Urol Res (1998) 26:89–95

ORIGINAL PAPER

Inge Højgaard · Hans Göran Tiselius

The effects of citrate and urinary macromolecules on the aggregation of hydroxyapatite crystals in solutions with a composition similar to that in the distal tubule

Received: 6 March 1997 / Accepted: 22 October 1997

Abstract The effects of citrate and dialysed urine (dU) on the aggregation of hydroxyapatite (HAP) crystals in solutions with different pH and otherwise with an ion composition assumed to correspond to that in the distal part of the distal tubule were studied by spectrophotometric assessment of the rate of crystal sedimentation. When the concentration of dU was increased from 1% to 20% we recorded an increased inhibition of HAP crystal aggregation at pH 6.5. There were no differences in the inhibition accomplished by 10% dU when the pH was varied between 5.5 and 7.0, but a lower inhibition was recorded at pH 7.5. Citrate in the range 0.05–4 mmol/l had a concentration-dependent inhibitory effect on HAP crystal aggregation. In the presence of 10% dU the net inhibitory effect of citrate was reduced at all pH levels. In the pH interval 5.5–7.0 a higher inhibition was recorded with 0.5 mmol/l citrate than with 10% dU, but in the presence of dU there was only a minor additional effect of citrate at concentrations below 0.5 mmol/l. These findings indicate that urinary macromolecules present in dU strongly inhibit HAP crystal aggregation under solution conditions corresponding to those in distal tubular urine. At the same nephron level citrate might have a direct inhibitory influence on the aggregation of HAP crystals, but in the presence of normal urinary macromolecules the additive inhibitory effect of citrate is probably only marginal.

Key words Aggregation · Citrate · Dialysed urine · Hydroxyapatite · Inhibition · pH

I. Højgaard (✉) · H. G. Tiselius
Department of Urology, Clinical Research Center,
Faculty of Health Sciences, University Hospital,
S-581 85 Linköping, Sweden, fax: +46 13224574

Introduction

The formation of stones composed of a mixture of calcium oxalate (CaOx) and calcium phosphate (CaP) is a complex process that apparently requires several risk factors such as a high supersaturation with respect to the involved salts, a pH level that favours the crystallization, an increased promotion or a reduced inhibition of nucleation, crystal growth, crystal aggregation and crystal retention. It has been claimed that an enhanced CaOx crystal aggregation is thereby one of the most important factors [15, 17, 27, 29], and it has been shown that calcium stone formers excrete larger crystals and crystal aggregates than normal subjects [26, 40].

The influence of citrate and urinary macromolecules on CaOx crystal aggregation has been thoroughly examined [2, 7, 10, 15–18, 28, 29, 36, 37]. A direct effect of citrate on CaOx crystal aggregation in supersaturated solutions was recorded by Kok and co-workers [15, 17] as well as by Tiselius and co-workers [37]. It also was reported that citrate reduced the sedimentation rate of brushite and hydroxyapatite [36]. Although unable to demonstrate a direct effect of citrate on the aggregation of CaOx monohydrate crystals in equilibrated solutions [7], Hess and co-workers [10] showed that citrate in a concentration of 3.5 mmol/l converted a highly polymerized and aggregation-promoting Tamm-Horsfall glycoprotein (THP) from stone formers to an inhibitor of CaOx monohydrate crystal aggregation.

Urinary macromolecules are probably more important than citrate in regulating CaOx crystal aggregation [3, 7, 14, 16, 30, 33, 35] and their crystallization-modifying properties can be augmented or attenuated by physico-chemical changes [6, 9].

Although CaP is a major constituent of most calcium stones [19, 23], little attention has been directed towards factors of importance for the aggregation of CaP crystals. In view of the possibility that CaP might be the primary crystal phase in such stones [13, 22, 24], an increased knowledge of how the aggregation of CaP

crystals is modified might be important for a better understanding of how to prevent recurrent calcium stone formation.

It has been shown previously that CaP is the salt that most easily might form in the distal tubule [11, 20] as well as in the loop of Henle [1, 4] and that CaP crystals can induce nucleation of CaOx in the collecting duct [13, 38, 39]. On the basis of these findings this experimental study was undertaken to assess the influence of citrate, urinary macromolecules and pH on the sedimentation rate of hydroxyapatite crystals in solutions with an ion composition assumed to correspond to that in the distal part of the distal tubule.

Materials and methods

To a salt solution (A) that per litre contained 8.34 mmol phosphate, 0.08 mmol oxalate and 0.70 mmol citrate, hydroxyapatite crystals (HAP; Fluka Chemie, Buchs, Switzerland) were added to give a seed concentration of 1 mg/ml. The pH of solution A was adjusted to 5.5, 6.0, 6.5, 7.0 or 7.5. The pH was measured with a pHM 84 pH meter (Radiometer, Copenhagen, Denmark). A pH of 6.5 was assumed to represent the average pH in urine from the distal part of the distal tubule (DTd) [25]. We also prepared solutions without citrate but otherwise with the same composition as above. Following adjustment of the pH, the seeded solution A was placed in an ultrasound bath for 60 min and stored at least overnight. Another salt solution (B) with 2.08 mmol calcium, 0.82 mmol magnesium and 27.6 mmol sulphate per litre was prepared and pH-adjusted as above.

