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The effects of citrate on hydroxyapatite induced calcium oxalate crystallization and on the formation of calcium phosphate crystals

C. Berg and H.-G. Tiselius

Department of Urology, University Hospital, Linköping, Sweden

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Summary. The addition of different amounts of hydroxyapatite crystals (HAP) to a solution, metastably supersaturated with respect to calcium oxalate (CaOx) resulted in heterogenous crystallization at seed concentrations exceeding 0.2 mmol/l. The induction period varied between 1 and more than 8 h with the shortest period for a seed concentration of 2 mmol/l. Addition to the system of 1 and 2% of whole urine and citrate in concentrations corresponding to approximately 1% of that found in normal urine inhibited the crystallization for as long as 4 h. In a system supersaturated with respect to calcium phosphate (CaP) the total number of crystals was markedly reduced by citrate concentrations exceeding 0.5 mmol/l. The fractions of medium sized and large crystals were sharply reduced and small crystals predominated at higher citrate concentrations. This might indicate effects of citrate on both crystal growth and crystal aggregation. We conclude that increased citrate concentrations during treatment with alkali leads to a significant inhibition of CaOx growth on HAP as well as to a prevention of the formation of large CaP crystals from solutions supersaturated with respect to CaP.

Key words: Alkali – Calcium oxalate – Calcium phosphate – Citrate – Hydroxyapatite – Inhibition – Urine

Introduction

Because calcium oxalate (CaOx) occurs in the majority of renal stones [10, 18], elimination of risk factors for CaOx crystallization has become a mainstay in prevention of recurrent stone formation. Administration of alkaline citrate is a clinically promising alternative [16], which affects urinary citrate, calcium. and pH [3, 4, 6, 16, 21].

Urinary citrate is of particular importance because of its capacity to complex calcium [17] and to inhibit the crystallization of both CaOx [19] and calcium phosphate (CaP) [2]. Thus, it was shown that increased levels of urinary citrate reduced the formation of CaOx and CaP crystals in whole urine [5]. The observations of an inhibiting effect on CaP formation are very important because many calcium stones are composed of both CaOx and CaP and the increased pH during treatment with alkali will undoubtedly increase the supersaturation with CaP.

Crystallization of CaOx is considered to start as a heterogenous nucleation, a process requiring a lower supersaturation than homogenous nucleation. A heterogenous nucleation is facilitated by a good crystal lattice match for CaOx [9] and prevented by activity of inhibitors on the surface of crystallization.

In alkaline urine, the thermodynamically most stable CaP phase is hydroxyapatite (HAP), probably formed from dicalcium phosphate dihydrate (DCPD, brushite) with octacalcium phosphate (OCP) as a possible intermediate [13]. It was shown previously that CaOx-monohydrate (COM) will precipitate on the surface of seed crystals of DCPD [12, 15] and HAP [11, 14]. However, only CaOx-trihydrate (COT) could induce crystallization of HAP at low levels of supersaturation [8] and the latter phase was a suitable seed for further COM growth. Inasmuch as COT is a very unstable phase and DCPD is most stable in acid urine, HAP appears to be the most important phase for considerations of heterogenous crystallization in alkaline urine. The possibility of an increased precipitation of CaP and a subsequent heterogenous crystallization and growth of CaOx on the HAP crystal surface has to be considered before alkalinization of urine can be used in prevention of calcium stone formation.

The aim of the present study was to determine the risk of heterogenous crystallization of CaOx on HAP at different concentrations of HAP seed crystals in a

solution, metastably supersaturated with CaOx, and to study the effect of citrate and urine on this process. In addition we determined the effect of citrate on the size distribution of CaP crystals formed spontaneously in a system supersaturated with respect to CaP.

Materials and methods

Precipitation of CaP in urine

Determination of the amount of CaP that precipitated in whole urine was performed in clear urine samples from 8 healthy volunteers. Following filtration through Whatman filterpaper No. 3, pH was increased to 8.0 by addition of sodium hydroxide. The samples were centrifuged after an equilibration period of 30 min. The precipitate was dried at 37°C over night in a desiccator and weighed.

