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H. Bek-Jensen · A.-M. Fornander · M.-A. Nilsson
H.-G. Tiselius

Is citrate an inhibitor of calcium oxalate crystal growth in high concentrations of urine?

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Abstract The effect of citrate on calcium oxalate (CaOx) crystal growth was studied in a system in which series of samples containing [^{45}Ca]calcium chloride were brought to different levels of supersaturation with various concentrations of oxalate. The crystallization was assessed by measuring the amount of isotope remaining in solution 30 min after the addition of CaOx seed crystals to samples containing citrate in concentrations corresponding to those in final urine. The experiments were carried out both in pure salt solutions and in solutions with dialysed urine. Increased concentrations of citrate resulted in a reduced crystallization of CaOx in both the presence and absence of dialysed urine, but with the lowest rate of crystallization in the samples containing urine. The increased concentration of ^{45}Ca remaining in solution reflected a reduced crystallization, which could possibly be explained both by a reduced supersaturation and by an increased inhibition of CaOx crystal growth. The direct effects of citrate on CaOx crystal growth were assessed by calculating the ion-activity product of CaOx (AP_{CaOx}) at corresponding degrees of crystallization. The AP_{CaOx} recorded at a 30% reduction of the amount of isotope in solution increased with increasing concentrations of citrate between 1.0 and 1.5 mmol/l in samples both with and without dialysed urine. These findings indicate that citrate has a weak direct inhibitory effect on CaOx crystal growth, which adds to the reduced growth rate brought about by urinary macromolecules and a decreased supersaturation.

Key words Calcium oxalate · CaOx crystallization · Crystal growth · Inhibition · Citrate · Dialysed urine

H. Bek-Jensen (✉) · A.-M. Fornander · M.-A. Nilsson
H.-G. Tiselius
Department of Urology and Clinical Research Center,
Faculty of Health Sciences, University Hospital,
S-581 85 Linköping, Sweden

It has been demonstrated that citrate can affect the crystallization of calcium oxalate (CaOx) in several ways. The formation of strong complexes with calcium citrate affects the ion-activity product of both CaOx and calcium phosphate (CaP). Studies in seeded metastably supersaturated solutions showed that citrate, even in low concentrations, had an inhibitory influence on the growth of both CaOx and CaP crystals [17, 21]. Citrate furthermore inhibits the aggregation (agglomeration) of CaOx crystals.This effect of citrate might be attained both by a direct action on crystal aggregation [22] and by enhanced inhibitory strength of urinary macromolecules [7]. In addition to their effect on CaOx crystal aggregation, urinary macromolecules are powerful inhibitors of CaOx crystal growth [18, 19], and with the high concentration of macromolecules that is encountered in urine it has been assumed that the major effect of citrate in whole final urine is accomplished by a reduction of the CaOx supersaturation. The inhibitory effect of citrate on CaOx crystal growth might therefore be negligible.

The present study was undertaken to determine whether citrate under physiological conditions has a direct inhibitory influence on the growth of CaOx crystals apart from the growth reduction that results from the effect of citrate on the CaOx supersaturation.

Methods

Dialysed urine

Pooled urine collected from normal subjects was dialysed against water and 0.15 mol/l saline in Spectrapore No. 3 dialysis tubings as described in detail elsewhere [16]. During the dialysis any significant amounts of calcium (Ca), oxalate (Ox), citrate (Ci) or magnesium (Mg) were removed.

System for assessment of crystal growth

Ninety millilitres of dialysed urine or 0.15 mol/l sodium chloride solution was carefully mixed with 4 ml of 0.05 mol/l [^{45}Ca]calcium chloride and 5 ml 0.15 mol/l sodium chloride without citrate and with citrate necessary for giving concentrations in the final samples between 0.1 and 3.0 mmol/l. Aliquots of 4.9 ml of these solutions were distributed into two series of nine tubes. Different levels of CaOx supersaturation were established by adding 1 ml sodium oxalate solutions with concentrations between 10 and 80 mmol/l. A sample without oxalate was used as a control.