Immediately before assessing the crystal sedimentation, the pH of suspension A was checked and if necessary adjusted, after which the suspension was subjected to ultrasonication for 30 min. Following addition of dialysed urine (dU) or citrate, the two solutions A and B were mixed in a cuvette to produce a HAP-DTd solution with the following ion concentrations (mmol/l): calcium 1.04, magnesium 0.41, phosphate 4.17, oxalate 0.04, sodium 96, potassium 22.5, sulphate 13.8. The HAP seed concentration was 0.5 mg/ml.

Pooled dialysed bladder urine (dU) from normal men was used as a source of macromolecules; we ignored the fact that certain macromolecules might have been added to the urine as a result of its passage and storage in the lower parts of the urinary system. Urine was collected between 2200 and 0600 hours with sodium azide as preservative, and screened to exclude the presence of bacteria, protein and glucose before inclusion in the pool. The dialysis was carried out as described previously [20]. We did not quantify proteins in the dU, because the purpose of the experiment was to examine the relative effect that different concentrations of macromolecules from normal men had on the aggregation of HAP crystals.

The effect of dU on aggregation of HAP crystals was examined in HAP-DTd suspensions with citrate in a concentration of 0.35 mmol/l and a pH of 6.5. dU was added to give final concentrations of 1%, 2.5%, 5%, 10%, 15% and 20%. How pH in the range 5.5–7.5 affected the inhibitory properties of urinary macromolecules was assessed in HAP-DTd suspensions containing 10% dU without citrate.

Experiments also were carried out to study the effect of citrate on HAP crystal aggregation in the presence of 10% dU and in the absence of dU. To HAP-DTd suspensions without citrate and with or without dU, citrate was added to give final concentrations of 0.05, 0.1, 0.2, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 3.0 and 4.0 mmol/l. The sedimentation rate was assessed at pH 5.5, 6.0, 6.5, 7.0 and 7.5.

The aggregation of HAP crystals was derived from the rate of sedimentation in the HAP-DTd suspension mainly according to the principles described by Hess and coworkers [7, 37]. For this purpose 1.5-ml aliquots of the sample were transferred to a cuvette in a spectrophotometer (Perkin-Elmer Lambda 2) in which magnetic stirring at a rate of 1100 r.p.m. was started. The absorbance at 690 nm was recorded during 5 min of continuous stirring. The inhibition of aggregation (per cent) was calculated by comparing after 300 s the absorbance in a sample containing the test compound with that in a sample without the test compound (Fig. 1). The theoretical inhibition of aggregation of 100% thereby corresponded to the initial absorbance.

All experiments were carried out at least in triplicate.

To study the possibility of crystal growth in the supersaturated DTd solution we recorded the number, volume and size distribution of crystals in the size interval 2.4–45 μm in a Coulter Multiziser with a 100-μm capillary tube (Coulter Electronic, Luton, UK). These recordings were made in HAP-DTd suspensions without citrate and without dU in the pH interval 5.5–7.5. The mean crystal volume (MCV) was calculated as the quotient between the total crystal volume and the total number of crystals.

Statistical analysis

Wilcoxon's rank sum test was used for group comparison.

Results

A statistically significant increase both in the number of crystals and in the total crystal volume was recorded when the pH was increased, with the exception that there was no significant difference between samples with a pH of 6.5 and 7.0 (Fig. 2). As could be expected there was pronounced crystallization at the highest pH levels, but the crystal size distributions were similar with about 95% of the crystals recorded in the size interval 2.4–4.4 μm and about 5% in the size interval 4.4–10.0 μm, irrespective of the pH. The median MCV varied from 18 to 22 μm^3 with the lowest values recorded at pH 6.5 and 7.0. There were, however, no differences between MCV values at pH 5.5, 6.0 and 7.5.

