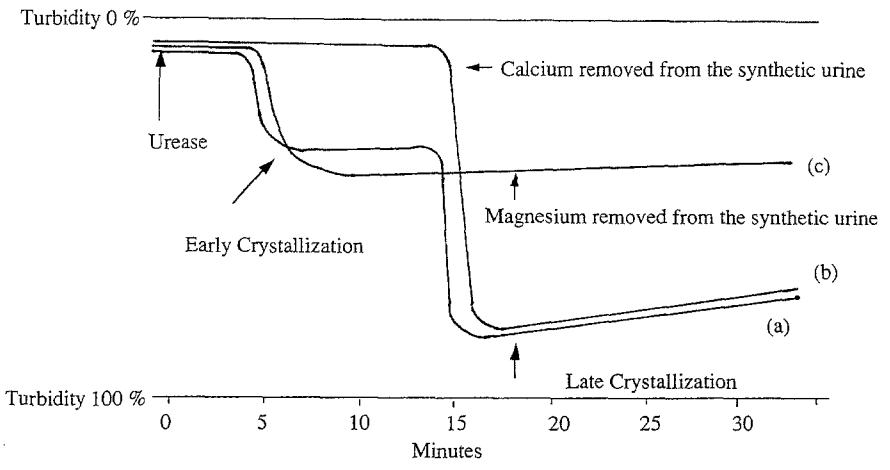

Fig. 1 a Typical two-phase turbidity curve for synthetic urine using the aggregometer technique. Firstly, mild turbidity progressed gradually up to approximately 15 min, and then the turbidity increased rapidly. We called the mild turbidity "early crystallization" and the rapid turbidity "late crystallization". **b** When calcium was removed completely from the synthetic urine, urease-induced crystallization only gave a rapid turbidity curve in the late phase ("late crystallization"). **c** In the synthetic urine without magnesium, the urease-induced crystallization only gave a mild turbidity curve in the early phase, "early crystallization"

Results

Figure 1 shows a typical turbidity curve for synthetic urine using the aggregometer method. Urease-induced crystallization gave a two-phase turbidity curve for synthetic urine. Firstly, mild turbidity appeared gradually up to approximately 15 min, and then the turbidity increased rapidly. Mild turbidity was called "early crystallization" and rapid turbidity was called "late crystallization." The pH changes induced by incubation with urease in the synthetic urine were monitored continuously, and "early crystallization" started at pH 7.3, while "late crystallization" occurred at pH 8.5 (Fig. 2). The crystallization products (both early and late) contained both calcium phosphate and magnesium ammonium phosphate, as shown by light microscopy (Fig. 3), and from the infrared spectrum.

The amount of "early crystallization" was completely dependent on the concentration of calcium in the synthetic urine, and "early crystallization" did not occur when the calcium content of the synthetic urine was less than 17.69 mg/l (10% of the calcium in the synthetic urine). When calcium was removed completely from the synthetic urine, urease-induced crystallization only gave a rapid turbidity curve in the late phase (Fig. 1), and this was shown to be magnesium ammonium phosphate (more than 95%) by infrared spectrometry and light microscopy (Fig. 4). The amount of "late crystallization" was dependent on the magnesium content, and "late crystallization" did not occur when the magnesium content of the synthetic urine was less than 6.97 mg/l (5% of the magnesium in the synthetic urine). In the synthetic urine without magnesium, the urease-induced crystallization only resulted in mild turbidity in the early phase (Fig. 1), and that was shown to be calcium phosphate. Therefore, "early crystallization" represented the crystallization of calcium phosphate and "late crystallization" represented the crystallization of magnesium ammonium phosphate, respectively.

