Urol Res (1995) 23: 53–62

ORIGINAL PAPER

I. R. Doyle · V. R. Marshall · C. J. Dawson · R. L. Ryall

Calcium oxalate crystal matrix extract: the most potent macromolecular inhibitor of crystal growth and aggregation yet tested in undiluted human urine in vitro

Received: 13 July 1994 / Accepted: 30 September 1994

Abstract Demineralization of calcium oxalate (CaOx) crystals precipitated from human urine in vitro yields an organic crystal matrix extract (CME) consisting predominantly of a single protein which we originally named crystal matrix protein but have subsequently shown to be a urinary form of prothrombin activation peptide fragment 1 (F1). The aim of this study was to determine whether CME is a promoter or inhibitor of CaOx crystallization. The effect of CME on CaOx crystal growth and aggregation was tested using a standard seeded crystallization system, and its effect quantified by use of particle size analysis and a computer model. In addition, the effect of CME on the crystallization of CaOx was tested in undiluted, ultrafiltered human urine using Coulter Counter analysis and scanning electron microscopy. It was shown that CME is a potent inhibitor of CaOx crystal growth and aggregation in a seeded metastable solution. However, of greater significance is that at a concentration of 10 mg/l it completely reversed the formation of large crystalline aggregates that form upon the removal of urinary macromolecules from undiluted urine. It was concluded that CME is the most potent macromolecular urinary inhibitor yet to be tested in urine in vitro. By preventing the aggregation of newly formed crystals, the components of CME may significantly reduce the probability of particle retention in vivo and therefore the occurrence of urolithiasis.

Key words Calcium oxalate urolithiasis
Crystal matrix extract · Crystal matrix protein
Prothrombin fragment F1 · Crystal growth
Crystal aggregation · Crystallization inhibitor

Irrespective of their mineral content, all renal stones contain an organic matrix [1]. The ubiquity of this matrix, and the fact that it is composed largely of protein, suggest that proteins fulfil an important determinant role in stone pathogenesis. However, attempts to define such a role have been thwarted by the complex chemical nature of stone matrix, its intractable insolubility, and alterations in its molecular structure resulting from chemical isolation procedures, ageing, and the crystallization process itself [5, 15, 24]. The problem is further compounded by the fact that proteins in matrix may derive both from those occurring normally in urine and others resulting from cellular trauma caused by the developing stone [5]. The analysis of stones is therefore of little practical value in assessing the function of proteins in the critical crystallization step of stone pathogenesis. However, the problems attendant upon stone analysis can be largely circumvented by studying proteins associated with crystals freshly precipitated from urine. Unlike that from stones, matrix derived from calcium oxalate (CaOx) crystals precipitated from human urine contains relatively few proteins [5, 19, 20], one of which is present in quantities exceeding that of any other. The disproportionate abundance of this protein, which we originally named crystal matrix protein [5], or CMP, but have since shown to be urinary prothrombin activation peptide F1 [40, 42], within the crystals suggests that it may influence their formation and consequently, may fulfil some decisive role in stone pathogenesis. Paradoxically, however, that role may equally well be as a promoter or as an inhibitor of crystallization.

The potential importance of promoters and inhibitors in renal stone formation cannot be overemphasized. Urinary supersaturation with respect to CaOx is not unusual, even in people who have never formed a stone [29], and all of us occasionally pass crystals in our urine. Those passed by recurrent stone formers, however, tend to be excreted in greater quantities and are clustered into larger particles more likely to be retained within the renal collecting system than those observed in healthy controls [27]. Therefore, any urinary molecule which promotes or inhibits the mass of CaOx deposited from urine, or the size of the crystal particles produced, has the potential to influence the likelihood of particle retention and thereby the development of stone disease.

I. R. Doyle · V. R. Marshall · C. J. Dawson · R. L. Ryall (✉)
Department of Surgery, Flinders Medical Centre, Bedford Park, South Australia 5042, Australia, Fax: +61 (8) 374 0832

Since levels of supersaturation required for spontaneous CaOx crystal nucleation are rarely found in urine, it is generally accepted that the precipitation of stone minerals in urine in vivo is a result of heterogeneous nucleation which may result from the induction of high localized concentrations of crystal ions brought about by the binding of certain urinary proteins [18, 25]. This is supported by reports that some urinary proteins promote CaOx crystallization both in inorganic solutions [eg., 6] and in concentrated whole urine [12]. The presence of proteins, particularly urinary F1, in CaOx crystals may thus be construed as evidence for their acting as promoters of nucleation and solute deposition. Equally, however, it can be interpreted as supporting an inhibitory role. A large number of studies have reported urinary macromolecules to be inhibitors of CaOx crystal growth [eg., 26, 28, 32] and aggregation [eg., 7, 16, 35, 37]. Inhibition is generally thought to result from binding of the macromolecule to the crystal surface, with the degree of inhibition being proportional to the concentration of inhibitor bound [2]. The presence of proteins within CaOx crystals may therefore be a consequence of their acting as incomplete inhibitors of growth and aggregation, causing them to become trapped within the crystal as deposition of calcium and oxalate ions proceeds over the surface.

Because the proteins contained within CaOx crystals may fulfil a directive role in stone formation, the aim of this study was to determine whether the organic matrix isolated from newly precipitated CaOx crystals inhibits or promotes crystallization of this salt both in a metastable, inorganic solution and in undiluted human urine.

Materials and methods

Materials

All reagents used were of the highest purity commercially available. Chemicals were supplied by Sigma (St Louis, Mo., USA), with the exception of EDTA (Univar; Ajax Chemicals, Auburn, NSW, Australia); [^{14}C]oxalic acid (NEN Research Products, Du Pont, Wilmington, Del., USA); and sodium oxalate (BDH Chemicals Australia, Kilsyth, Victoria, Australia). All solutions were prepared using the highest quality water from a Hi Pure water purification system fitted with a 0.2-μm filter (Permutit Australia, Brookvale, NSW, Australia).