A suspension of crystal seed was prepared by mixing 100 mg CaOx monohydrate (BDH Chemicals, Poole, UK) with 100 ml water. This suspension was stored for at least 24 h before use in the experiments. The crystallization was started by adding 1 ml of the crystal suspension to each tube in the experimental series. This resulted in a final sample volume of 6 ml in which the concentrations of oxalate thus varied between 0.17 and 1.33 mmol/l. The crystallization was allowed to proceed for 30 min, after which the process was interrupted by passing the solution through a Millipore filter (Molsheim, France; pore size 0.22 μm).

The isotope concentration remaining in solution was determined in a scintillation spectrometer (LKB, Wallac, 1217, Turku, Finland). Correction for quenching was made automatically by means of an external standard.

The crystal growth followed the kinetics of a second-order equation and as demonstrated in semilogarithmic plots the decrease in soluble calcium was linear beyond 30 min. The amount of isotope remaining in solution after 30 min thus reflected the rate of crystal growth and a comparison of the growth rate in different samples was made from the oxalate concentrations, corresponding to a 30% reduction in the isotope concentration at this point in time.

It needs to be emphasized that the corresponding samples with and without dialysed urine had the same concentrations of calcium and oxalate and the same amount of seed crystals.

Calculations

The ion-activity product of CaOx (AP_{CaOx}) was calculated from the ion composition in each sample by means of the EQUIL2 program [24]. A comparison was subsequently made between the AP_{CaOx} levels recorded with different concentrations of citrate at a 30% reduction of the isotope concentration 30 min after the addition of seed crystals. The concentrations of oxalate in the solutions that were added to the different samples and the corresponding AP_{CaOx} in the experimental system without citrate are shown in Fig. 1.

The total inhibition, expressed as a percentage, was calculated according to the formula:

$$100 \times (70 - A)/(100 - A)$$

where A is the percentage isotope remaining in solution in the control sample at the oxalate concentration resulting in a 30% reduction of isotope in the experimental system.

Results

Citrate in concentrations up to 3.0 mmol/l resulted in a marked reduction in precipitation of CaOx crystals during the first 30 min after addition of the seed crystals. The effects on crystal growth in saline without citrate and with citrate in concentrations of 0.25, 1.0 and 2.0 mmol/l (Fig. 2A) clearly demonstrate this effect of citrate. As is evident from Fig. 2B, the presence of dialysed urine resulted in a slower rate of crystal growth than that observed in the corresponding samples without urine. Increments in the citrate concentration in the presence of urine brought about a further reduction in the rate of CaOx precipitation. In these experiments the crystallization was approximately the same in samples containing dialysed urine without citrate as in a urine-free salt solution with citrate in a concentration of 1 mmol/l (Fig. 3).

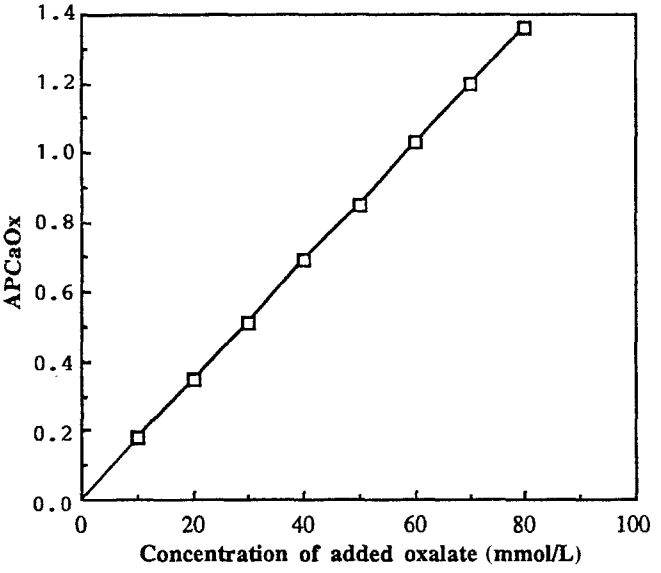

Fig. 1 Relationship between concentration of added oxalate and AP_{CaOx} in the different samples without citrate