Crystallization in solutions supersaturated with CaOx

The crystallization of CaOx on seed crystals of HAP was monitored from the decrease in soluble [^{14}C]-oxalate [20] in a solution, metastably supersaturated with CaOx and saturated with HAP.

The crystallization system contained per litre: 1 mmol of calcium chloride, 0.2 mmol of sodium oxalate, 10 mmol of a sodium cacodylate buffer adjusted to pH 6.5, and 0.15 mol of sodium chloride. The system was prepared by mixing stock solutions of calcium chloride and sodium oxalate previously saturated with HAP. Immediately before use the final solution was passed through a Millipore filter with a pore size of 0.22 μm.

Suspensions of HAP seed crystals were prepared from the commercially available salt (Merck AG) and were characterized by infrared spectrography, which disclosed a spectrum identical to that obtained with HAP crystals prepared according to Nancollas and Mohan [13]. After ultrasonication for 30 min the diameter of the HAP crystals varied up to 12.5 μm, as determined in a Coulter Counter (Model ZBI) with Channelyzer. No attempts were made to measure the crystal surface area.

Crystallization experiments were performed in 50 ml of the crystallization system, to which was added 0.5 ml of [^{14}C]-oxalate (0.5 μCi/ml. The Radiochemical Centre, Amersham, England) and 2 ml of an HAP seed crystal suspension. The samples were kept at 37° C in a water bath with continous magnetic stirring. HAP seed crystals in concentrations of 0.05, 0.5, 5, 10, 25, and 50 mg/ml were used corresponding to concentrations: 0.002, 0.02, 0.2, 0.4, 1.0, and 2.0 mmol/l in the system. A system with 2 ml of CaOx seed crystals (1 mg/ml) resulting in a system concentration of 0.3 mmol/l was run as a control. Within 1 min after addition of seed crystals and then at regular intervals aliquots of 1.5 ml were withdrawn from the system, immediately passed through a Millipore filter with a pore size of 0.22 μm and analysed for isotope content in a liquid scintillation spectrometer.

The inhibitory capacity of citrate and urine on the heterogenous crystallization was studied in systems with 2 mmol/l of HAP seed crystal concentration. Citrate was added to the system to give final concentrations of 0.01, 0.02, and 0.03 mmol/l. Urine from a healthy person was added in concentrations of 1 and 2%.

Crystallization in solutions supersaturated with CaP

In a solution, metastably supersaturated with respect to CaP, the number of crystals and their size distribution was determined in the Coulter Counter.

The crystallization system contained per litre: 4.5 mmol of calcium chloride, 4 mmol of sodium hydrogen phosphate (NaH_2PO_4), 0.15 mol of sodium chloride and 3 mmol of sodium azide (NaN_3). The system was prepared by mixing stock solutions of calcium chloride and sodium hydrogen phosphate. Immediately before mixing they were passed through Millipore filters with a pore size of 0.22 μm.

Crystal counting was performed after 60 minutes in 50 ml of the crystallization system adjusted to pH 7.0 with 1 ml of 0.15 mol/l sodium hydroxide. Each sample in addition contained 1 ml of 0.15 mol/l sodium chloride or 1 ml of dialysed urine and 1 ml of a citrate solution. The final citrate concentration in a total volume of 53 ml thus varied between 0.02 and 1.89 mmol/l. The samples were kept at 37°C in a water bath with continous magnetic stirring until crystal counting.

Crystal size distribution was evaluated by counting the crystals in the three intervals 3.5–5 μm (small crystals), 6.5–14 μm (medium sized crystals), and 15.5–27.5 μm (large crystals).

Calculation of supersaturation

The ion-activity products of CaOx and HAP as well as the crystallization driving force (DG) with respect to these salts were calculated by means of the EQUIL 2 programme [22].

Results

Calcium phosphate precipitation by alkalinization to pH 8 in urines from healthy subjects showed crystal amounts corresponding to a HAP concentration in the range of 0.1–2.2 mmol/l.