Collection of urine samples

Urine samples were collected without preservative and refrigerated for the duration of the collection. Each urine sample was checked for microscopic haematuria using Multistix test strips (Miles Laboratories, Australia): none showed any evidence of blood.

Isolation of crystal matrix extract

During the same 24 h period, 12.9 l of urine was collected without preservative from five healthy women aged between 18 and 43 years of age. Crude extract was isolated as described in detail elsewhere [5]. In summary, crystallization was induced in the urine by the addition of an oxalate load. The crystals were isolated, washed, and dried under vacuum, yielding 2.40 g. They were then demineralized in EDTA and the extract electrodialysed over 7 days with the water changed twice a day. The dialysate was filtered through a 0.22-μm Millipore filter (Millipore Corp., Bedford, Mass., USA; GSWP04700) and the filtrate lyophilized to give colourless flocculant material, weighing 24.0 mg. The crude extract, henceforth referred to as crystal matrix extract or CME, was shown by electrophoretic analysis to consist principally of urinary F1 [40], and was used without further purification.

Effect of CME on crystal growth and aggregation in a seeded metastable solution of CaOx

Measurement of crystal growth and aggregation

The method used for recording the rates of growth and aggregation when seed crystals of CaOx are added to a metastable solution of this salt have been described elsewhere [31]. Briefly, portions of a suspension (1 g/l) of calcium oxalate monohydrate seed crystals were added to a metastable solution of the same salt. The size distribution of the particles was recorded using a Coulter Counter Model TAII particle sizer, fitted with a 70-μm orifice and a Population Count Accessory (Coulter Electronics, Harpenden, Herts, UK). The effect of CME on calcium oxalate monohydrate crystal growth and aggregation was tested at five concentrations, namely, 1.250, 0.625, 0.313, 0.156, and 0.078 mg/l. Metastable solution without CME was routinely included as a standard control in each incubation. Solutions were incubated at 37C and the crystal size distributions recorded at 10-min intervals for 1.5 h. To pre-empt the possibility that any inhibitory effects observed might be attributable to EDTA remaining as a consequence of incomplete dialysis, the effects of EDTA at a final concentration of 1.250 mg/l were tested in a separate experiment.

Each experiment was performed 6 times, and from the averaged crystal numbers the extents of crystal growth and aggregation were quantified independently by use of a computer model [34]. Crystal growth was expressed as the cumulative increase in crystal diameter (D) and crystal aggregation as the net proportional change (N) in crystal number from time zero.

Calculation of inhibitory activity

In order to calculate inhibitory activity, data were transformed to linear form and initial rates of growth (T/D) and aggregation (T/N) were calculated from plots of $1/D$ and $1/N$ versus $1/T$, where T=time. Inhibition was then expressed as the percentage of the rate occurring in a saline control as described elsewhere [32], with 0% inhibition occurring in the control and values of 100% indicating complete inhibition.

Effect of CME on CaOx crystallization in ultrafiltered urine

Assessment of crystallization by particle analysis

Urine was collected for 24 h from 3 healthy males 30, 44, and 52 years of age. Sample preparation was performed at room temperature. The urine specimens were pooled and centrifuged at 10 000 × g for 30 min in a Beckman J2-21 M/E centrifuge (Beckman Instruments, Palo Alto, Calif., USA). Microscopic examination of the sediment revealed only organic material. After filtration through 0.22-μm Millipore filters, the urine samples were divided. One portion was stored at 4C for further study (spun and filtered urine) and the other (ultrafiltered urine) was ultrafiltered using an Amicon hollow fibre bundle (Amicon, Danvers, Mass., USA; HlP10-20) with an M_r cut-off of 10 kDa. The urine samples were used immediately after processing.

The experimental system for measuring CaOx crystallization in urine has been described previously [33]. The effect of CME on CaOx

crystallization in ultrafiltered urine was measured at four concentrations, namely 1.25, 2.50, 5.00, and 10.00 mg/l. Portions of the ultrafiltered urine and the spun and filtered urine were routinely included in each incubation as controls. Solutions were incubated at 37C and the crystal size distributions recorded at 10 min intervals for 2 h using the Coulter Counter.

Once again, to take account of the possibility that any inhibitory effects observed might be caused by remnant EDTA in the lyophilized crystal matrix extract, the effects of EDTA were tested in a separate experiment at a final concentration of 10.00 mg/l.

Each experiment was repeated to give a total of six replicates. At the end of every alternate experiment the urine samples were examined by scanning electron microscopy.

Sample preparation and scanning electron microscopy (SEM)

At the end of the 2-h incubation period, 1-mL portions of the urine specimens were passed though 0.22-μm Millipore filters. The filters were air dried, mounted on aluminium stubs, and coated with gold (210 s; SEM Autocoating Unit E5200, Polaron Equipment). The stubs were examined using an ETEC Auto Scan electron microscope (Siemens) at an operating voltage of 20 kV.

Comparison of crystal deposition by [^{14}C]oxalate deposition and Coulter Counter analysis

It has previously been shown that aggregated proteins in whole urine can interfere with the assessment of crystallization using the Coulter Counter [35]. To check whether particle volume recorded by the Coulter Counter in the presence of CME actually reflected the amount of solute deposited, a comparison of crystal deposition by [^{14}C]oxalate and Coulter Counter analysis was made.

Mid-morning urine specimens of 500 ml were collected from five healthy men aged between 25 and 45 years, and on a second occasion from five healthy women aged between 18 and 45 years. Each urine sample was subjected to 10-kDa ultrafiltration using an Amicon hollow fibre bundle and divided for assessment by Coulter Counter analysis and by [^{14}C]oxalate deposition.

The urine samples were further divided; one portion was retained as a control (ultrafiltered urine) while CME was added to the other to a final concentration of 10 mg/l (ultrafiltered urine+CME). The metastability of the samples ("cold" urines) was determined and crystallization induced by the addition of a standard load of oxalate above the metastable limit as described above.