At a citrate concentration of 2.0 mmol/l there appeared to be a small additional effect of citrate in the presence of dialysed urine. The total inhibition of CaOx crystal growth – that is the net effect of the driving force of the supersaturated solution and the CaOx crystal growth rate – is shown for different citrate concentrations in Fig. 4. Although the total inhibition increased up to a citrate concentration of 3 mmol/l, the most pronounced effect was observed at the lowest concentrations of citrate.

The AP_{CaOx} recorded for a 30% reduction of the isotope concentration in saline increased from 0.40×10^{-8} to 0.53×10^{-8} M^2 when the citrate concentration was increased from 0.10 to 3.0 mmol/l. In solutions containing urine the corresponding increment in AP_{CaOx} was from 0.45×10^{-8} to 0.53×10^{-8} M^2. It should be observed, however, that in the concentration range of 1.5–3 mmol/l citrate, the AP_{CaOx} remained at a plateau in both saline and urine (Fig. 5). For citrate concentrations up to 1 mmol/l, a slightly higher AP_{CaOx} was necessary to obtain the same extent of crystallization in urine as in saline.

Discussion

Citrate is of crucial importance for the crystallization of calcium salts. By complex formation the activity of

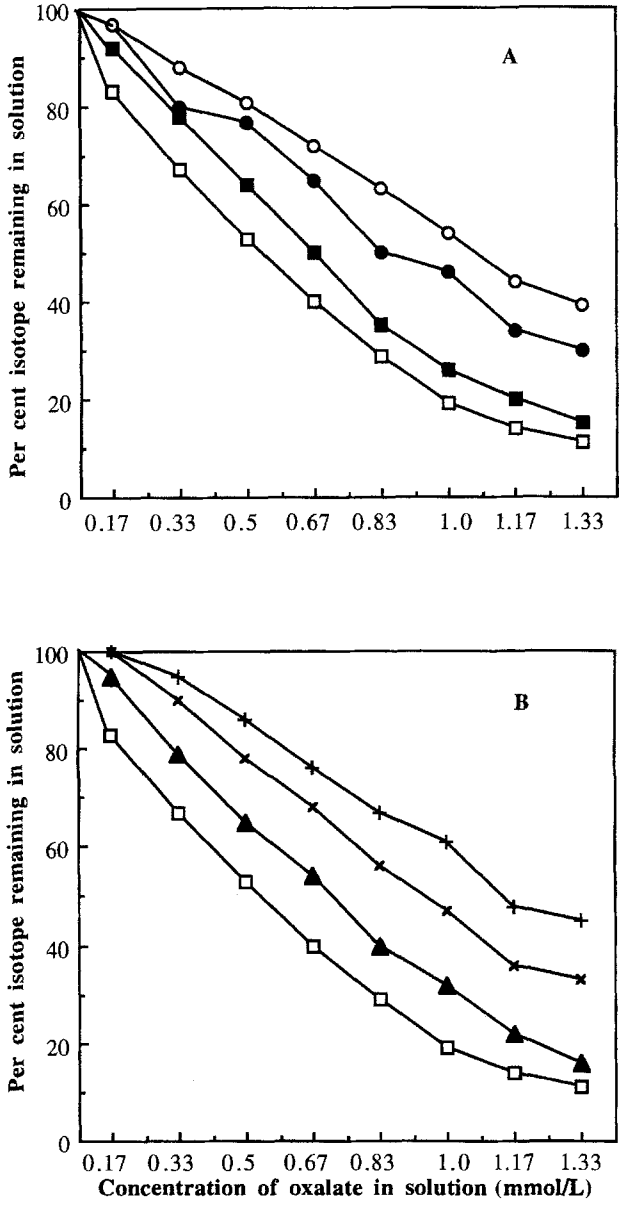