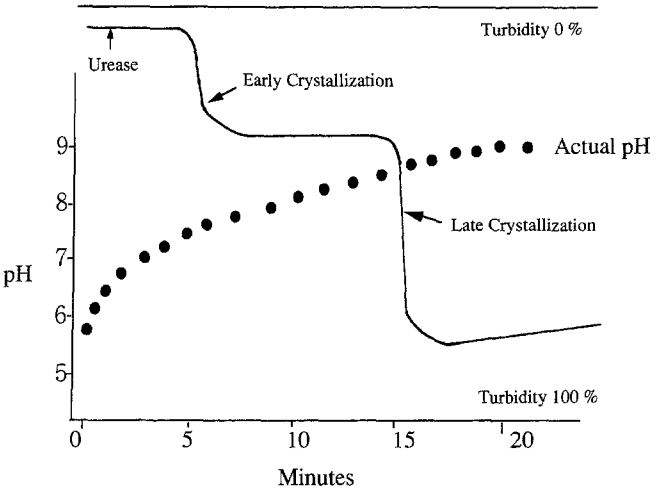

Fig. 2 Actual pH changes induced by incubation with urease in the synthetic urine were monitored continuously, and showed that "early crystallization" started at pH 7.3, while "late crystallization" occurred at pH 8.5

In addition, the concentration of urea in the synthetic urine, which is decomposed into ammonia and carbon dioxide by urease, was also evaluated in our system. When the concentration of urea was decreased in steps, the lag time in the occurrence of the "late crystallization" increased concentration dependently. Furthermore, "late crystallization" was not observed if the urea content was less than 1.25 g/l (5% of the urea in the synthetic urine), and both crystallizations ("early and late") did not occur when the concentration was less than 0.5 g/l (2% of the urea in the synthetic urine).

The turbidity curves derived from human urine demonstrated various patterns, and these were divided into four major types: type 1 showed no turbidity, type 2 showed only "early crystallization," type 3 only showed "late crystallization" without "early crystallization" and type 4 showed both early and late crystallizations. These typical patterns are shown in Fig. 5. Concerning the

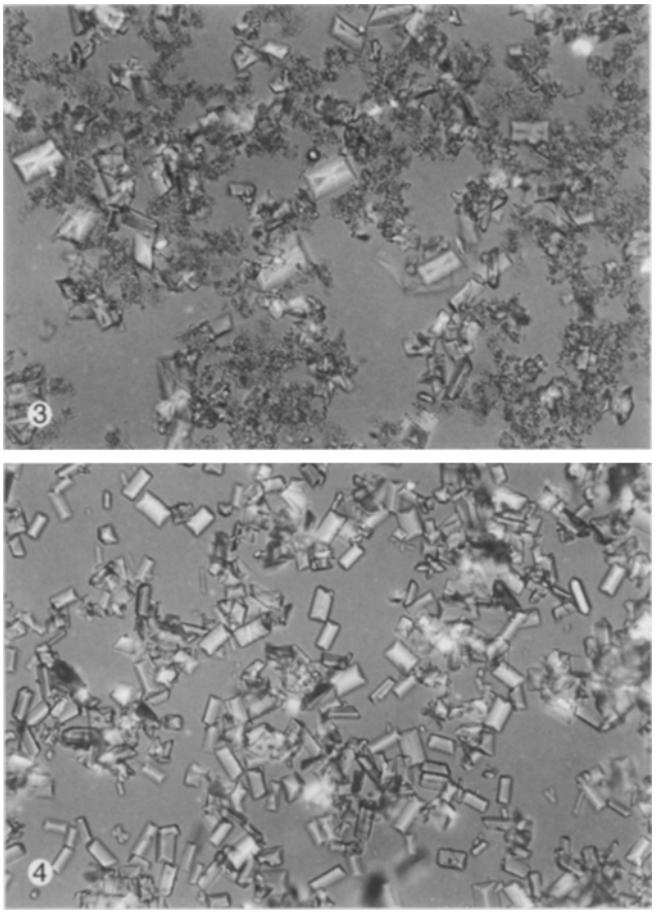

Fig. 3 Light microscopic analysis of the precipitates composed of calcium phosphate (small amorphous precipitates) and magnesium ammonium phosphate (various-sized X-shaped crystal), after incubating urease and synthetic urine

Fig. 4 Crystallization of synthetic urine lacking calcium; the various sized X-shaped crystals were shown to be magnesium ammonium phosphate. The turbidity curve is shown in Fig. 1b