Crystal deposition by [^{14}C]oxalate analysis was performed in tandem with Coulter Counter analysis. The samples ("hot" urines) were treated identically except that the oxalate load was dosed with [^{14}C]sodium oxalate (1.546 Ci/100 ml urine). The experiments were performed in duplicate, and to correct for possible interference caused by precipitated protein, controls containing CME, but no exogenous oxalate, were included.

Coulter Counter analysis

At the end of 2 h incubation the particle distributions in the "cold" urines were recorded using the Coulter Counter.

[^{14}C]oxalate deposition

After the 2-h incubation, 15 ml of the "hot" urines were filtered through 0.22-μm Millipore filters and the crystals washed 3 times with 5 ml water to remove extraneous [^{14}C]oxalate. The retained crystals were dissolved in 1.5 ml of 1 M HCl and 1 ml of this solution added to 10 ml PCS scintillation fluid (Amersham, Oakville, Ontario, Canada; 196385) and counted for 10 min in a liquid scintillation counter (Searle 6880 Liquid Scintillation System).

Statistical methods

For the sake of clarity results of the growth studies are presented as the mean of the six replicates. Statistical analysis of results, however, was made using the Wilcoxon Matched-Pairs Signed-Rank Test.

Results

Effect of CME on crystal growth and aggregation in a seeded metastable solution of CaOx

EDTA at a final concentration of 1.25 mg/l had a negligible effect on the crystallization parameters measured. Figure 1 illustrates the effect of CME on crystal aggregation, expressed as the net proportional change in crystal number from time zero. In the control, particle number decreased rapidly from 100% at zero time to 49% during the first 20 min, followed by a smaller decline to 31% at completion of the 90-min experiment. At a concentration of 0.078 mg/l, the addition of CME caused a small reduction in the degree of crystal aggregation; particle number decreased to 34% but followed a similar time course to that of the control. At the maximum concentration of CME tested, 1.25 mg/l, crystal aggregation was strongly inhibited and particle number decreased only 9% over the 90-min incubation period. At intervening concentrations the effect of the CME was dose-dependent ($P< 0.01$).

Figure 2 illustrates the effect of CME on crystal growth, expressed as the cumulative increase in crystal diameter. In the control, crystal diameter increased rapidly during the first 20 min to 1.0 μm when, presumably as a result of solute depletion, the rate started to wane. At 0.078 mg/l the effect of CME was marginal, the cumulative increase in crystal size being only slightly smaller (2.6 μm) than that of the control (2.7 μm) at the end of 90 min incubation. At the maximum concentration of 1.25 mg/l, however, CME

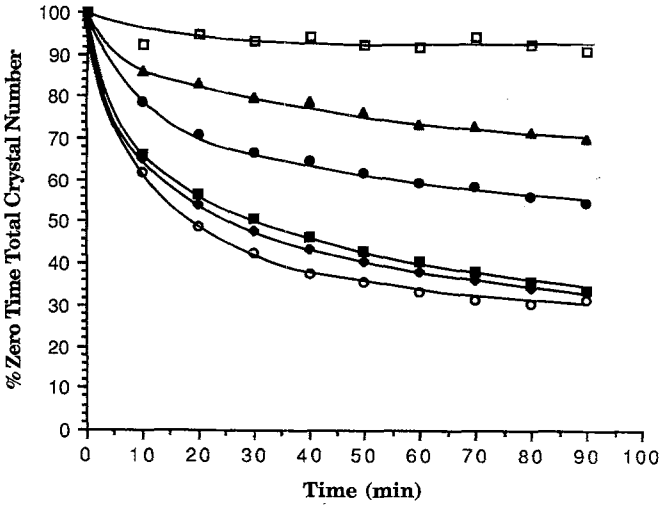

Fig. 1 Effect of CME on crystal aggregation, expressed as the percentage change in crystal number during 90 min incubation (○ control, ◆ 0.078, ■ 0.156, ● 0.313, ▲ 0.625, □ 1.25 mg/l), in a seeded metastable solution

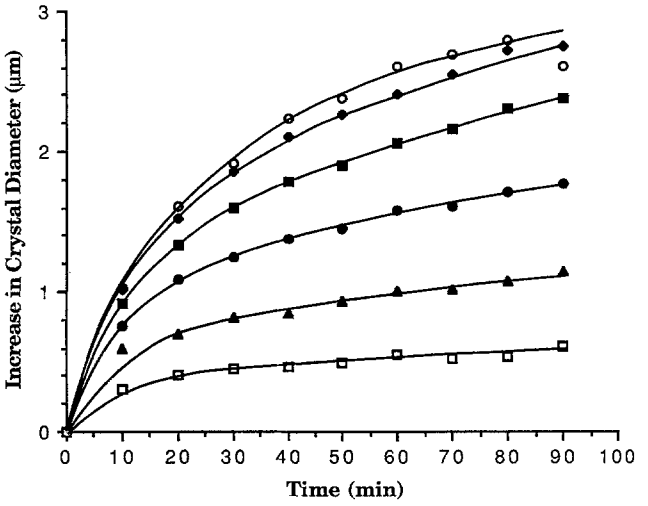

Fig. 2 Effect of CME on crystal growth, expressed as the cumulative increase in crystal diameter calculated during 90 min incubation (○ control, ◆ 0.078, ■ 0.156, ● 0.313, ▲ 0.625, □ 1.25 mg/l), in the metastable solution depicted in Fig. 1