Fig. 2 **A** Percentage ^{45}Ca remaining in solution 30 min after the addition of calcium oxalate seed crystals to urine-free samples with different levels of supersaturation, without citrate (□) and with citrate concentrations of 0.25 mmol/l (■), 1 mmol/l (●) and 2 mmol/l (○). **B** Percentage ^{45}Ca remaining in solution 30 min after the addition of calcium oxalate seed crystals to samples containing dialysed urine without citrate (▲) and dialysed urine together with citrate in concentrations of 1 mmol/l (×) and 2 mmol/l (+). The isotope remaining in solution in a sample without both dialysed urine and citrate is shown (□)

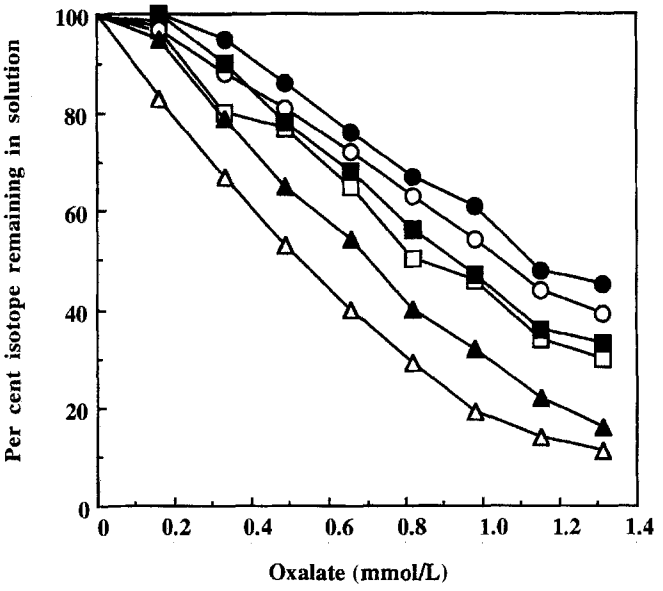

Fig. 3 Percentage ^{45}Ca remaining in solution 30 min after the addition of calcium oxalate seed crystals to samples with different levels of calcium oxalate supersaturation (concentrations of oxalate).The samples were prepared without urine and without citrate (△), without urine but with citrate in concentrations of 1 mmol/l (□) and 2 mmol/l (○), with urine without citrate (▲) and with urine together with citrate in concentrations of 1 mmol/l (■) and 2 mmol/l (●)

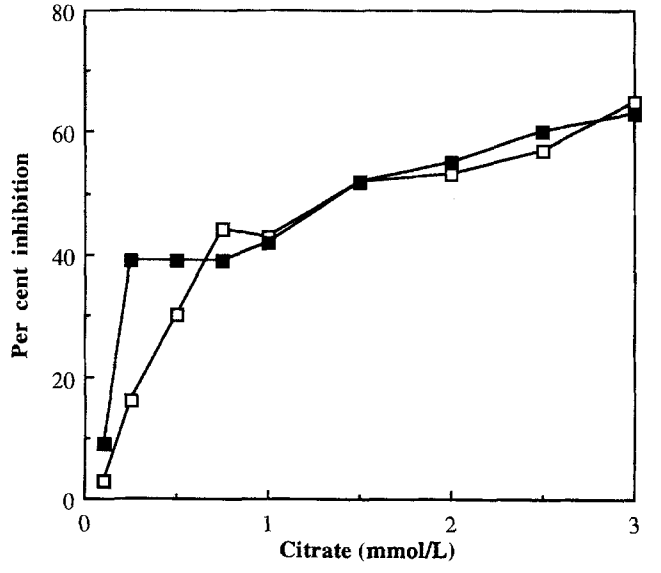

Fig. 4 Total inhibition of CaOx crystal growth at increasing concentrations of citrate in 0.15 mol/l saline (□) and dialysed urine (■). For a detailed explanation of the calculation, see "Methods"

calcium ions and thereby the supersaturation with CaOx as well as with CaP are reduced [21]. The inhibition of growth of CaOx and CaP crystals has been clearly demonstrated in systems without urine [12, 21]. Furthermore citrate inhibits the aggregation of CaOx and CaP [4, 9, 21]. In the presence of urinary macromolecules citrate might influence the stucture of Tamm-Horsfall protein [7] and in this way affect the aggregation. Because of the several ways in which citrate can thus affect the different components of the crystallization of CaOx, it is experimentally difficult to assess its relative contribution to the crystallization brought about by a reduced supersaturation [11, 15], an increased inhibition of aggregation [3, 12] and a direct inhibitory effect on crystal growth [12, 15].

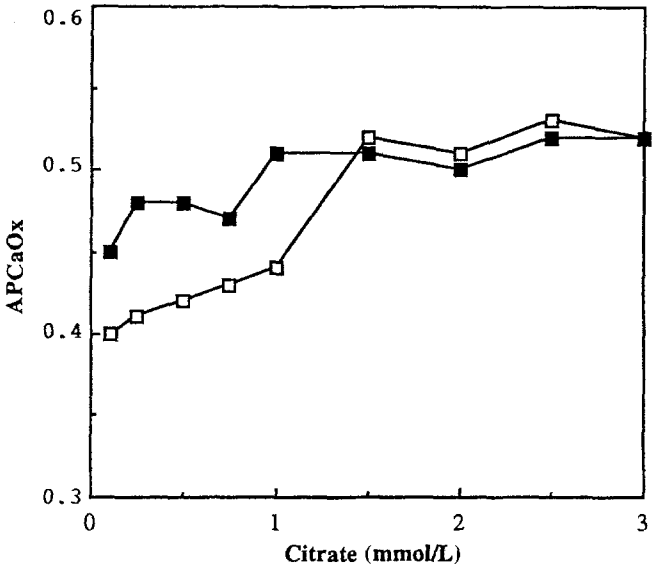

Fig. 5 The AP_{CaOx} required for a 30% precipitation of calcium at different concentrations of citrate in samples with (■) and without (□) dialysed urine

Whereas the inhibitory properties of citrate on crystal growth and crystal aggregation can be fairly easily measured in samples of diluted urine, this is definitely not so for whole urine. Low concentrations of citrate inhibit CaOx crystal growth in metastably supersaturated solutions [22], but the inhibitory role in the presence of macromolecules is far from clear [23]. In order to overcome this problem we used a series of samples with a calcium concentration and an ion-strength approximately corresponding to that in whole urine. Dialysed urine was used as a possible source of macromolecules to add to the crystallization system in whole urine concentrations without adding any small ions. The ion composition was thus similar in the systems with and without urine. The crystallization was started by adding seed crystals to solutions with different concentrations of oxalate and citrate.

Attributable to the crystallization kinetics under these experimental conditions, the decreased concentration of [^{45}Ca] remaining in solution 30 min after the addition of seed crystals was considered to reflect the growth rate of CaOx in samples both with and without urinary macromolecules. The recorded rate of crystallization is, however, the net effect of a decreased driving force attributable to a reduced AP_{CaOx}, a direct inhibition of the crystal growth and possibly an increased rate of crystal growth resulting from an increased inhibition of crystal aggregation. A reduced aggregation results in a greater surface area and therefore an increased rate of growth. A discrimination between these two effects was not possible in our experiments and the recorded reduction of growth is therefore the net effect of a direct inhibition of citrate on the growth rate probably by citrate adsorption to growth sites on the crystal surface, and an increased growth rate attributable to the increased inhibition of aggregation. The latter effect might thus obscure the direct inhibition of CaOx crystal growth.

One way to assess whether citrate causes a direct inhibition on CaOx crystal growth is to determine the AP_{CaOx} for corresponding degrees of ^{45}Ca precipitation at different concentrations of citrate. Citrate in whole urine concentrations markedly retarded the consumption of calcium during the first 30 min after addition of CaOx seed crystals, and higher AP_{CaOx} levels were required to obtain the same extent of calcium precipitation when the citrate concentration was increased from low to normal levels. This observation indicates that citrate, in addition to the effect on CaOx supersaturation, also directly inhibits crystal growth, even though the latter effect appears to be weak.