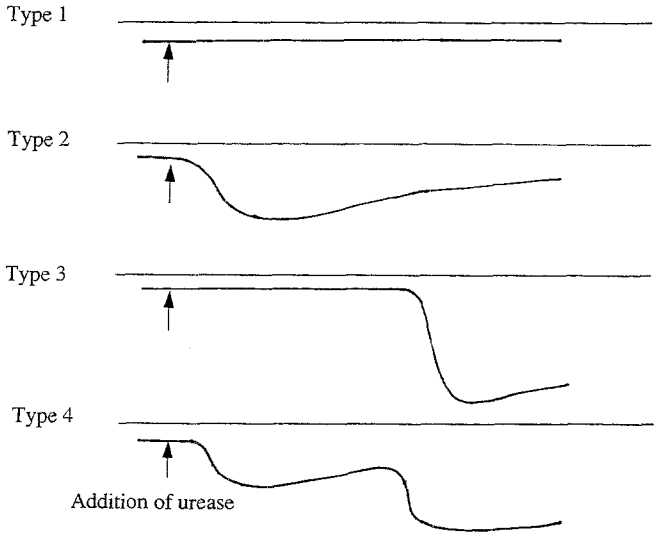

Fig. 5 Four types of turbidity curve for human urine; type 1 showed no turbidity, type 2 showed only "early crystallization", type 3 showed only "late crystallization," and type 4 showed both crystallizations

Table 1 Classification of the turbidity types, patient profiles and urinary parameters. Data are expressed as means ± SD

	Type of turbidity curve	Number (male)	Duration of immobilization (y)	UTI[a] [number %]	History of urolithiasis [number (%)]	Urinary concentration				Urinary pH	
						Phosphate (mg/dl)	Ca (mg/l)	Mg (mg/l)	Ca/Mg	Initial	Final
SCI patients	Type 1	10 (9)	20.6 ± 11.6	4 (40%)	2 (20%)	42.6 ± 48.4	38.1 ± 33.2	36.0 ± 28.0	1.19 ± 1.02	8.06 ± 0.84	9.30 ± 0.15
	Type 2	3 (3)	1.3 ± 0.47	0	0	33.9 ± 6.23	87.4 ± 43.2	54.0 ± 21.0	1.65 ± 0.51	6.42 ± 0.48	9.37 ± 0.02
	Type 3	2 (1)	9 ± 2	1 (50%)	0	74.0 ± 2.0	70.7 ± 7.7	89 ± 2.2	0.80 ± 0.11	6.42 ± 0.48	9.37 ± 0.02
	Type 4	13 (11)	8.7 ± 8.4*	3 (23%)	2 (15%)	71.2 ± 27.3	210 ± 102**	143 ± 80*	1.58 ± 0.75	5.96 ± 0.62**	9.28 ± 0.13
Controls	Type 2	1 (0)	–	–	–	85.3	182	69	2.63	5.15	9.34
	Type 4	14 (9)	–	–	–	89.5 ± 38.6	160 ± 46.5**	94 ± 33**	1.80 ± 0.47	5.92 ± 0.64**	9.31 ± 0.11

[a] Large numbers of bacteria in unstained sedimented specimens indicate patients with severe urinary tract infections, as shown by microscopic examination
*$P < 0.05$, **$P < 0.01$ compared with type 1 from SCI patients

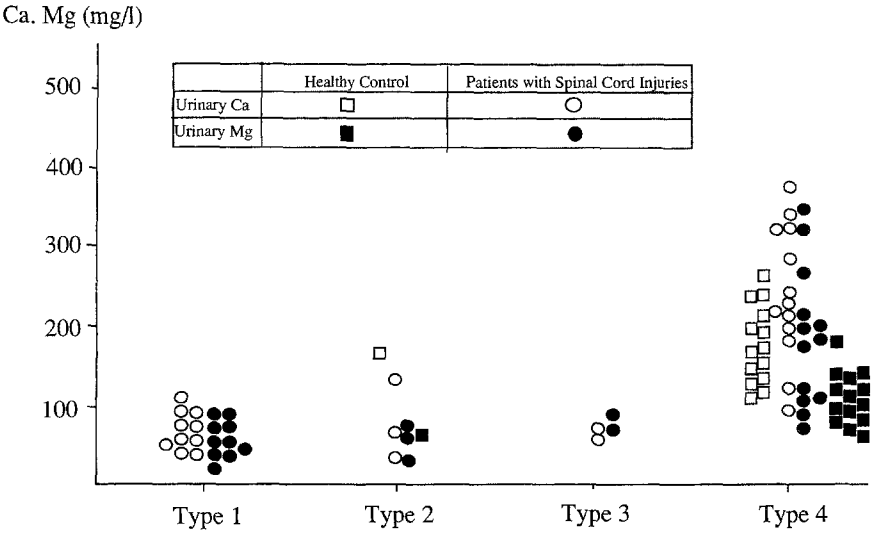

Fig. 6 Relationships between the turbidity patterns and the concentrations of calcium and magnesium. The variations in the patterns of the turbidity curve appeared to arise from differences in the urinary calcium and magnesium concentrations

reproducibility of the represented type, the evaluation of individual patterns did not change during triplicate assays with the same materials. Furthermore, daily variations were not observed in urine samples of five patients and five healthy subjects. Individuals urine samples were classified into four types from the typical patterns. The patterns of the turbidity curves from human urines, important patients profiles and urinary parameters are summarized in Table 1. All the urine samples, except for one subject, from the healthy control group had the type 4 pattern, while the urine samples from the group with spinal cord injuries had the type 1 (10/28, 36%) and type 4 pattern (13/28, 46%). The patients showing the type 1 turbidity pattern had suffered from spinal cord injuries for long periods. Four urine samples of type 1 (40%) and three of type 4 (23%) had severe urinary tract infection, respectively. Moreover, the initial urinary pH in the patients with type 1 turbidity pattern was significantly higher than that in those showing type 4 from both patients and healthy controls. However, the final pH in each urine sample was alkalinized (pH > 9.0) after 30 min of incubation with urease. Amorphous salt was observed in four urine samples showing type 1 pattern (40%).

The relationships between these turbidity patterns and the concentrations of calcium and magnesium are shown in Fig. 6. The variations in the patterns of the turbidity curves appeared to be mainly due to differences in urinary calcium and magnesium levels. Urinary calcium, magnesium and the Ca/Mg ratio in each turbidity type from the patients and healthy subjects are also summarized in Table 1. The concentrations of calcium and magnesium in the type 1 urines from the patients were significantly lower than that in type 4 urines from patients and healthy subjects. The type 1 urines from the patients had lower Ca/Mg ratios than type 4 urines from both patients and healthy controls, but this was not significant. The Ca/Mg ratio in type 2 and type 3 is not mentioned because those samples were scarce in this series.

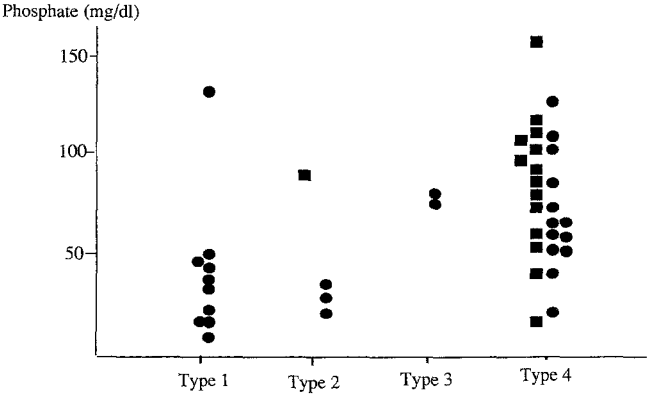

Fig. 7 Relationship between the turbidity patterns and the urinary concentration of phosphate. There was no correlation between the turbidity patterns and phosphate excretion (■ Healthy control, ● Patients with spinal cord injuries)

The relationship between these turbidity patterns and the urinary concentration of phosphate is shown in Fig. 7. There was no correlation between the turbidity patterns and phosphate excretion, because urinary phosphate level varied between individuals. The concentration of phosphate in the type 1 urines from patients seemed to be lower than the type 4 urines from both patients and healthy controls (Table 1). However, there were no significant differences.

Discussion

Some turbimetric techniques [8,10], including the aggregometer technique that we devised [1], have been used to estimate calcium oxalate crystallization, or to measure the inhibition of calcium oxalate crystallization in human urine. A Coulter counter technique could also be used when investigating urease-induced precipitation [13]. However, the Coulter counter may not be suitable when simultaneously estimating the crystallization of

two different kinds of crystals. With the aggregometer technique, the comparative turbidity is mainly indicated by the initial nucleation of crystallization. The formation of calcium phosphate and struvite crystals in urine can be individually monitored with the aggregometer.

It is well known that urease-induced crystallization is especially pronounced for magnesium ammonium phosphate. However, calcium phosphates are usually co-deposited with struvite as shown by the analysis of the constituents of many stones containing struvite [11]. Experiments in vitro have also shown that urease alkalinized synthetic urine, resulting in the crystallization of struvite and calcium phosphate [6,7]. However, the different phases involved in urease-induced crystallization of calcium phosphate and magnesium ammonium phosphate have not been completely elucidated. Hedelin et al. [6] showed that the precipitation of ammonium magnesium phosphate occurred at a higher pH than calcium phosphate. This phenomenon was also confirmed in our system using the aggregometer, whereby urease-induced crystallization yielded a two-phase turbidity curve with synthetic urine. "Early crystallization," the precipitation of calcium phosphate, occurred at pH 7.3, while the "late crystallization," the precipitation of magnesium ammonium phosphate, occurred at pH 8.5.

We found a report that human urine added to synthetic urine had a strong inhibitory effect on urease-induced crystallization [4]. In our investigation, the turbidity patterns for human urines could be classified into four types. All urine samples had been alkalinized (pH > 9.0) after 30 min of incubation, and the degree of alkalization was almost constant because of the addition of excess urease. The increased pH of the urine samples showing the type 1 turbidity pattern was verified after incubation with urease. It could be denied that type 1 urines had considerable inhibitory activity against urease. However, the initial pH of the type 1 urines was significantly higher than type 4 urines in both patients and healthy controls. Therefore, the slopes of the pH change curves in type 1 urines, by incubation with urease, were gentler than those of the other three types, and the smaller changes in the pH might not be enough to initiate the precipitations of calcium phosphate and struvite. We assumed that one factor in the variation in the patterns of the turbidity curves might be the difference in the original pH of the urine.

The results of this study showed that the turbidity patterns of healthy subjects and patients with chronic spinal injuries differed distinctly. All the healthy controls, except one subject, had the type 4 curve, while patients with spinal cord injuries had type 1 (36%) and type 4 (46%) curves. Variability in the turbidity curves seemed to be mainly due to differences in the urinary concentrations of calcium and magnesium. Thus, type 4 urine has a high concentration of calcium and magnesium, while type 1 urine has a low concentration of both calcium and magnesium. Similar results were reported by Huggosson et al. [9]. It is not clear why urinary calcium and magnesium concentrations in the patients are significantly lower than in healthy individuals. In this study, the duration of the immobilization of the type 1 patients was much longer than that of type 4. We speculate that the lesser amounts of calcium and magnesium excretion might be dependent on the low physical activity of the patients, including dietary habits. There was a report showing that calcium regulatory systems were affected by long-standing spinal cord injuries [12].

The relationship between urinary phosphate and urease-induced crystallization is still controversial. Our results showed that phosphate levels in the urine varied between individuals, and there was no correlation between the turbidity pattern and phosphate excretion. Moreover, the concentration of urinary phosphate in the type 1 group of patients seemed to be lower than that shown by type 4, but not significantly. Two other reports [7,9] gave similar results to ours. Edin-Lilegren et al. [2] showed the pH increase induced by urease was more dependent on urinary phosphate, as it is the major buffer in human urine. The role of phosphate ion in the crystallizations of calcium phosphate and magnesium ammonium phosphate is still unknown and requires further detailed investigation.

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