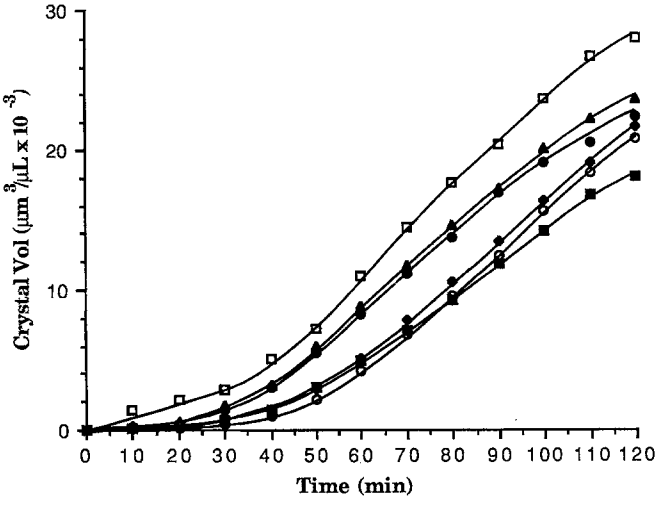

Fig. 4 Change in the total volume of calcium oxalate crystal particles with a diameter greater than 2 μm after a given load of oxalate above the metastable limit. The crystal particles were precipitated during 2 h incubation from pooled spun and filtered urine before (□) and after (○) ultrafiltration, and from the same ultrafiltered urine in the presence of CME (◆ 1.25, ■ 2.5, ● 5, ▲ 10 mg/l)

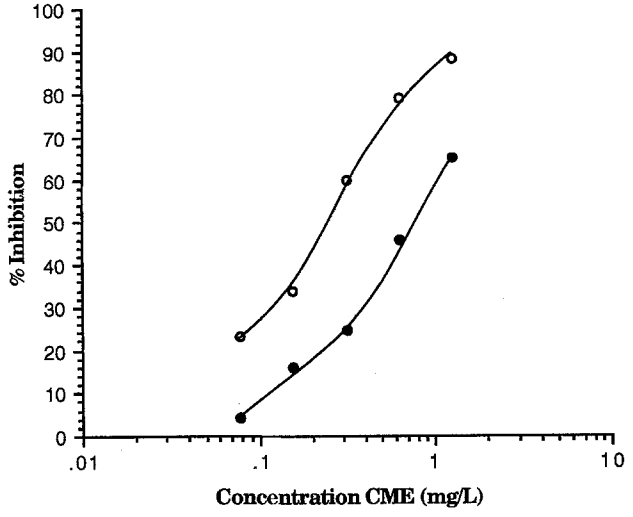

Fig. 3 Effect of CME on the percentage inhibition of calcium oxalate crystal growth (●) and crystal aggregation (○), in the metastable solution depicted in Figs. 1 and 2

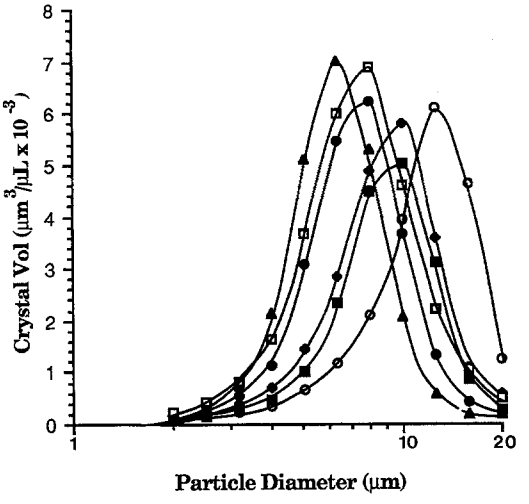

Fig. 5 Effect of CME (◆ 1.25, ■ 2.5, ● 5, ▲ 10 mg/l) after 2 h incubation on the size distributions of calcium oxalate crystal particles precipitated from the ultrafiltered urine samples depicted in Fig. 4. The particle size distributions are compared with those obtained from the spun and filtered urine before (□) and after (○) ultrafiltration

strongly inhibited crystal growth and the cumulative diameter increased by only 0.6 μm over the same time period. Again, as with crystal aggregation, at intervening concentrations the effect of CME on crystal growth was dose-dependent ($P< 0.01$).

In Fig. 3 the percentage inhibition of crystal growth and of aggregation is plotted in relation to the logarithm of CME concentration. It can be seen that, as with a number of macromolecules previously tested [8, 32], CME had a greater effect on crystal aggregation than on crystal growth. Aggregation was inhibited by 88% at a final CME concentration of 1.25 mg/l, while growth was inhibited by 65% at this concentration.

Effect of CME on CaOx crystallization in ultrafiltered urine

Neither the removal of macromolecules by ultrafiltration nor the subsequent addition of CME affected the metastability of the urine; identical amounts of oxalate were therefore added to each aliquot. EDTA at a final concen-