It is well known that urinary macromolecules such as glycosaminoglycans [1, 2, 13], glycoproteins [8] and Tamm-Horsfall protein [5, 25] are inhibitors of CaOx crystal growth. This effect is most certainly accomplished by a binding or adsorption of these macromolecules to the surface of the crystals. The inhibitory effect observed with citrate is probably also the result of a binding to growth sites on the CaOx crystals [10]. In a system which like urine contains both citrate and macromolecules, it is thus reasonable to assume that these two constituents compete for the same binding sites. We have not carried out any experiments to assess any differences between citrate and macromolecules in their affinity to the CaOx crystals, but it is evident from our experiments that citrate even in the presence of dialysed urine caused an inhibition of CaOx crystal growth.

A comparison between samples with and without urine in terms of AP_{CaOx} cannot easily be made, because the effects of macromolecules on the ion activities [14] have not been accounted for in our calculations with the EQUIL2 program. It was assumed, however, that the relative effect of macromolecules on supersaturation was the same in all samples containing dialysed urine.

It has previously been shown that citrate might interfere with urinary macromolecules in terms of inhibition of CaOx crystal aggregation [17]. In the concentration range of citrate between 0 and 1 mmol/l there appeared to be an additive effect of citrate and macromolecules on the inhibition of CaOx crystal growth (Fig. 5), an effect that was not observed with higher concentrations of citrate. The hypothesis of possible competition between citrate and macromolecules for the same binding sites was further supported by the fact that the supersaturation required for the same degree of CaOx precipitation approached that of pure citrate solutions when the citrate concentration was increased from 1.5 to 3.0 mmol/l.

The clinical importance of an interaction between macromolecules and citrate is difficult to predict from

these experimental results, inasmuch as the amount of crystals as well as the AP_{CaOx} differs from that in whole urine. Although urinary macromolecules were present in whole urine concentrations, there were no small molecular weight inhibitors present except citrate. There appeared to be an optimal concentration of citrate above which a further increment of the citrate concentration did not result in any additional growth inhibition. This effect is, however, determined by the size of the crystal surface area and the amount of seed crystals. In our experimental system the concentrations of calcium oxalate monohydrate crystals and oxalate in solution at the beginning of the crystallization corresponded to a total oxalate concentration of 1.3–2.4 mmol/l, which is unphysiological. It therefore stands to reason that any clinical importance of a reduced growth inhibition only might occur at low concentrations of citrate.

In view of the possible interaction between citrate and macromolecules in the calcium salt crystallization process [20, 21], it appears important to determine the optimal physiological concentration of citrate at different levels of the nephron in order to obtain a basis for the most efficient design of preventive treatment with alkaline citrate. With the smaller area of crystals formed in the nephron and the much lower concentration of macromolecules in tubular urine, where the initial CaOx crystallization most likely takes place, it is possible that the growth-inhibiting effect of citrate is more important than our measurements indicate. In a situation where the macromolecular inhibition is either quantitatively inferior or structurally inefficient [6], it is thus possible that the role of citrate becomes more important. An increased concentration of citrate then to some extent might compensate for or correct such deficiencies and exert a prevention of CaOx crystallization, by adding a direct growth-inhibition to the effects on supersaturation and aggregation.

The effect of citrate on CaOx crystal growth that was recorded in this series of experiments gives further support to the importance of a low citrate concentration as a risk factor for CaOx stone formation and to the potential usefulness of citrate in treating of patients with hypocitraturic CaOx stone disease.

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References

1. Angell AH, Resnick MI (1989) Surface interaction between glucosaminoglycans and calcium oxalate. J Urol 141:1255
2. Bek-Jensen H, Tiselius HG (1991) Inhibition of calcium oxalate crystallization by urinary macromolecules. Urol Res 19:165
3. Grases F, Costa-Bauza A (1990) Study of factors affecting calcium oxalate crystalline aggregation. Br J Urol 66:240
4. Grases F, Masárová L, Söhnel O, Costa-Banzá A (1992) Agglomeration of calcium oxalate monohydrate in synthetic urine. Br J Urol 70:240
5. Hess B (1992) Tamm-Horsfall glucoprotein-inhibitor or promoter of calcium oxalate monohydrate crystallization processes? Urol Res 20:83
6. Hess B, Nakagawa Y, Parks JH, Coc FL (1991) Molecular abnormality of Tamm-Horsfall glycoprotein in calcium oxalate nephrolithiasis. Am J Physiol 260:F569
7. 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
8. Kaiser ET, Bock SC (1989) Protein inhibitors of crystal growth. J Urol 141:750
9. Kok DJ, Papapoulos SE, Bijvoet OLM (1990) Crystal agglomeration is a major element in calcium oxalate urinary stone formation. Kidney Int 37:51
10. Minero C, Maggiorotti P, Barbeni M, Pelizzetti E, Tizzani A, Casetta G, Piana P, Giona C (1987) Effect of inhibitors on the crystal growth of calcium oxalate. Contr Nephrol 58:65
11. Ogawa Y (1994) Impact of sodium-potassium citrate on the diurnal variations in urinary calcium oxalate and calcium phosphate saturation levels in normal individuals. Br J Urol 73:136
12. Ryall RL, Marshall VR (1981) The effect of urine and other inhibitors on the growth and aggregation of calcium oxalate crystals in vitro. In: Urolithiasis (eds) Smith LH, Robertson WG, Finlayson B. Plenum Press, New York
13. Ryall RL, Marshall VR (1984) The relationship between urinary inhibitory activity and endogenous concentration of glucosaminoglycans and uric acid: comparison of urines from stone-formers and normal subjects. Clin Chim Acta 141:197
14. Sheinfeld J, Finlayson B, Reid F (1978) Ultrafiltration evidence of ion binding by macromolecules in urine. Invest Urol 15:462
15. Shirane Y, Kagawa S (1993) Scanning electron microscopic study of the effect of citrate and pyrophosphate on calcium oxalate crystal morphology. J Urol 150:1980
16. Tiselius HG (1985) Measurement of the risk of calcium oxalate crystallization in urine. Urol Res 3:297
17. Tiselius HG, Fornander AM (1981) Evaluation of a routine method for determination of calcium oxalate crystal growth inhibition in diluted urine samples. Clin Chem 27:565
18. Tiselius HG, Fornander AM, Nilsson MA (1987) Inhibition of calcium oxalate crystallization in urine. Urol Res 15:83
19. Tiselius HG, Fornander AM, Nilsson MA (1990) Effects of urinary macromolecules on the crystallization of calcium oxalate. Urol Res 18:381
20. Tiselius HG, Fornander AM, Nilsson MA (1993) The effects of citrate and urine on calcium oxalate crystal aggregation. Urol Res 21:363
21. Tiselius HG, Berg C, Fornander AM, Nilsson MA (1993) Effects of citrate on the different phases of calcium oxalate crystallization. Scan Microscopy 7(1):381
22. Tiselius HG, Fornander AM, Nilsson MA (1993) The effects of citrate and urine on calcium oxalate crystal aggregation. Urol Res 21:363
23. Wang YH, Grenabo L, Hedelin H, McLean RJC, Nickel JC, Pettersson S (1993) Citrate and urase-induced crystallization in synthetic and human urine. Urol Res 21:109
24. Werness PG, Brown CM, Smith LH, Finlayson B (1985) EQUIL2: a basic computer program for the calculation of urinary saturation. J Urol 134:1242
25. Yosihioka T, Koide T, Utsunomiya M, Itatani H, Oka T, Sonoda T (1989) Possible role of Tamm-Horsfall glycoprotein in calcium oxalate crystallization. Br J Urol 64:463