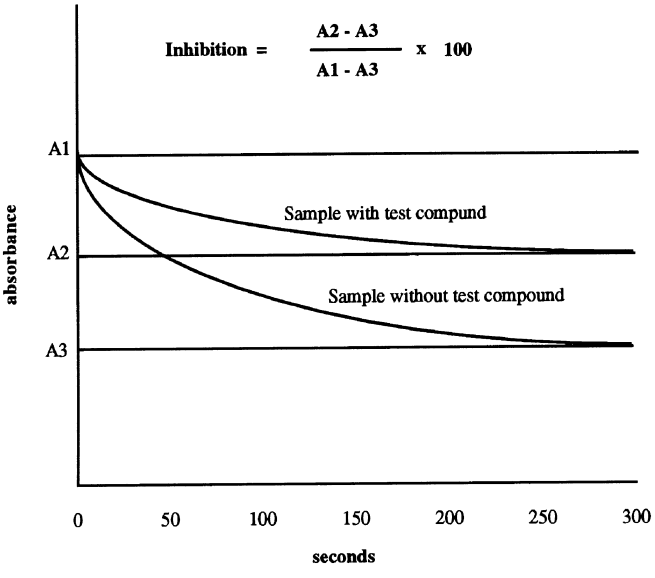

Fig. 1 Principles for calculation of the inhibition of aggregation from the absorbance recorded at 690 nm after 300 s

When the concentration of dU was increased in HAP-DTd suspensions with a pH of 6.5 and 0.35 mmol/l of citrate, an increased inhibition of crystal aggregation was recorded with concentrations of dU above 5% (Fig. 3). The median (range) inhibition of crystal aggregation in the presence of 1%, 2.5% and 5% dU was 4.5 (3.71–19.5), 7.9 (5.69–19.8) and 12.0 (3.34–19.5), respectively ($P > 0.05$). Neither was there any statistically significant difference between the inhibition recorded in the presence of 10% and 15% dU [median (range): 36.0 (25.1–47.6) and 45.0 (38.8–54.5), respectively; $P > 0.05$]. The inhibition recorded with 10% and 15% dU was, however, significantly higher than that in samples with 1%, 2.5% and 5% dU ($P < 0.05$) and significantly lower than the inhibition of 56.0 (47.5–71.9) recorded with a dU concentration of 20% ($P < 0.05$).

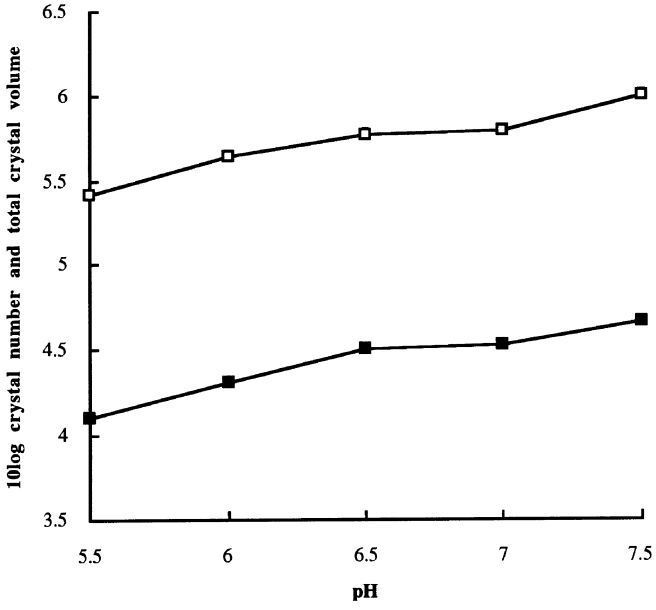

Fig. 2 The relationship between the pH and the 10 log crystal number (*filled squares*) and volume (*open squares*) in hydroxyapatite–distal tubule (HAP-DTd) suspensions without citrate and dialysed urine (dU)

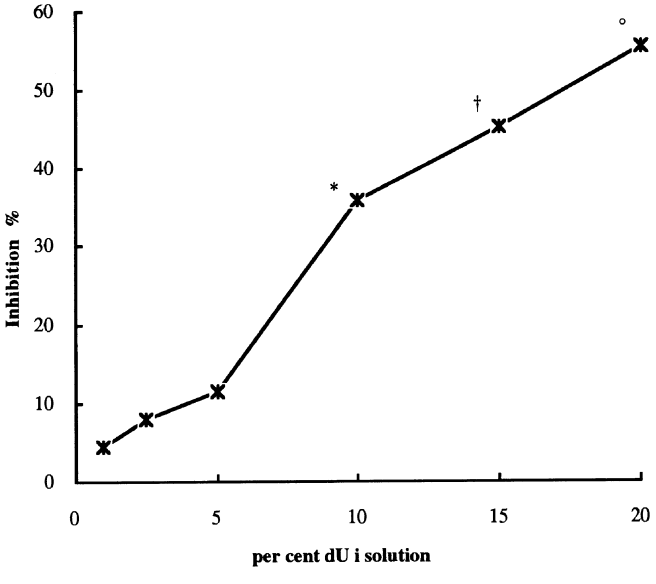

Fig. 3 The inhibition of crystal aggregation recorded at different concentrations of dU; $*P < 0.05$ when 10% dU was compared with 1%, 2.5%, 5% and 20% dU; $\dagger P < 0.05$ when 15% dU was compared with 1%, 2.5%, 5% and 20% dU; $^{\circ}P < 0.05$ when 20% dU was compared with 1%, 2.5%, 5%, 10% and 15% dU

The presence of citrate in HAP-DTd suspensions without dU brought about an increased inhibition of HAP crystal aggregation (Fig. 4). This increase was most pronounced with concentrations of citrate up to 2 mmol/l. At citrate concentrations between 0.05 and 0.2 mmol/l there was only a small effect on the inhibiting activity when pH was varied between 5.5 and 7.0. In the concentration range from 0.05 to 1.5 mmol/l the inhibition recorded in samples with a pH of 7.5 was significantly lower ($P < 0.05$) than that observed at the other pH levels. For samples containing citrate in concentrations between 0.75 and 4.0 mmol/l the inhibition of crystal aggregation increased when the pH was reduced from 7.0 to 6.0 ($P < 0.05$). The inhibition of HAP crystal aggregation thus apparently increased with increased concentrations of citrate. There was a similar pattern in terms of citrate effects in the pH range 6.0–7.0, but at pH 7.5 the values were significantly lower for citrate in concentrations up to 1.5 mmol/l.

The addition of 10% dU to HAP-DTd suspensions without citrate (Fig. 5) resulted in an inhibition of HAP crystal aggregation of between 29% and 41%, with a significantly lower inhibition at pH 7.5 than at 5.5–7.0. Whereas the inhibition of HAP crystal aggregation varied between 48% and 57% in solutions without dU, at a citrate concentration of 0.5 mmol/l and a pH of 5.5–7.0, the presence of 10% dU resulted in an inhibition

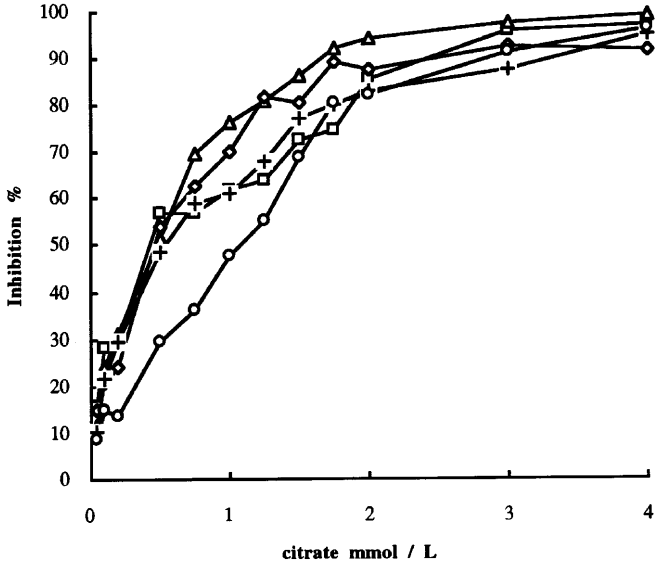

Fig. 4 The inhibition of crystal aggregation recorded in samples without dU but with different concentrations of citrate at pH 5.5 (*squares*), 6.0 (*triangles*), 6.5 (*diamonds*), 7.0 (*crosses*) and 7.5 (*circles*)

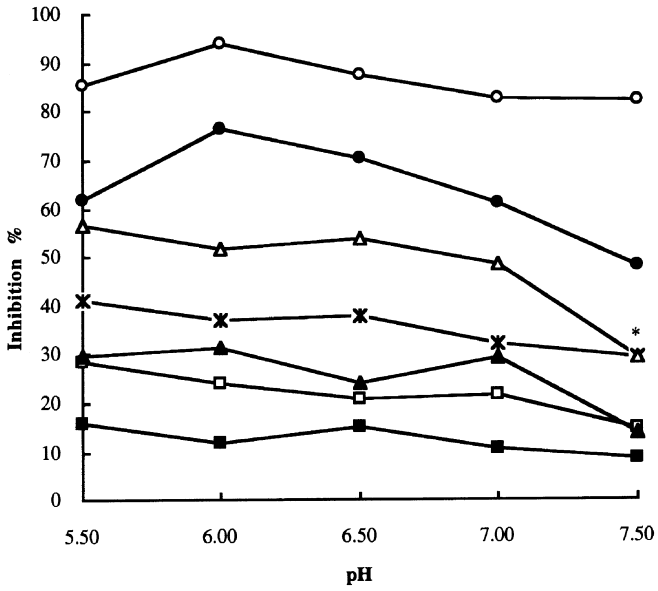

Fig. 5 The inhibition of crystal aggregation recorded in HAP-DTd suspensions without dU at different pH levels and with citrate in concentrations of: 0.05 mmol/l (*filled squares*), 0.1 mmol/l (*open squares*), 0.2 mmol/l (*filled triangles*), 0.5 mmol/l (*open triangles*), 1 mmol/l (*filled circles*), 2 mmol/l (*open circles*); and in HAP-DTd-suspensions without citrate and 10% dU (*crosses*). $*P < 0.05$ difference between citrate-free samples with 10% dU at pH 7.5 and samples with pH 5.5–7.0

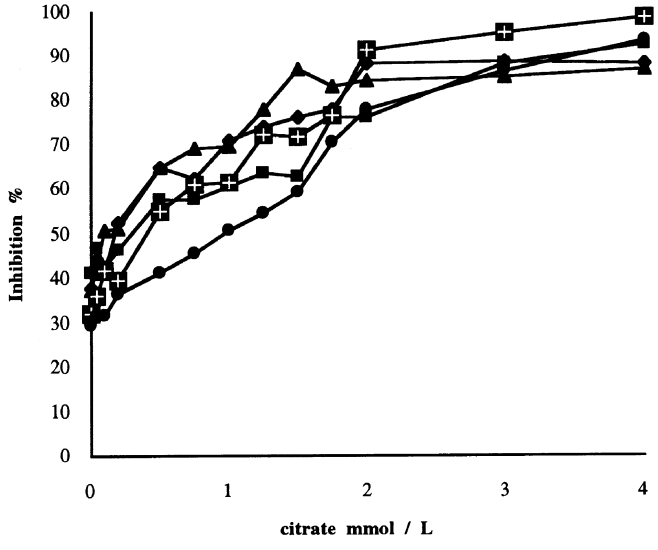

Fig. 6 The inhibition of crystal aggregation recorded in samples with 10% dU and with different concentrations of citrate at pH 5.5 (*squares*), 6.0 (*triangles*), 6.5 (*diamonds*), 7.0 (*crosses in squares*) and 7.5 (*circles*)

between 55% and 65% (Fig. 6). This result should be considered in light of the inhibition of between 32% and 41% achieved with solutions containing 10% dU but no citrate. The net effect of citrate in solutions also containing dU was thus only 23–24% as compared with 48–57% in the dU-free solutions. In solutions with a pH of 7.5 the inhibition recorded with either 10% dU alone or 10% dU together with citrate in concentrations up to 1.5 mmol/l was much lower than in solutions with lower pH levels (Figs. 4, 6). In the presence of dU, together with citrate in concentrations above 2.0 mmol/l, no important effects of pH on the inhibition of aggregation were demonstrated.

Discussion

It needs to be emphasized that the DTd solutions used in these experiments will reflect only roughly the solution environment in the distal part of the distal tubule. Our knowledge of the average composition of DTd urine is incomplete and furthermore most certainly subject to considerable intraindividual and interindividual variation. dU, which was used as a source of macromolecules in our system, was prepared from bladder urine and no attention was paid to the fact that some macromolecular species might thereby have been added to the urine at levels below the distal tubules during its passage and storage. We made the probably oversimplified assumption that macromolecules of importance for the aggregation of CaP crystals appeared in the dU sample at relative concentrations corresponding to those in the distal tubule. The activity and composition of the urinary macromolecules in the dU might also have been affected by the preparation technique [29]. Without knowledge of which macromolecule or macromolecules are of importance for CaP crystal aggregation there is, however, no easy way to address this problem. There is presently a considerable amount of knowledge emerging on the role urinary proteins, glycoproteins and glycosaminoglycans in calcium salt crystallization, and it is likely that the future will provide us with better techniques for isolation of the most relevant macromolecules in this respect.

Our DTd solutions were also supersaturated with CaP. The perfect experimental condition for isolated measurement of crystal aggregation is undoubtedly a solution at equilibrium as used by Hess and co-workers [10]. Such a system also will reflect the in vivo situation provided the crystals move through the nephron in the same urine bolus in which they were nucleated. We assume, however, that crystals might be retained and thus move at a slower speed than the urine and, if so, the equilibrated solution does not reflect the crystallization conditions met in the distal tubule. The drawback of using a supersaturated DTd solution is the obvious risk of having a growth process simultaneous with the aggregation. If this occurs to a significant extent conclusions regarding crystal aggregation will be incorrect.

Approximately 90% of the crystal sedimentation was completed within the first 100 s and it is not likely that crystal growth, which is a fairly slow process, started so rapidly that it would have affected the sedimentation rate, at least not in samples with a pH below 7.0. Although the risk of growth cannot be neglected at pH levels as high as 7.5 or 7.0, analysis of the crystal size

distribution suggested that new crystal formation (nucleation) rather than crystal growth accounted for the pH-related increment in total crystal volume in the most alkaline samples. Definitive proof is, however, difficult to provide because of our inability to detect crystals with a diameter below 2.4 μm. Our preliminary data (unpublished) on the rate of crystallization in seeded DTd solutions as measured with an isotope technique gave support to our assumption that the crystallization of CaP was a slow process under the experimental conditions we used. Although some degree of crystal growth cannot be excluded, we believe that crystal aggregation was the major process accounting for the observations described in this paper and that the designation "inhibition of aggregation" was therefore appropriately used.

Commercial HAP crystal might behave differently from CaP crystals formed in urine and this should be kept in mind when the results are interpreted. The reason for not using CaP crystals produced in urine, as was done previously [38, 39], was a desire to have a standard batch of seed crystals that was unaffected by differences in the concentration of solutes and macromolecules.

Our experiments show that urinary macromolecules strongly reduce the sedimentation rate of HAP crystals in solutions with a composition similar to that in the distal tubule and thus might act as important inhibitors of aggregation. In a recent study with volume reduction by evaporation technique, we found that increased concentrations of urinary macromolecules (dialysed urine) at pH 6.45 under conditions similar to those in the distal tubule resulted in an increased number of small crystals, but there was no difference between the results obtained with 10% and 20% dU [11]. In the present study, however, we found a significant increase in the inhibitory effect on the aggregation of HAP crystals in the DTd solution when the concentration of dU was increased from 10% to 20%. The effect of volume reduction on the urinary macromolecules might to some extent explain this, inasmuch as the evaporation process resulted in a much higher concentration of dU and a much higher ion strength in the solutions, particularly in the most concentrated samples. It has previous been shown that the effect on CaOx crystal aggregation of nephrocalcin and Tamm-Horsfall protein (THP) is affected by such physico-chemical changes [7, 31, 32]. When solutions with an ion composition assumed to correspond to that in the distal tubule were evaporated, a pH reduction from 6.45 to 5.8 resulted in an increased number of small crystals [12]. This is in accordance with the present results, where an increased inhibitory effect of citrate was observed when pH was reduced from 6.5 to 6.0 at citrate concentrations above 0.50 mmol/l. This also was found in the concentration range of citrate between 0.1 and 0.5 mmol/l when the pH was reduced from 6.5 to 5.5. There was no important difference in the inhibitory effect of 10% dU when the pH was reduced from 6.5 to 6.0 or 5.5, whereas a pH increment to 7.5 resulted in a statistically significant decrease in the inhibitory effect of 10% dU. Following volume reduction of solutions containing 20% dU, a pH increase from 6.45 to 7.28 also generated a greater mean crystal volume [12].

The dissociation of citrate increases with increasing pH and it has been demonstrated that the inhibition of CaOx crystal growth increased when the pH was increased [34]. An increased inhibition of aggregation would thus be expected when the pH is increased. In contrast a slightly higher inhibition was recorded with citrate at the lower pH levels. With the exception of the results recorded at pH 7.5 the differences were generally small and probably without clinical significance. They can probably be explained at least to some extent by a slight HAP crystal dissolution at the lower pH levels due to decreased CaP supersaturation.

The inhibitory effect of citrate on HAP crystal aggregation was less pronounced in the presence of 10% dU, but it should be noted that 10% dU resulted in a considerable inhibition. The fact that citrate had a more pronounced inhibitory effect on the aggregation in the lower concentration range up to 0.5 mmol /l in dU-free solutions compared with solutions containing dU might be due to competition between citrate and urinary macromolecules for binding sites on the HAP crystals. Inasmuch as the concentration of 10% dU resulted in a pronounced inhibition of HAP crystal aggregation, low concentrations of citrate may therefore have a negligible effect in the presence of normal macromolecular inhibitors of aggregation. These findings are in accordance with the effect of citrate and dU on the aggregation of CaOx crystals reported by Tiselius and co-workers [37].

There was no major difference in the inhibitory effect on HAP crystal aggregation of dU and citrate in the pH interval between 5.5 and 7.0. Raising the pH to 7.5, however, resulted in a pronounced reduction in the inhibition brought about by both dU and citrate. The lower inhibition recorded at pH 7.5 can probably be explained by a nucleation of CaP at this pH level. Such a crystallization makes comparison with the aggregation at other pH levels impossible. In a previous study on the effect of THP on CaOx crystal aggregation Hess and coworkers [7] found that the inhibitory effect of THP was reduced when the pH was decreased from 7.2 to 5.7. In our experiments we used dU as a source of urinary macromolecules, and although THP is one of the main macromolecules in urine and also a strong inhibitor of CaOx crystal aggregation [7], there is no doubt that urinary macromolecules other than THP play a great role in modifying the crystallization of both CaP and CaOx [5].

The normal concentration of citrate in the distal tubule can be assumed to be around 0.35 mmol/l [20], and the concentration of urinary macromolecules would correspond to a dU concentration of approximately 10–20%. Citrate apparently does not play a large role in the inhibition of CaP in the distal tubule provided the concentration and function of urinary macromolecules are normal. It has, however, been shown that urinary macromolecules might be structurally and functionally

abnormal in recurrent stone formers [8, 21]. Hess and coworkers [10] found that citrate in a concentration of 3.5 mmol/l could change abnormal aggregation-promoting THP to inhibitory THP. Citrate therefore possibly has a more important role in distal tubular urine of stone formers with abnormalities in their aggregation-protective macromolecules. From a therapeutic point of view it seems reasonable to increase a low concentration of citrate to a level that exceeds 0.5 mmol/l in the distal tubule, inasmuch as it had an additive inhibitory effect to that of dU on CaP crystal aggregation when the concentration was increased above 0.5 mmol/l. This citrate concentration in DTd would roughly correspond to a citrate concentration of 3 mmol/l in final urine. If we assume that CaP is the primary nucleus in the development of mixed calcium stones it thus might be of value to raise the citrate concentration in the distal tubule, to counteract the aggregation of CaP crystals and in that way get smaller CaP crystals that can easily dissolve or pass before heterogeneous nucleation of CaOx is induced. At a pH of 7.5 nucleation of CaP might counteract the beneficial effect on the aggregation inhibition brought about by a higher citrate excretion. How this influences the further course of calcium stone formation is not known, but an extreme alkalinization probably should be avoided.

The experimental findings reported above suggest that urinary macromolecules in dU act as efficient inhibitors of HAP crystal aggregation. Although a direct effect on crystal aggregation was recorded also with citrate, the latter effect is probably marginal in the presence of normal macromolecular inhibitors.

Acknowledgements This study was supported by grants from the Wibergs Foundation and the Foundation of the Linköping Medical Society. The skilful technical assistance of Mrs. Anne-Marie Fornander and Mrs. Mari-Anne Nilsson is highly appreciated.

References

1. Asplin J, Mandel NS, Coe FL (1996) Evidence for calcium phosphate supersaturation in the loop of Henle. Am J Physiol 270: F604
2. Azoury R, Robertson WG, Garside J (1987) Observations on in vitro and in vivo calcium oxalate crystalluria in primary calcium stone formers and normal subjects. Br J Urol 59: 211
3. Coe FL, Parks JH, Nakagawa Y (1991) Protein inhibitors of crystallization. Semin Nephrol 11: 98
4. Deganello S, Asplin J, Coe FL (1990) Evidence that the tubule fluid in the thin segment of the loop of Henle normally is supersaturated and forms poorly crystallized hydroxyapatite that can initiate renal stones. Kidney Int 37: 472
5. Doyle IR, Marshall VR, Dawson CJ, Ryall RL (1995) Calcium oxalate crystal matrix extract: the most potent macromolecular inhibitor of crystal growth and aggregation yet tested in undiluted human urine in vitro. Urol Res 23: 53
6. Grases F, Millan A, Söhnel O (1992) Role of agglomeration in calcium oxalate monohydrate urolith development. Nephron 61: 145
7. Hess B, Nakagawa Y, Coe FL (1989) Inhibition of calcium oxalate monohydrate crystal aggregation by urine proteins. Am J Physiol 257: F99
8. Hess B, Nakagawa Y, Parks JH, Coe FL (1991) Molecular abnormality of Tamm-Horsfall glycoprotein in calcium oxalate nephrolithiasis. Am J Physiol 260: F569
9. Hess B (1992) Tamm-Horsfall glycoprotein: inhibitor or promoter of calcium monohydrate crystallization processes? Urol Res 20: 83
10. Hess B, Zipperle L, Jaeger P (1993) Citrate and calcium effects on Tamm-Horsfall glycoprotein as a modifier of calcium oxalate crystal aggregation. Am J Physiol 265: F784
11. Højgaard I, Fornander AM, Nilsson MA, Tiselius HG (1996) Crystallization during volume reduction of solutions with an ion-composition corresponding to that in the distal tubuli. Scanning Microsc, 10: 487
12. Højgaard I, Fornander AM, Nilsson MA, Tiselius HG (1996) The effect of pH changes on the crystallization of calcium salts in solutions with an ion-composition corresponding to that in the distal tubule. Scanning Microsc, in press
13. Højgaard I, Fornander AM, Nilsson MA, Tiselius HG (1996) Crystallization during volume reduction of solutions with a composition corresponding to that in the collecting duct: the influence of hydroxyapatite seed crystals and urinary macromolecules. Scanning Microsc, in press
14. Koide T, Takemoto M, Itatani H, Takaha M, Sonoda T (1981) Urinary macromolecular substances as natural inhibitors of calcium oxalate crystal aggregation. Invest Urol 18: 382
15. Kok DJ, Papapoulos SE, Bijvoet OLM (1986) Excessive crystal agglomeration with low citrate excretion in recurrent stone formers. Lancet I: 1056
16. Kok DJ, Papapoulos SE, Blomen LJMJ, Bijvoet OLM (1988) Modulation of calcium oxalate monohydrate crystallization kinetics in vitro. Kidney Int 34: 346
17. Kok DJ, Papapoulos SE, Bijvoet OLM (1990) Crystal agglomeration is a major element in calcium oxalate urinary stone formation. Kidney Int 37: 51
18. Kok DJ (1991) The role of crystallization process in calcium oxalate urolithiasis. Doctorate thesis. University of Leiden, Netherlands, p 58
19. Leusmann D, Blaschke R, Schwandt W (1990) Results of 5035 stone analyses: a contribution to epidemiology of urinary stone disease. Scand J Urol Nephrol 24: 205
20. Lupták J, Jensen HB, Fornander AM, Højgaard I, Nilsson MA, Tiselius HG (1994) Crystallization of calcium oxalate and calcium phosphate at supersaturation levels corresponding to those in different parts of the nephron. Scanning Microsc 8: 47
21. Nakagawa Y, Ahmed MA, Hall SL, Deganello S, Coe FL (1987) Isolation from human calcium oxalate renal stones of nephrocalcin, a glycoprotein inhibitor of calcium oxalate crystal growth: evidence that nephrocalcin from patients with calcium oxalate nephrolithiasis is deficient in γ-carboxyglutamic acid. J Clin Invest 79: 1782
22. Nancollas GH (1983) Crystallization theory relating to urinary stone formation. World J Urol 1: 131
23. Ohkawa M, Tokunaga S, Nakashima T, Yamaguchi K, Orito M, Hisazumi H (1992) Composition of urinary calculi related to urinary tract infection. J Urol 148: 995
24. Pak CYC (1969) Physicochemical basis for formation of renal stones of calcium phosphate origin: calculation of the degree of saturation of urine with respect to brushite. J Clin Invest 48: 1914
25. Rector FC Jr (1983) Acidification of the urine In: Geiger SR, Orloff J, Berliner RW (eds) Renal physiology, sect 8, Handbook of physiology. American Physiological Society, Washington, DC, p 431
26. Robertson WG, Peacock M, Nordin BEC (1969) Calcium crystalluria in recurrent renal-stone formers. Lancet II: 21
27. Robertson WG, Peacock M (1972) Calcium oxalate crystalluria and inhibitors of crystallization in recurrent renal stone-formers. Clin Sci (Colch) 43: 499
28. Ryall RL, Harnett RM, Marshall VR (1981) The effect of urine, pyrophosphate, citrate, magnesium and glycosamino-

glycans on the growth and aggregation of calcium oxalate crystals in vitro. Clin Chim Acta 112: 349
29. Ryall RL, Harnett RM, Hibberd CM, Edyvane KA, Marshall VR (1989) The effect of macromolecules on the crystallization of calcium oxalate in human urine. In: Walker VR, Sutton RAL, Cameron ECB, Pak CYC, Robertson WG (eds) Urolithiasis. Plenum Press, New York, p 133
30. Ryall Rl, Harnett RM, Hibberd CM, Edyvane KA, Marshall VR (1991) Effects of chondroitin sulphate, human serum albumin and Tamm-Horsfall mucoprotein on calcium oxalate crystallization in undiluted human urine. Urol Res 19: 181
31. Scurr DS, Robertson WG (1986) Modifiers of calcium oxalate crystallization found in urine. II. Studies on their mode of action in an artificial urine. J Urol 136: 128
32. Scurr DS, Robertson WG (1986) Modifiers of calcium oxalate crystallization found in urine. III. Studies on the role of Tamm-Horsfall mucoprotein and of ionic strength. J Urol 136: 505
33. Springman KE, Drach GW, Gottung B, Randolph AD (1986) Effects of human urine on aggregation of calcium oxalate crystals. J Urol 135: 69
34. Tiselius HG (1981) The effect on pH on the urinary inhibition of calcium oxalate crystal growth. Br J Urol 53: 470
35. Tiselius HG, Fornander AM, Nilsson MA (1990) Effects of urinary macromolecules on the crystallization of calcium oxalate. Urol Res 18: 381
36. Tiselius HG, Berg C, Fornander AM, Nilsson MA (1993) Effect of citrate on the different phases of calcium oxalate crystallization, Scanning Microsc 7: 381
37. Tiselius HG, Fornander AM, Nilsson MA (1993) The effects of citrate and urine on calcium oxalate crystal aggregation. Urol Res 21: 363
38. Tiselius HG, Fornander AM, Nilsson MA (1996) Studies on the crystallization process following addition of calcium phosphate crystals to solutions with a composition corresponding to that in the collecting duct. Scanning Microsc, in press
39. Tiselius HG, Højgaard I, Fornander AM, Nilsson MA (1996) Is calcium phosphate the natural promoter of calcium oxalate crystallization? In: Pak CYC, Resnick MI, Preminger GM (eds) Urolithiasis 1996. Millet the Printer, Dallas, p 238
40. Werness PG, Bergert JH, Smith LH (1981) Crystalluria. J Crystal Growth 53: 166