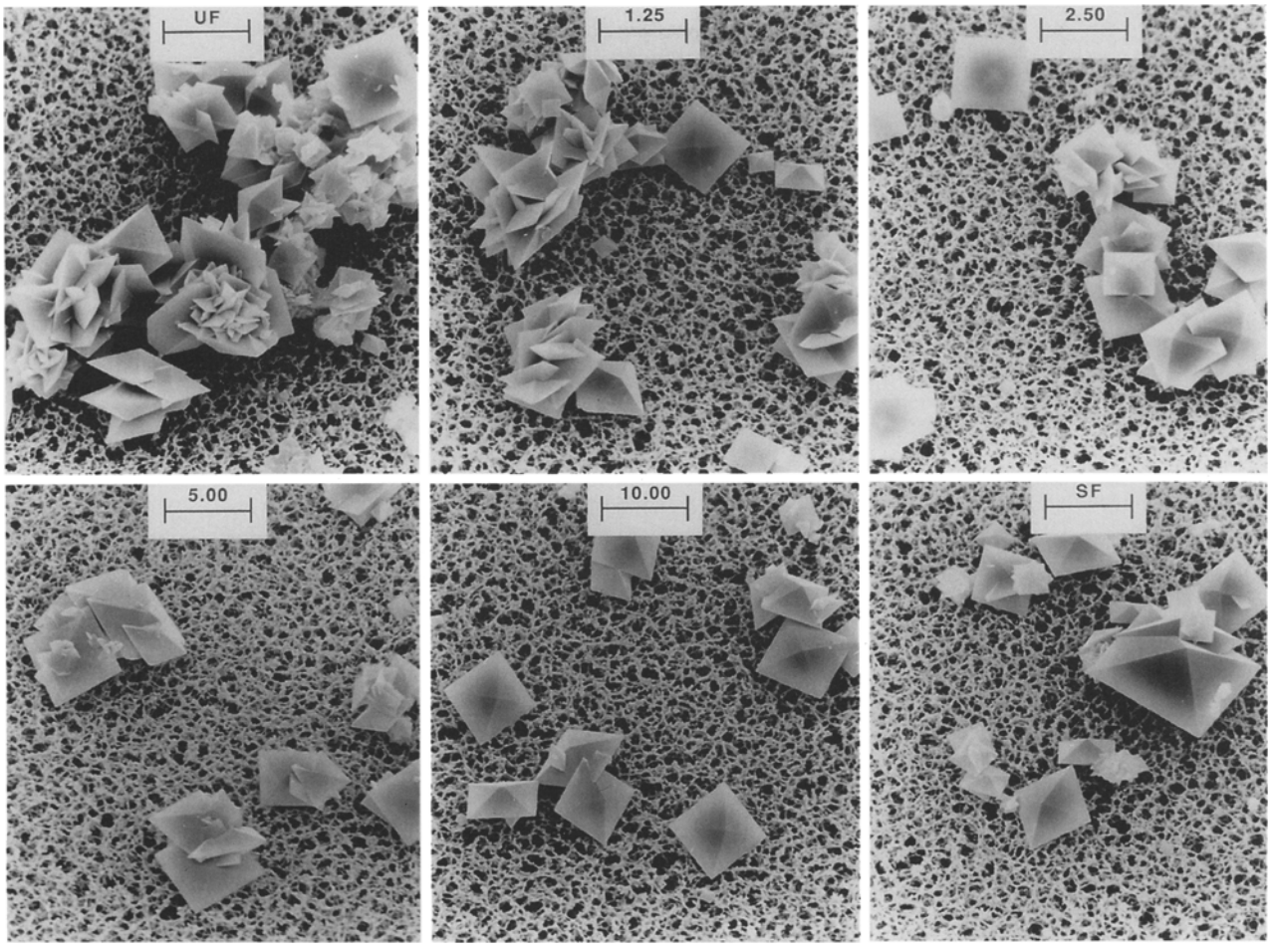

Fig. 6 Scanning electron micrographs of crystalline material typically deposited during the 2 h incubation period. The crystals were precipitated from urine which had been spun and filtered, before (SF) and after (UF) ultrafiltration, and from the same ultrafiltered urine supplemented with 10, 5, 2.5 and 1.25 mg/l CME (10.00, 5.00, 2.50 and 1.25). The *bar* in each micrograph represents 10 μm

tration of 10 mg/l had a negligible effect on the crystallization parameters measured.

Particle analysis

The dose response effect of CME on calcium oxalate particle volume in ultrafiltered urine during the 2-h incubation period is illustrated in Fig. 4. After an initial lag phase crystal volume increased linearly until 110 min where, presumably as a result of oxalate depletion, the rates of deposition started to decrease. It is noteworthy that after the first 50 min, with the exception of the ultrafiltered urine dosed with 2.5 mg/l CME, the growth curves ran approximately parallel to each other, suggesting that total solute deposition occurred at a constant rate.

Particle volume was greatest in the spun and filtered urine. In the remaining urines, with the exception of the ultrafiltered urine dosed with 2.5 mg/l CME, crystal volume paralleled the urinary content of CME. That is, the addition of CME caused an increase in total particle volume in a dose response manner ($P< 0.01$).

Figure 5 shows the particle volume distributions of the samples recorded by the Coulter Counter after 2 h incubation. The addition of CME to ultrafiltered urine evoked a dose response shift in the distributions to smaller diameters. At the maximum concentration tested, 10 mg/l, CME caused the mode of the volume distribution curve to shift from 12.7 μm in the ultrafiltered urine to a diameter of 6.35 μm. This was considerably smaller than in even the spun and filtered urine, where the mode of the volume distribution curve corresponded to a diameter of 8.00 μm, perhaps suggesting that the use of this amount of CME (10 mg/l) resulted in greater concentrations of its active inhibitory components than were present in the corresponding spun and filtered urine sample.

Scanning electron microscopy

Figure 6 illustrates typical crystals isolated after 2 h incubation from the ultrafiltered urine (UF), ultrafiltered urine which had been dosed with the various concentrations of CME, and spun and filtered urine (SF). Neither ultrafiltration nor addition of CME had any effect on the morphology of the crystals precipitated from the urine samples. In

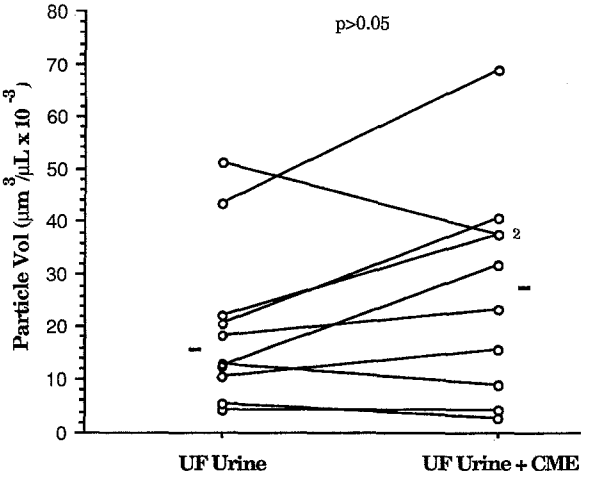

Fig. 7 Total volume of calcium oxalate crystal particles with a diameter greater than 2 μm precipitated from single ultrafiltered urine samples and from subsamples of the same urine samples which had been supplemented with 10 mg/l CME, 2 h after a given load of oxalate above the metastable limit

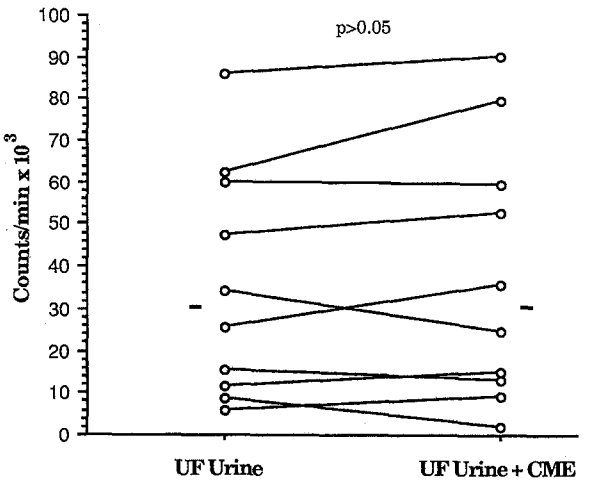

Fig. 9 Effect of the addition of 10 mg/l CME on deposition of [^{14}C]oxalate, expressed as counts per minute, precipitated after 2 h incubation from the ultrafiltered urine samples depicted in Figs. 7 and 8

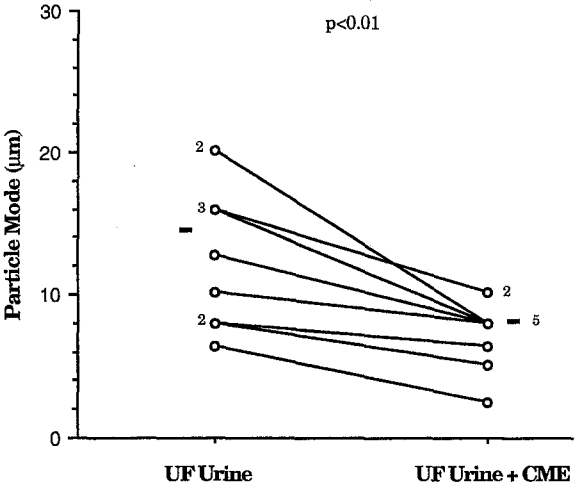

Fig. 8 Effect of the addition of 10 mg/l CME on the size of crystal particles precipitated from single ultrafiltration urine samples. The size of the crystal particles precipitated is expressed as the position of the mode of the volume distribution curve obtained from the Coulter Counter after 2 h incubation from the ultrafiltered urine samples depicted in Fig. 7.

all cases calcium oxalate dihydrate crystals were the only phase present.

It can be seen that while the size of the individual crystals precipitated from the different urines did not perceptibly differ, the variation in the modes of the volume distribution curves (Fig. 5) could clearly be attributed to the extents of crystal aggregation. The crystals derived from the ultrafiltered urine were extensively aggregated, while those from the spun and filtered urine were single crystals or small aggregates. Most importantly, however, the addition of CME to ultrafiltered urine greatly reduced the degree of crystal aggregation and, at a concentration of 10 mg/l, almost completely prevented the process: the crystals precipitated from this urine consisted mostly of single crystals or twins.

Comparison of crystal deposition in ultrafiltered urine by [^{14}C]oxalate deposition and Coulter Counter analysis

Again, the addition of CME had no effect on the metastability of the ultrafiltered urine samples; therefore, the amounts of oxalate added to the ultrafiltered urine controls were the same as those added to the aliquots dosed with CME.

Coulter Counter analysis

The effect of the addition of 10 mg/l CME to ultrafiltered urine on crystal particle volume is shown in Fig. 7. The median particle volume was increased from 15 380 μm^3/μl (range 4253–50 897) to 27 389 μm^3/μl (range 2552–68 799). However, this difference was not significant ($P > 0.05$; $n=10$). Particle volume did not increase in the no-oxalate controls during the 2-h incubation period.

The effect of the addition of 10 mg/l CME on the mode of the crystal volume distribution curves in ultrafiltered urine is illustrated in Fig. 8. CME reduced the median mode of the crystal volume distribution curves from a diameter of 14.35 μm (range 6.35–20.16) to 8.00 μm (range 2.52–10.08) ($P < 0.01$; $n=10$).

[^{14}C]oxalate deposition

As is illustrated in Fig. 9, the addition of 10 mg/l CME to ultrafiltered urine had no effect on the deposition of

[^{14}C]oxalate. The counts per minute after 2 h incubation increased only slightly from the median value of 29 838 (range 5627–86 087) in the ultrafiltered urine samples to 30 110 (range 2011–90 221) in those dosed with CME. This difference was not significant ($P>0.05$; $n=10$).

Discussion

Experimental systems used to assess the effects of an agent on CaOx crystallization fall into two broad categories – those based on urine, and those performed in inorganic solutions. Although it has been acknowledged for some years [10] that urine is the preferred medium for determining an inhibitor's effect on crystallization, since results are more likely to reflect its potency in vivo, *both* types of model were used in the present study. There were two reasons for this. First, the effects of CME on CaOx crystallization had not previously been examined, and it was important to assess its action under controlled experimental conditions free of interfering ions and molecules. Second, the inhibitory effects of very few urinary macromolecules have been tested in urine; most have been studied in inorganic solutions. It was therefore important to obtain similar data for CME, to enable comparisons with other known macromolecular inhibitors to be made.

The findings were conclusive; CME is a potent inhibitor of CaOx crystallization both in human urine and in a defined inorganic solution. However, CME is not a single macromolecule; it consists of a mixture of proteins [5], and it is therefore not possible to attribute its potent inhibitory effect to any single component. Despite the fact that urinary F1 comprises by far the major portion of the proteins detected in CME and may therefore be primarily responsible for the latter's inhibitory properties, it is only one of a number of urinary proteins which have been reported to inhibit CaOx growth, and in some cases aggregation. These include a 26-kDa protein isolated by Resnick et al [25,26], crystal surface binding substance with a molecular weight of 70 kDa [17], inter-α–trypsin inhibitor with an M_r of 40 kDa [39], a 30 kDa protein or proteoglycan described by Nishio et al. [23], and nephrocalcin. Of these, only nephrocalcin has been studied in detail. It was isolated some years ago by Nakagawa and colleagues [21] and described as a potent inhibitor of CaOx crystal growth, and later by the same group [13] as an inhibitor of CaOx crystal aggregation, in a seeded metastable crystallization system. Any or all of these proteins may be present in CME and may therefore contribute to its inhibitory properties. It is therefore germane to compare the effects of CME with those of other individual macromolecules.

In the inorganic assay system used here CME inhibited both crystal growth and aggregation in a dose-dependent manner, with its effect on the latter being more pronounced. In this respect it is similar to a number of macromolecules which have been shown [8, 32] to inhibit aggregation strongly and crystal growth only weakly under identical experimental conditions. At the highest concentration tested, 1.25 mg/l, CME inhibited crystal aggregation by around 88%. Using a similar seeded, inorganic crystallization system, nephrocalcin has been shown by Hess and coworkers to have a like effect [13]. Using data published in their paper [13], it is possible to calculate that nephrocalcin inhibits CaOx crystal aggregation by approximately 90% at a final concentration of 1.5 mg/l. This is not surprising: a number of proteins, including human serum albumin, α-globulin, and a mixture of α and β-globulins inhibit crystal aggregation to an equivalent degree in a similar experimental system [8]. However, it is its effect on crystal growth that distinguishes CME from these other macromolecules. Typically, these retard crystal growth by less than 5% at concentrations around 1 mg/l [8]. In contrast, CME strongly inhibits crystal growth by 65%. In a similar crystallization system nephrocalcin retards growth by around 70% at a concentration of 36 mg/l and after 4 h incubation [21]. Thus, insofar as it strongly inhibits both aggregation *and* growth, CME differs markedly from other urinary macromolecules, including nephrocalcin, whose inhibitory activities have been reported, suggesting that, individually, they cannot account for the properties of CME. The disproportionate abundance of urinary F1 within CaOx crystals would suggest that this protein is responsible for the major portion of the activity of CME. If this is so, then urinary F1 is unique among the urinary proteins whose effects in inorganic crystallization systems have been measured to date.

More recently, uropontin, a protein possessing marked molecular homology with osteopontin, has been purified from human urine and shown to inhibit CaOx crystal growth, though its effect on aggregation has not been described [38]. No relationship between uropontin, urinary F1, and nephrocalcin has been identified to date, but the inhibition data described above would suggest that nephrocalcin and urinary F1 are different proteins. Furthermore, both appear to be distinct from uropontin. The three proteins have different molecular weights [5, 21, 38] and immunohistochemical distributions within the human nephron [14, 22, 41]. However, although it is clear that all these proteins inhibit CaOx crystal growth and/or aggregation in an inorganic assay system, they do not necessarily fulfil a definitive function in CaOx stone pathogenesis. As stated above, an inhibitor's effect on CaOx crystallization in a defined inorganic milieu cannot be used to predict its likely effects under physiological conditions. A more reliable indication of the respective roles of urinary F1, uropontin, and nephrocalcin in stone formation might therefore be provided by evidence that their inhibitory potencies are retained in human urine. However, to date, the effects of none of them have been determined in undiluted urine, and thus their roles in stone formation and relationship to each other await further clarification.

Like other urinary proteins previously examined, CME did not alter the metastable limit of the urine with respect to CaOx [7, 9, 35]. It did, however, prove to be the most potent inhibitor of CaOx crystal aggregation induced in vitro in undiluted urine that has yet been reported. It reduced

crystal aggregation in a dose-dependent manner, and at a concentration of 10 mg/l almost completely reversed the formation of large CaOx aggregates that occurs when crystallization is induced by addition of oxalate to urine from which the macromolecules have been removed by ultrafiltration [7, 35]. In contrast, a concentration of 20 mg/l human serum albumin exerts virtually no effect in the same experimental system [35] – despite its potent effect on aggregation in a CaOx metastable solution [8]. This discrepancy, and the fact that protein concentrations approximately 10 times greater are required to inhibit aggregation in urine, compared with inorganic assay systems, emphasise the enormous disparity between inorganic solutions and human urine and affirm the need to test the effects of inhibitors in the latter.

The only other natural urinary protein whose effects have been tested with the undiluted urine technique used in the present study, Tamm-Horsfall glycoprotein (THG), inhibits crystal aggregation at a concentration of 20 mg/l [35]. Unlike CME, however, THG probably retards crystal aggregation by physical interference, rather than by binding to the crystal surface and altering the zeta potential. THG exists in urine in a polymerized state, forming discrete particles which may prevent newly formed single crystals from becoming close enough to allow aggregate formation [35]. Such a mode of action is supported by the fact that, despite its abundance in urine, the protein cannot be immunologically detected in CaOx crystals precipitated from urine [5]. However, although THG would be expected to reduce crystal aggregation in urine in vivo, it cannot be the sole agent responsible for preventing the clustering of CaOx crystals into large aggregates in urine. THG is almost completely removed from urine by both centrifugation and filtration [4]. Yet CaOx crystals precipitated from urine which has been subjected to these procedures tend to be either single, or aggregated into small clumps, indicating that at least one other urinary macromolecule, perhaps urinary F1, must be responsible for preventing the transition to large crystal aggregates that occurs upon ultrafiltration [7, 35].

Although CME clearly inhibited both crystal growth and aggregation in the inorganic assay system, and aggregation in the urine model, its influence on crystal growth in urine was less easily interpreted. Increasing concentrations of CME in the dose response experiments actually increased the volume of material deposited during the incubation period, suggesting that it was promoting crystal growth. However, experiments performed with an additional 10 urine specimens using [^{14}C]oxalate showed that CME had no effect on the net amount of CaOx precipitated, thus demonstrating that the increase in volume in the dose response experiments was not the result of a promotion of oxalate deposition. In fact, the results strongly suggest that CME is an inhibitor of crystal growth. Any inhibitor of crystal aggregation which does not also directly inhibit crystal growth must indirectly promote the amount of CaOx precipitated by increasing the crystal surface area available for deposition of calcium and oxalate ions [36]. Thus, because CME inhibited crystal aggregation, had it not also inhibited crystal growth the amount of radioactive oxalate precipitated would have increased. The fact that it was identical in its presence and absence indicates that CME must have also reduced the amount of CaOx deposited *per crystalline particle*; CME is therefore also an inhibitor of crystal growth in urine. The proportional increase in particle volume observed in the dose response experiments is therefore worthy of further comment.

When CME is added to ultrafiltered urine particles of protein cannot be detected by the Coulter Counter, as occurs with THG [11]. Furthermore, precipitation of protein does not occur during the incubation period, since controls to which CME but no oxalate load was added showed no increase in particle volume during the experiment. Incompletely dissolved or precipitated protein in CME cannot therefore explain the proportional increase in total volume that occurred with the addition of increasing quantities of CME to the urine samples. On the other hand, entrapment of protein within the crystalline architecture can provide an explanation for the phenomenon. The Coulter Counter estimates a particle's volume and derives its size from the volume of electrolyte it displaces as it passes through the counting orifice: it cannot account for differences in particle density. CaOx crystals precipitated from urine contain considerable amounts of urinary protein [5, 19, 20], and SEM studies of such crystals which have been partly dissolved show that this organic material can occupy a significant portion of the intracrystalline space [3]. Inclusion of larger amounts of proteins, notably urinary F1, within the CaOx crystal structure in response to increasing concentrations of CME will therefore be recorded by the Coulter Counter as greater particle volume, even when the amount of CaOx deposited is exactly the same – as occurred here.

In summary, this study has demonstrated conclusively that CME inhibits CaOx crystal growth in both aqueous inorganic solution and in undiluted human urine. The fact that it is present in CaOx crystals indicates that its inhibitory effect results from binding of its component proteins to the crystal surface. In particular, the disproportionately high concentration of urinary F1 in CaOx crystals relative to the urine whence it was derived [5] is an indication of the extraordinary strength of this association. Prima facie, the association of potent inhibitory activity with the presence of urinary F1 and other proteins within the crystal might seem paradoxical; foreign molecules will become embedded within a crystalline structure only if the growth front envelops them after they have bound to the exposed crystal surface. The presence of large concentrations of a macromolecule within CaOx crystals might therefore suggest that it has only a minor, or no, inhibitory effect on the deposition of calcium and oxalate ions – otherwise growth would not have occurred. However, even the most potent of crystal poisons, despite binding avidly to a crystal surface, would not be able to prevent solute deposition in the face of overwhelming levels of supersaturation [2]. Therefore, though CME is unlikely to prevent CaOx precipitation in vivo, it may nonetheless reduce the deposition of CaOx after crystal formation has occurred.

But it is the effect of CME on CaOx crystal aggregation which has the more vital implications for stone pathogenesis. By preventing the aggregation of newly formed CaOx crystals in the kidney tubules, CME might significantly lessen the probability of particle retention, allowing them to be flushed harmlessly from the urinary tract. Therefore until such time as experimental evidence satisfactorily proves that other urinary proteins such as nephrocalcin or uropontin also inhibit CaOx crystal aggregation in undiluted human urine, the results presented here demonstrate that CME is the most potent inhibitor of CaOx crystallization in human urine yet described, and a potentially important determinant of stone formation. Prothrombin fragment F1 is known to inhibit the growth of hydroxyapatite crystals in inorganic solution [30] and the fact that urinary F1 is the predominant component of CME strongly suggests that it may account for most, if not all, of its inhibitory activity – a possibility whose confirmation must await repetition of the present study using pure urinary F1 isolated from CME. It is imperative that future studies clarify the role of the constituent proteins of CME in CaOx urolithiasis, and in particular address the question of their relationship to other urinary proteins such as nephrocalcin and uropontin, which may also influence the pathogenesis of the disease.

Acknowledgements This work was supported by grants 910864 and 940283 from the National Health and Medical Research Council of Australia.

References

1. Boyce WH, Garvey FK (1956) The amount and nature of the organic matrix in urinary calculi: a review. J Urol 76:213
2. Cabrera N, Vermilyea DA (1958) In: Doremus RH, Roberts BW, Turnbull D (eds) Growth perfection of crystals. Wiley, New York, p 393
3. Doyle IR. The role of urinary proteins in calcium oxalate urolithiasis. PhD thesis, The Flinders University of South Australia 1992
4. Doyle IR, Ryall RL, Marshall VR (1989) The effect of low-speed centrifugation and millipore filtration on the urinary protein content. In: Walker VR, Sutton RAL, Cameron ECB, Pak CYC, Robertson WG (eds) Urolithiasis. Plenum Press, New York, p 593
5. Doyle IR, Ryall RL, Marshall VR (1991) The incorporation of urinary proteins into calcium oxalate crystals precipitated from human urine: A highly selective phenomenon. Clin Chem 37:1589
6. Drach GW, Thorson S, Randolph A (1980) Effects of urinary organic macromolecules on crystallization of calcium oxalate: enhancement of nucleation. J Urol 123:519
7. Edyvane KA, Hibberd CM, Harnett RM, Marshall VR, Ryall RL (1987) Macromolecules inhibit calcium oxalate crystal growth in whole human urine. Clin Chim Acta 167:329
8. Edyvane KA, Ryall RL, Marshall VR (1986) The influence of serum and serum proteins on calcium oxalate crystal growth and aggregation. Clin Chim Acta 157:81
9. Edyvane KA, Ryall RL, Mazzachi RD, Marshall VR (1987) The effect of serum on the crystallization of calcium oxalate in whole human urine: inhibition disguised as apparent promotion. Urol Res 15:87
10. Fleisch H (1978). Inhibitors and promoters of stone formation. