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Studies on the crystallization of magnesium ammonium phosphate in urine

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Summary. The crystallization of magnesium ammonium phosphate (MAP) was studied in salt solutions and dialysed urine at similar levels of MAP supersaturation. At pH levels of 7.1 or higher crystallization occurred to the same extent in solutions with and without urinary macromolecules. Whereas crystals in the size range 3.5–5 μm were observed in the salt solution at pH 7.0, this was not so in dialysed urine. When the crystal size distribution was determined after 30 min larger crystals were observed in dialysed urine, indicating a promoting effect by urinary macromolecules on the formation of MAP crystals.

A modified AP(MAP) index was formulated based on calculations with the EQUIL 2 programme [8] in order to improve the relationship between this simplified estimate and the ion-activity product of MAP (AP_{MAP}). This index had the following form:

$$\frac{3.8 \times 10^{-4} \times Mg^{1.06} \times NH_4^{0.98} \times P^{0.71} \times (pH\text{-}4.5)^{6.30}}{V^{2.39}}$$

for 24-h values of magnesium (Mg), ammonium (NH_4), phosphate (P), pH and urine volume (V).

The AP_{MAP} required for the formation of 2,000 crystals in the size range 3.5–5 μm varied between 226×10^{-15} and 293×10^{-15} $(mmol/l)^2$ in dialysed urine. An experimental system was designed based on the measurement of pH during the addition of NH_4OH. At the point assumed to correspond to the start of crystallization, AP(MAP) index values between 409 and 903 were recorded. Such a test might provide useful information on the crystallization properties in urine.

Key words: Magnesium ammonium phosphate – AP-(MAP) index – EQUIL 2 programme – Crystallization risk – Inhibition – Promotion

Most staghorn stones that form in patients from the industrialized part of the world are composed of magnesium ammonium phosphate (MAP) and carbonate apatite (CarbAp) [6]. Although some animal species might form MAP crystals under non-infection conditions [12], crystallization in human urine is presumed to occur only in the presence of infection with urease-producing bacteria [7]. The urease-catalysed splitting of urea results in an alkaline urine with high concentrations of ammonium, bicarbonate and carbonate ions. This is an environment favourable for the crystallization of MAP and CarbAp. Owing to the large stone volume and the presence of infection, this type of stone constitutes a particular problem. An incrased understanding of the mechanisms behind MAP crystallization is therefore of great value for an appropriate evaluation, design of efficient recurrence-preventive therapy and rational follow-up of these patients.

This paper describes some experiments carried out in order to study the formation of MAP in the absence of calcium, whereby the otherwise compulsory precipitation of calcium phosphate was avoided.

Material and methods

Effect of pH on crystallization

The crystallization of MAP at different pH levels was studied in calcium-free solutions. The experiments were performed either in solutions of 0.15 *M* NaCl, containing 3 mmol of magnesium (Mg), 25 mmol of ammonium (NH_4) and 20 mmol of phosphate (PO_4; P) per litre or in dialysed urine, equilibrated with 0.15 *M* NaCl and with the same electrolyte composition as in the salt solution. The sample volume was adjusted to 100 ml and the pH to 4.0 after which the solutions were passed through Millipore filters (0.22 μm). The pH was increased to 6.0 with 8 *M* NaOH and subsequently stepwise to 6.9, 7.0, 7.1, 7.2 and 7.3 with 1 *M* NaOH. Six samples were investigated at each pH level. Crystallization was measured in a Coulter Counter (model Z_B) equipped with a Channelyzer at 3-min intervals. The time required for the formation of 100 particles in the size range 3.5–50 μm was recorded. The measurements were stopped after 120 min if no particles occurred. The samples were subsequently kept on a magnetic stirrer at 34°–37 °C for determination of the crystal size distribution 30, 60 and 120 min later, when changes in particle number and size were recorded. The macroscopic appearance of the crystals was studied under a light microscope.

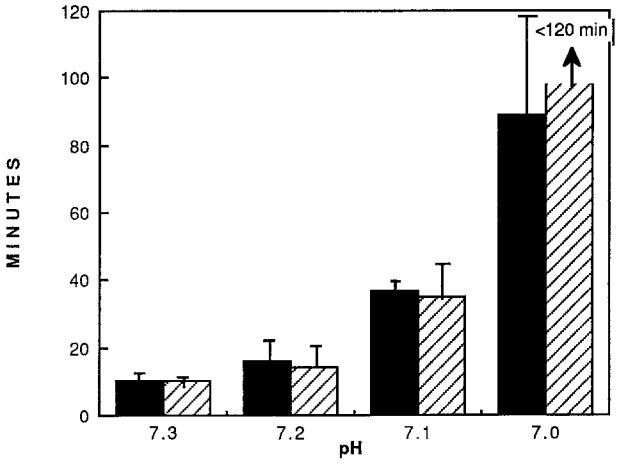

Fig. 1. The time lag before magnesium ammonium phosphate (MAP) crystals were recorded in sodium chloride (■) and dialysed urine (▨)

Effects of magnesium, ammonium and phosphate on crystallization

Dialysed urine, prepared as above, was used to study the effect of different concentrations of Mg, NH_4 and P on the crystallization of MAP. The sample volume was adjusted to 25 ml. The concentration of Mg was increased stepwise from 2 to 4 m*M*, of NH_4 from 20 to 80 m*M* and of P from 20 to 40 m*M*. The variables were changed one at a time, while the others were kept constant at 3.0 m*M* for Mg, 25 m*M* for NH_4 and 20 m*M* for P. After pH adjustment to 6.8, 7.0 and 7.25 with 8 m*M* NaOH, the samples were stirred for 30 min at room temperature. They were then analysed in the Coulter Counter and studied under the light microscope.

Crystallization risk in urine samples

Night urine collected between 10 p.m. and 6 a.m. from eight healthy subjects was used for studies on crystallization properties in whole urine. Urinary concentrations of calcium (Ca), phosphate (P) and magnesium (Mg) were analysed by methods described elsewhere [18]. Citrate was analysed with lyase [19] and ammonium (NH_4) as described by Henry et al. [9]. A sample of 200 ml was adjusted to pH 4.0 with 4*M* HCl and then passed through a Millipore filter (0.22 µm). The pH was subsequently adjusted to 6.0 with 2*M* NH_4OH. From this stage each sample was stirred magnetically and the pH further increased by adding 0.1 ml of a 2*M* solution of NH_4OH every minute. Starting at pH 6.5 and then at 0.5 pH intervals, 5 ml aliquots were collected and studied under the light microscope. The addition of NH_4OH was interrupted when MAP crystals were observed.

Measurements of pH were carried out with a pH meter, PHM84 (Radiometer, Copenhagen), equipped with a glass electrode.

A modified estimate of the ion-activity product

We have previously formulated an AP(MAP)-index [17], as a simplified estimate of the ion-activity product for MAP:

$$\frac{7.5 \times 10^{-5} \times Mg \times NH_4 \times P^{0.88} \times (pH\text{-}4.5)^{6.95}}{V^{2.4}}$$

This index was calculated from the 24-h excretion of Mg, NH_4, P and urine volume (*V*). This index was modified by means of data obtained with the EQUIL 2 programme [20]. The ion-activity product for MAP (AP_{MAP}) thereby was calculated by an iterative approximation based on information on urine concentrations of Ca, Mg, sodium, potassium, oxalate, citrate, sulphate, P, NH_4 and urinary pH. For determination of the effect of each variable on AP_{MAP}, we used the following basic 24-h excretion in our theoretical calculations: Ca 6.5 mmol, Mg 4.5 mmol, sodium 200 mmol, potassium 70 mmol, oxalate 0.3 mmol, citrate 3.0 mmol, sulphate 30 mmol, P 25 mmol, NH_4 35 mmol, pH 6.0 and urine volume 1.5 l. By changing the value of only one variable while keeping the others constant, it was possible to formulate a modified AP(MAP) index by adjusting the factors and exponents in the formula to obtain values that numerically approximately corresponded to $10^{15} \times AP_{MAP}$.

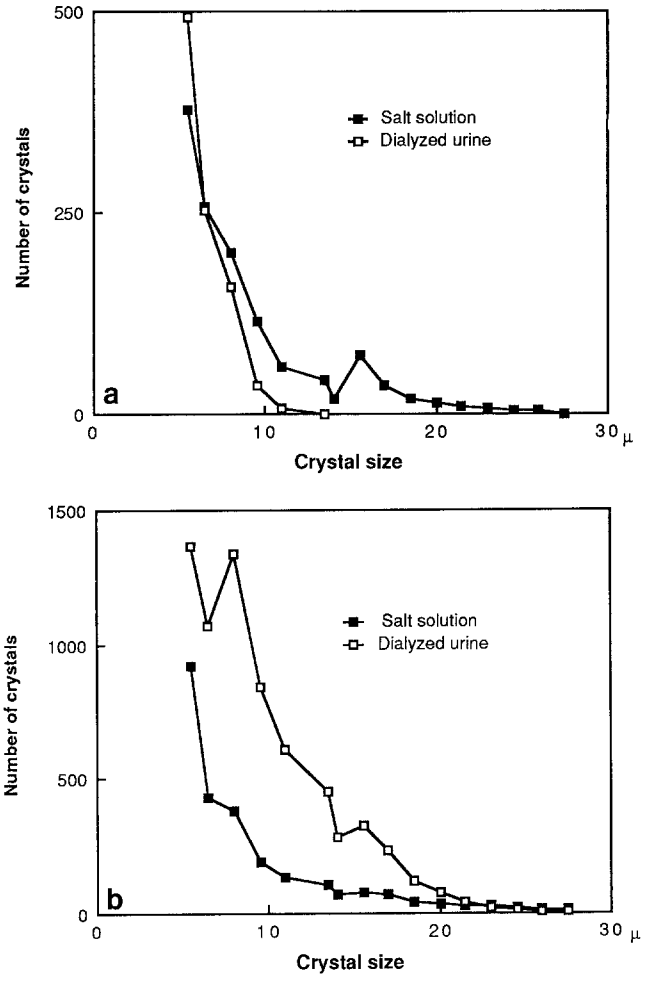

Fig. 2. a Crystal size at crystallization moment in dialysed urine and salt solution at pH 7.2. **b** Crystal size 30 min after crystallization in dialysed urine and salt solution

Results

When calcium-free solutions with constant concentrations of Mg, NH_4 and P were alkalinized by the addition of NaOH, crystals developed at a lower pH in the salt solution than in dialysed urine (Fig. 1). However, when the pH was increased up to or above 7.1, the same number of crystals was observed at similar periods of time after the pH adjustment.

The crystal size distribution at pH 7.2 when crystals were first observed and after 30 min is shown in Fig. 2.

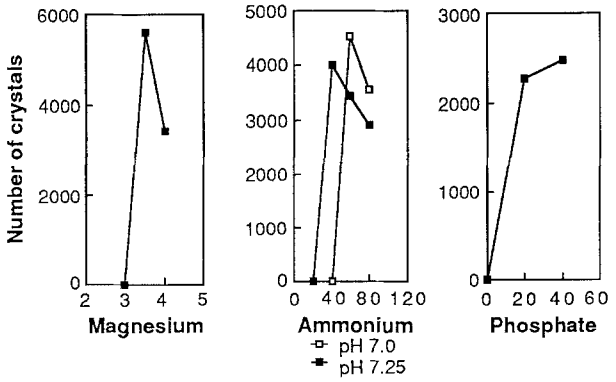

Fig. 3. Effect of magnesium, ammonium, phosphate and pH on MAP crystallization

Table 1. Value of (AP_{MAP}) calculated with the EQUIL 2 programme when 2,000 particles were found in the samples

Magnesium (m*M*)	3.15	3	3	3
Ammonium (m*M*)	25	30	48	25
Phosphate (m*M*)	20	20	20	17
pH	7.25	7.25	7.0	7.25
Volume (ml)	1,500	1,500	1,500	1,500
$10^{15} \times AP_{MAP}$	258	293	242	226

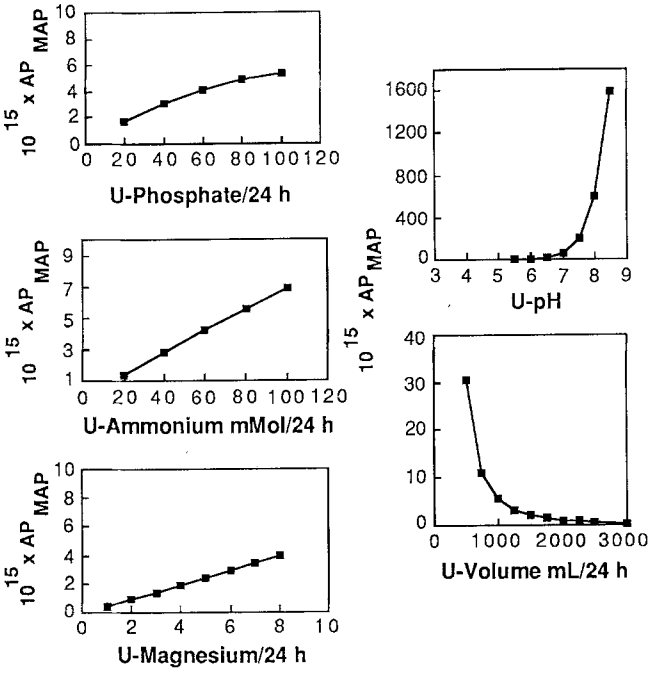

Fig. 4. Relationship between variable excretions of magnesium, ammonium, phosphate and pH and 24-h urine volume on ion-activity product (AP_{MAP})

Initially, the sodium chloride solution and the dialysed urine had similar numbers of crystals with approximately the same size distribution, but later there were more particles in the size range between 10 and 20 µm in the dialysed urine than in the salt solution.

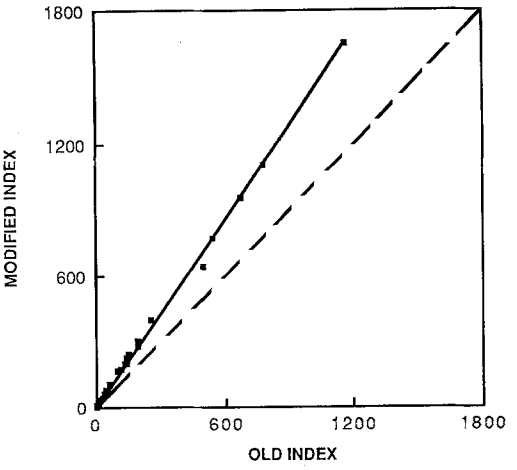

Fig. 5. Correlation between the old and the modified AP(MAP) indices

The effect of increased concentrations of Mg, P and NH_4 at different pH levels is shown in Fig. 3. At pH 6.8 no crystallization was observed, despite high concentrations of the different constituents. At pH 7.0 increment of the NH_4 concentration to 60 m*M* started the crystallization, but no effects were observed when the concentration of the other ions was increased to a similar extent. At pH 7.25, however, crystallization was observed at low concentrations of Mg, P and NH_4. AP_{MAP} corresponding to the formation of 2,000 crystals gave values that varied between 226×10^{-15} and 293×10^{-15} (mmol/l)^2 (Table 1).

Similar to our previously reported results [17], the effects of Ca, sodium, potassium, citrate and sulphate on AP_{MAP} appeared to be small when calculations were carried out with the EQUIL 2 programme. The most pronounced influence was accomplished by Mg, NH_4, P, pH and *V*, which is also in agreement with our previous observations. The relative effects of these variables on AP_{MAP} (Fig. 4), however, differed slightly, with the most important deviation recorded for pH. The best fit between $10^{15} \times AP_{MAP}$ and AP(MAP) index was obtained when the latter expression took the following form:

$$\frac{3.8 \times 10^{-4} \times Mg^{1.06} \times NH_4^{0.98} \times P^{0.71} \times (pH\text{-}4.5)^{6.30}}{V^{2.39}}$$

The relationship between the previously formulated index and this modified AP(MAP)-index is shown in Fig. 5. Evidently, there was a very good correlation ($r = 0.99$). The value of the modified index was, however, slightly higher than that of the old index. The high levels of supersaturation at which the deviation is most evident are probably not very often encountered clinically.

The effects on pH of NH_4OH added to a number of normal urine samples are shown in Fig. 6. The end-point of each curve represents the pH at which MAP crystals were first observed under the light microscope. The effect of NH_4OH on urine pH differed considerably between the samples. In four samples MAP crystals did not occur until a pH of 8.0 was reached. Sample no. 8 showed a high buffering capacity allowing for a high NH_4 concentration,

Table 2. AP(MAP) index calculated at different stages of MAP crystallization

No.	Ca	Mg	NH_4	P	Citrate	pH	AP(MAP) index
			86.8			6.0	14.3
1	3.07	3.66	113.8	23.2	2.05	7.15	673[a]
			127.8			7.50	1,648[b]
			34.9			6.0	1.9
2	1.47	1.70	60.9	14.6	0.68	7.85	510[a]
			74.9			8.00	823[b]
			56.5			6.0	5
3	1.38	2.16		23.3	1.31		–
			95.6			8.00	1,878[b]
			30.6			6.0	1
4	1.50	1.68	51.6	8.1	0.51	8.15	484[a]
			59.6			8.50	992[b]
			43.2			6.0	2.3
5	2.18	1.44	70.2	18.7	1.16	7.70	439[a]
			78.2			8.00	858[b]
			21.7			6.0	0.6
6	1.11	1.25	40.7	9.5	0.89	8.45	476[a]
			43.7			8.50	599[b]
			23.3			6.0	0.5
7	0.78	1.35	41.3	6.2	0.49	8.75	665[a]
			50.3			9.00	1,157[b]
			74.2			6.0	14.6
8	3.08	4.40	101.2	22.6	1.47	7.25	904[a]
			112.2			7.50	1,730[b]

[a] Moment of estimated crystallization
[b] Moment when MAP crystals were first microscopically visible

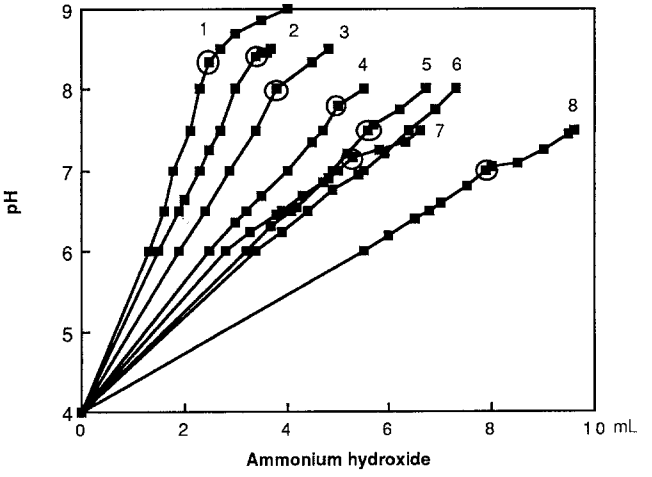

Fig. 6. Effects on pH by addition of NH_4OH to 8 normal urine samples. The addition of alkali was stopped when crystals were demonstrated microscopically. The deviation of the curve (O) presumably represents the start of crystallization

and crystals were observed at pH 7.5. On the other hand, sample no. 1 showed a rapid pH increase, but MAP crystals did not occur below pH 9.0. An altered slope of the curve just before microscopic detection of crystals was observed in all samples except one (no. 7). We assumed that this point reflects the start of crystallization.

The AP(MAP) index was calculated for the composition of urine at pH 6.0, at the point where the pH increment was retarded (start of crystallization?) and when MAP crystals appeared under the light microscope (Table 2). The AP(MAP) index at the point assumed to correspond to the start of crystallization varied between 439 and 904, whereas no crystals were detected under the microscope below an AP(MAP) index of 599. However, there was a wide range of AP(MAP) index values corresponding to the visualization of crystals, with the highest being 1878.

The samples with the highest AP(MAP) index values at pH 6.0 required the largest addition of NH_4OH to start crystallization. The same samples produced MAP crystals at the lowest pH levels. The reason for this might be a combination of a high buffering capacity, a pronounced complexing of phosphate and a higher concentration of urinary macromolecules in the more concentrated urine.

Discussion

Much knowledge has accumulated on the crystallization of MAP and CarpAp in artificial salt solutions [1, 2, 10, 14], whereas limited information is available on these processes in urine [3, 4]. The experiments reported in this paper were carried out to study the crystallization of MAP. Such investigations can be performed in samples of

whole urine, but conclusions might thereby be invalidated by the inevitable precipitation of calcium phosphate at high pH levels. It is doubtful, however, whether observations in salt solutions are valid for processes that take place in urine. In an attempt to role out such uncertainties we performed our crystallization experiments in solutions containing urinary macromolecules. This was accomplished by adding different constituents to and/or by manipulating the pH of dialysed urine samples. To maintain a reasonable ion strength, all samples were given a sodium chloride concentration of 0.15 *M*.

When the rate of MAP crystallization was compared in physiological saline and dialysed urine, the time lag before crystals were detected was similar in the two solutions at a pH of 7.1 or higher. At pH 7.0, however, there were no crystals observed in the dialysed urine despite observation periods as long as 2 h.

There are three possible explanations for such a phenomenon. The urinary macromolecules might either exert a direct effect on MAP suersaturation, inhibit nucleation and early crystal growth or promote the formation of many small crystals with diameters below the detection limit of the Coulter Counter.

It is well recognized that the negatively charged macromolecules in urine bind calcium [11, 13, 15, 16], and it is reasonable to assume that magnesium ions are bounds in a similar way. Nevertheless, such a mechanism is unlikely to affect supersaturation other than marginally, and the similarity in rates of crystallization at pH levels above 7.0 supports this assumption.

A direct inhibitory effect of dialysed urine cannot be excluded from the experimental results summarized in Fig. 1. Whereas other authors have shown that urease-induced precipitation of mainly CaP was inhibited by normal urine, the effects on MAP were not particularly studied [5]. However, neither glycosaminoglycans nor pyrophosphate inhibited the precipitation of MAP [8]. The difference in crystal size distribution between the salt solutions and the dialysed urine after 30 min is noteworthy, and the greater number of large crystals in the presence of macromolecules appears to be more in line with a promoting than an inhibitory effect.

The presence of macromolecules might thereby provide nucleating sites for MAP by facilitating crystallization either directly through the molecules or by forming zones with a high magnesium concentration. According to the data in Fig. 2 the crystals were apparently slightly smaller in dialysed urine during the initial phase of crystallization. It is possible that a large number of small crystals by further growth and probably aggregation will result in the different crystal size pattern in dialysed urine. Evidently, the crystal volume increased with time both in the salt solution and the dialysed urine, but this increment was most pronounced in the latter solution.

Although we carefully checked that calcium was efficiently removed from the urine by dialysis, we cannot exclude the presence of small residual amounts bound to the macromolecules. Areas of the macromolecules with high calcium concentrations might result in precipitation of calcium phosphate acting as nuclei for heterogenous MAP crystallization.

When the EQUIL 2 programme was used to calculate AP_{MAP} corresponding to the formation of 2,000 crystals in the size range 3.5–5 μm, the values obtained were consistent with those previously reported in the literature for salt solutions [2, 14]. A pronounced effect of the macromolecules on supersaturation is therefore unlikely.

These preliminary observations on MAP crystallization in the presence of urinary macromolecules are insufficient to explain fully the mechanism of the process. The results indicate that the macromolecules although possibly slightly reducing the supersaturation, act both as promotors and inhibitors of the crystallization of MAP. Additional experiments are necessary to shed further light on this process.

For design of an appropriate therapeutic regimen and for adequate follow-up of patients with infection stone disease, it is of importance to have methods for the estimation of the level of AP_{MAP}. We have previously formulated a simplified estimate, the AP(MAP) index, based on the analysis of Mg, NH_4P and pH [17]. More recent information on the stability constants and ion interactions [20] made it necessary to revise this index. The new and modified index presented above more closely relates to the AP_{MAP} obtained by the EQUIL 2 programme. Although we strongly propose use of the modified index, the correlation between this and the old index was very good. Furthermore, the numerical difference is small in the range which is most interesting from a clinical point of view.

A simple test system was set up to determine the risk of MAP crystallization by the stepwise addition of NH_4OH. The behaviour of normal urine samples showed a great variability most certainly explained by differences in buffer capacity, electrolyte composition, ion strength and probably also concentration of macromolecules. Although crystals were not microscopically demonstrated at the point where the slope of the pH response curve changed direction, it was assumed that this point indicates the start of crystallization. Nevertheless, even in this apparently early phase of crystallization the AP(MAP) index level was higher than expected from the results with dialysed urine. The reason for this cannot be established with certainty but might be due to an initial precipitation of small calcium phosphate crystals as a result of the increased pH. Although these crystals were too small to be detected under the light microscope, their formation might reduce the phosphate concentration and thereby cause an overestimation of the AP(MAP) index at the start of crystallization. An additional effect of small molecular inhibitors, albeit unlikely, cannot be completely excluded.

Continuous measurement of pH during titration with NH_4OH might nevertheless be an easy method for the determination of the crystallization properties of urine. In normal urine samples the addition of large quantities of NH_4OH was necessary to those samples that were most concentrated and thus had the highest initial AP(MAP) index. In the same samples crystallization started at lower pH levels than in more diluted urines, most likely because of the higher NH_4 concentration.

From a therapeutic point of view these results support the value of a high fluid intake even in the treatment of

patients with infection stone disease. The disadvantage of a reduced buffer capacity might thereby be counteracted by a favourable reduction of the macromolecular promotion of MAP crystallization.

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