Increased amounts of HAP seed crystals in solutions, which were metastably supersaturated with respect to CaOx resulted in decreased concentrations of soluble [^{14}C]-oxalate (Figs. 1 and 2). Addition of CaOx seed crystals started an immediate crystal growth process with less than 50% of the isotope remaining in solution after 8 h. In the absence of seed crystals the solution remained stable for 8 h, but after 24 h crystallization was observed, reducing the isotope concentration to 54%. HAP seed in concentrations up to 0.4 mmol/l caused no significant decrease in soluble isotope during the first 4 h, whereas HAP seed concentrations of 1 and 2 mmol/l reduced the levels of isotope after about 3 and 1 h respectively.

Figure 3 shows the inhibitory effect of 1 and 2% urine concentrations on the HAP induced crystallization of CaOx. Despite a HAP seed concentration as high as 2 mmol/l there was no crystallization demonstrable during the first 4 h. Between 4 and 6 h there was a slight reduction in soluble oxalate, whereby the higher urine concentration appeared to result in a more pronounced inhibition.

Addition of citrate to the system with final concentrations of 0.01, 0.02, and 0.03 mmol/l (Fig. 4) resulted in an inhibition by the latter concentration comparable

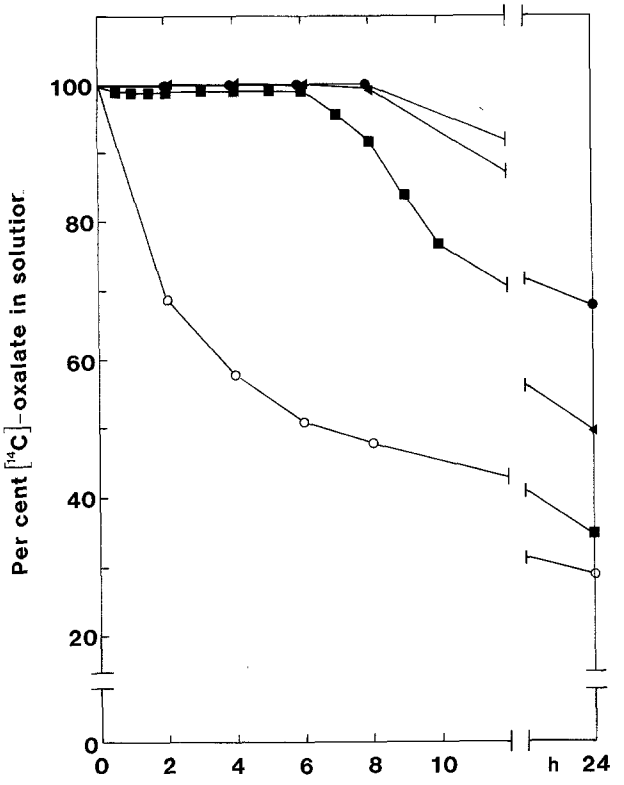
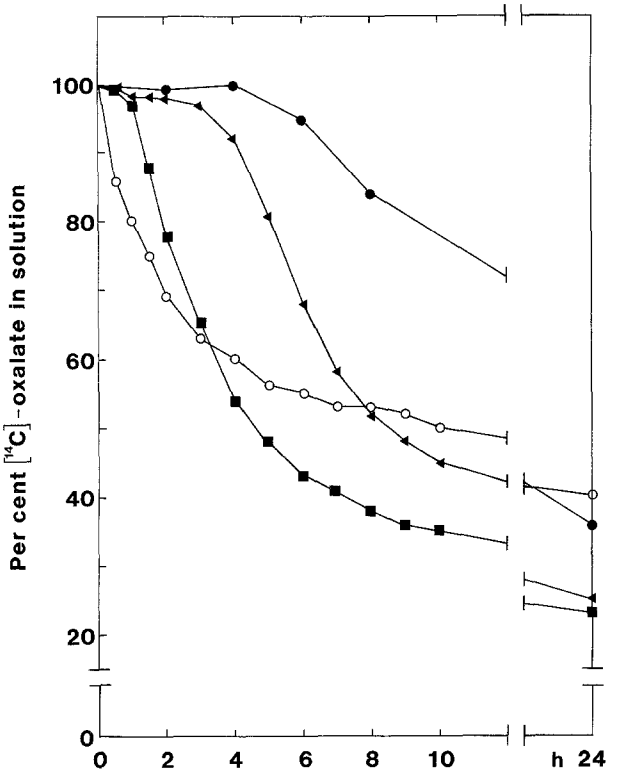


Fig. 1. Per cent of [^{14}C]-oxalate remaining in solution at different times after the addition of seed crystals to a solution, metastably supersaturated with CaOx and saturated with HAP. Seed crystals of HAP in concentrations of 0.002 (●), 0.02 (▲), and 0.2 (■) mmol/l and of CaOx in a concentration of 0.3 mmol/l (○) were used



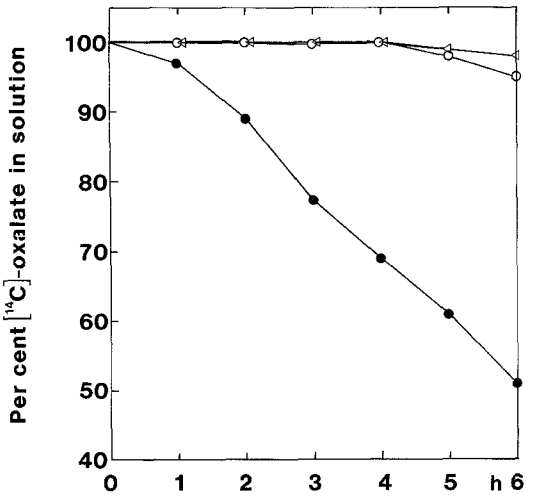


Fig. 3. Per cent of [^{14}C]-oxalate remaining in solution at different times after the addition of HAP seed crystals at a concentration of 2 mmol/l to a solution, metastably supersaturated with CaOx and saturated with HAP. The experiment was performed without urine (●) and with urine in concentrations of 1 (○) and 2 (△) %

to that observed with 1% of urine. Even with a citrate concentration as low as 0.01 mmol/l the crystallization process was retarded.

In a system supersaturated with respect to CaP the total number of crystals was markedly reduced by citrate concentrations exceeding 0.5 mmol/l both in samples without (Fig. 5a) and with dialysed urine (Fig. 5b). This course evidently occurred despite an increased pH-level with increased concentrations of citrate. There was also a shift in crystal size distribution with a sharp reduction of medium sized and large crystals and a corresponding increment in the fraction of small crystals (Fig. 6). The presence of dialysed urine in concentrations of about 2% had no significant influence on the effect of citrate on crystal size distribution in these experiments.

A standardized amount of sodium hydroxide was added to the samples aiming at a pH of 7.0. Measurement after 60 minutes disclosed an increasing pH with increasing concentrations of citrate. Thus the pH in solutions with no or low concentrations of citrate was 6.9–7.0. The highest citrate concentrations were associated with pH values as high as 7.3. Whereas the driving force (DG) for HAP decreased from 8.02 to 7.75 in the citrate concentration range of 0 to 0.001 at a pH of 7.0 the corresponding range calculated for the

Fig. 2. Per cent of [^{14}C]-oxalate remaining in solution at different times after the addition of seed crystals to a solution, metastably supersaturated with CaOx and saturated with HAP. Seed crystals of HAP in concentrations of 0.4 (●), 1.0 (▲), and 2.0 (■) mmol/l and of CaOx in a concentration of 0.3 mmol/l (○) were used

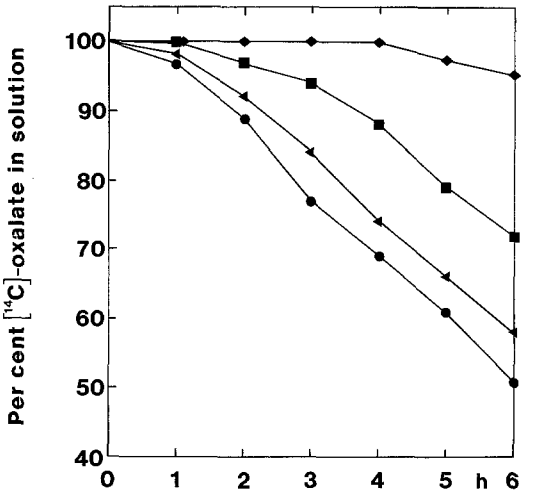


Fig. 4. Per cent of [^{14}C]-oxalate remaining in solution at different times after the addition of HAP seed crystals at a concentration of 2 mmol/l to a solution, metastably supersaturated with CaOx and saturated with HAP. The experiment was performed with citrate in concentrations of 0.01 (▲), 0.02 (■), and 0.03 (◆) mmol/l and without citrate (●)

pH values measured after 60 min was 7.71 to 8.35. Although the pH might vary slightly during the process of CaP precipitation, the higher citrate concentrations which were assumed to reduce the driving force, in fact might have increased the supersaturation. However, despite this the number of crystals and the fraction of large crystals decreased.

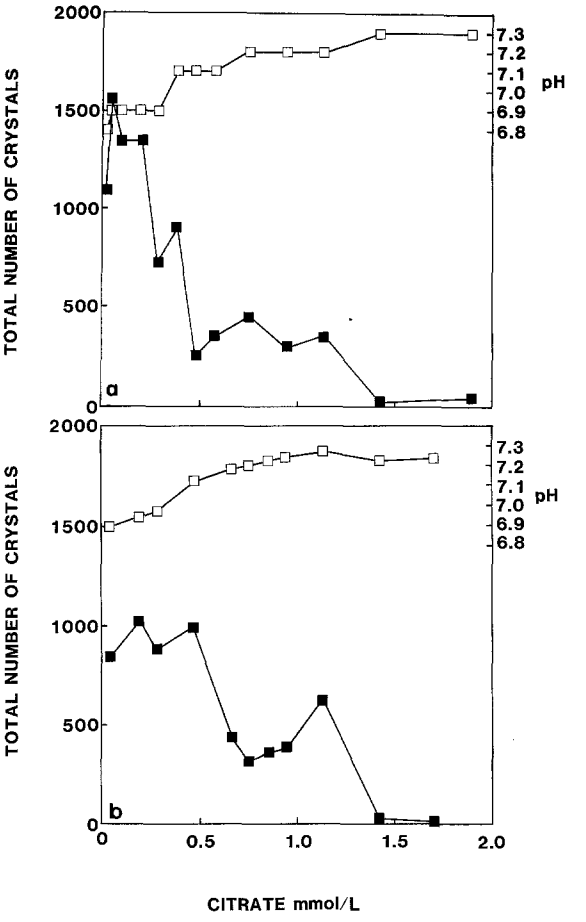


Fig. 5a and b. Total number of CaP crystals (■) 60 min after supersaturation with respect to CaP at different concentrations of citrate in a system without (*a*) and with (*b*) dialysed urine. The pH (□) was measured at the time of crystal counting

Discussion

The crystallization of CaOx is thought to play a central role in the formation of a majority of calcium containing renal stones. However, the frequent concomitant occurrence of CaP, usually in the form of HAP, clearly indicates that in a large number of patients the precipitation of both salts has to be considered.

The common use of prophylactic treatment with alkaline citrate [4, 6, 16], from a thermodynamic point of view, theoretically should result in a considerable crystallization of CaP. Therefore, increased knowledge of the effects of CaP on CaOx crystallization is of utmost importance.

Alkalinization of urine to a pH above 5.5 results in a reduced CaOx crystallization risk [1], which occurs at the expense of an increased formation of CaP crystals, at least for a pH above 6.5. Although alkaline citrate appears beneficial in preventing CaOx crystallization it might imply a risk of heterogenous crystallization of CaOx on CaP. Addition of seed crystals of HAP to our system, metastably supersaturated with CaOx did not result in a heterogenous crystallization until the amount of seed exceeded that corresponding to a HAP concentration of 0.2 mmol/l. With this amount of seed there was a long induction period and apparently the crystallization did not start until after 6 h. However, a more rapid crystallization was recorded at higher concentrations of HAP crystals. In comparison with the rapid growth of CaOx seed crystals at concentrations of 0.3 mmol/l, HAP crystals corresponding to a concentration of 2 mmol/l required an induction period of about 1 to 1.5 h to start a heterogenous crystallization.

A previous study on the ability of seed crystals of HAP and COM to induce epitaxial growth of COM crystals from a metastable solution supersaturated with CaOx showed that the rate of growth was dependent on the surface area of the seed material [11]. Comparison on the basis of surface area showed that the rate of growth on COM seed was 100 times higher than on HAP seed, whereas on a weight basis the rate was only 10 times higher. This indicates that a small

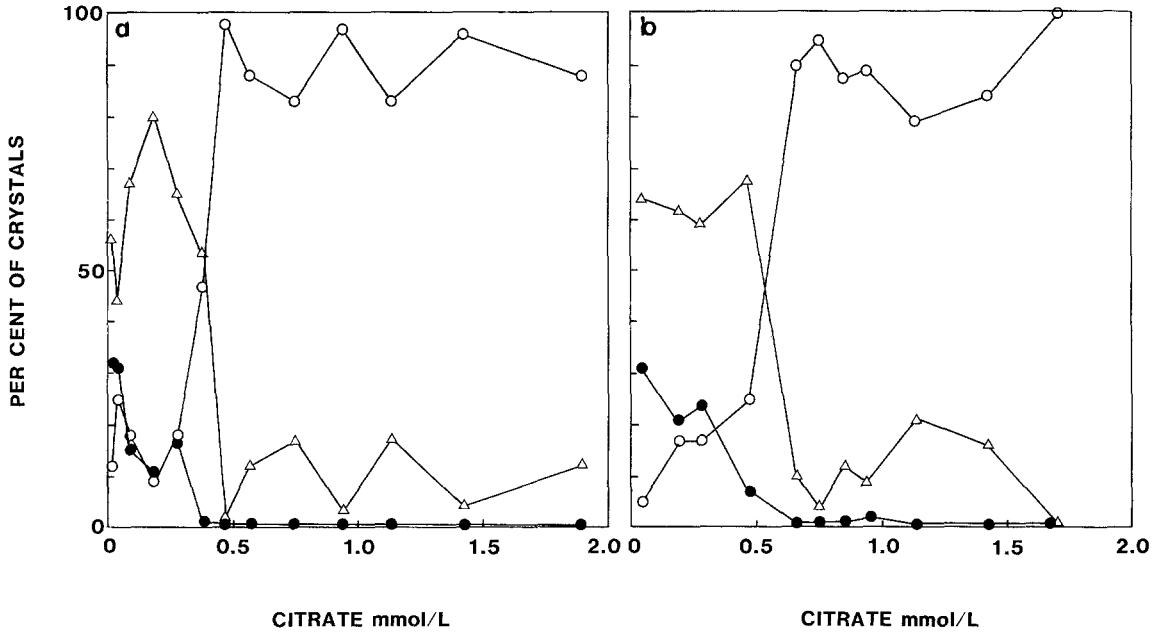


Fig. 6a and b. Crystal size distribution 60 min after supersaturation with respect to CaP at different concentrations of citrate in a system without (*a*) and with (**b**) dialysed urine. The ranges of crystal size were 3.5–5 (○), 6.5–14 (△), and 15.5–27.5 (●) μm

mass of HAP presents a relatively large surface for heterogenous nucleation compared with COM and that COM has a much greater number of sites for nucleation than HAP on the same surface area. Although we made no attempts to determine the surface area of the HAP and CaOx seed crystals, the results in our crystallization system are in agreement with these observations. It is possible that a smaller amount of HAP crystals might be sufficient for heterogenous crystallization at higher levels of CaOx supersaturation, but even with the highest HAP concentrations in our experiment the lag phase was considerable, even in the absence of inhibitors.

Nevertheless there is a heterogenous crystallization of CaOx on HAP and its importance in stone formation cannot be completely disregarded. The risk of this heterogenous crystallization might be particularly pronounced with seed crystals fixed to a surface of the collecting system. Although the amount of CaP precipitated from urine samples varied considerably the largest amount did correspond to the level of HAP crystal concentration used in our experiments. Therefore, urine from hypercalciuric subjects will certainly produce larger amounts of crystals with shorter induction periods. However, our results show that citrate at low concentrations and urine in concentrations of only about 1% were able to inhibit heterogenous crystallization of CaOx on HAP seed crystals for as long as 4 h.

The inhibition experiments were performed in a metastably supersaturated solution following addition of citrate in low concentrations. The citrate addition corresponded to concentrations at about 1% of that in normal urine. At higher concentrations the crystallization might be seriously affected by complex formation between calcium and citrate, markedly influencing the ion-activity product of CaOx. However, the inhibiting potential of citrate is apparently very high and extrapolation to whole urine concentrations between 1 and 2 mmol/l shows that about 60 to 120 mmol of HAP per litre would be protected from a heterogenous CaOx deposition at the same supersaturation level and crystal size as in our system. Although this relationship certainly is not linear it might be sufficient to conclude that the increased citrate concentration during treatment with alkali in addition to a favourable effect on supersaturation will provide a significant inhibitory activity regarding CaOx growth on HAP, even with smaller crystals than those in our seed preparation. In addition, other constituents of urine may also contribute to such an inhibitory effect.

It has been shown that about 50% of the inhibiting capacity of whole urine as regards CaP precipitation is attributable to citrate [2], and that it is likely that the inhibitory action depends on binding of the inhibitor to sites of growth on the surface of the seed crystals. There is also previous experimental evidence that increments in urinary citrate concentration of 40–50% will reduce CaP crystal formation at pH 6.8 by 42% [5].

Our results indicate that a major part of the inhibition of CaOx crystallization on HAP in urine is brought about by citrate.

It needs to be emphasized that an aggregation between CaOx and HAP crystals by no means has been excluded but according to our previous findings the risk of CaOx crystallization is low at high pH levels [1]. Theoretically there might also be a risk of CaOx induced crystallization of CaP in samples supersaturated with respect to the latter salt. Previous experiments have shown that this probably is an unlikely event in urine [8,11]. Although we performed some experiments to demonstrate this the metastable zone in our crystallization system was too narrow to give reproducible results.

The findings in our supersaturated CaP system show that CaP precipitates even in the presence of citrate, but at a markedly reduced rate with fewer crystals of which those of small size predominated at citrate concentrations corresponding to the level found in normal urine. This might be explained by a combined effect of a formation of citrate-calcium complexes, an inhibited crystal growth for CaOx and a reduced crystal agglomeration, which was recently shown for CaOx crystals [7].

The risk associated with alkalinization of urine appears to be of importance only at pronounced CaP precipitation, which might occur at very high levels of CaP supersaturation and especially with fixed crystals. In alkaline urine crystals of CaP will form as an effect of a pH-determined CaP supersaturation. These crystals appear to be of small size in the presence of citrate in normal concentrations. An increased citrate concentration will also favourably affect supersaturation with CaOx as well with CaP. Inhibitors in urine, of which citrate might be one of the most important, prevent the heterogenous growth of CaOx on HAP. In most patients with calcium stone disease it therefore appears to be safe to continue treatment with alkaline citrate provided they are regularly and carefully supervised. However, it is probably wise to reduce excessive calcium excretion during this form of treatment.

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Dr. Hans-Göran Tiselius
Department of Urology
University Hospital
S-58185 Linköping
Sweden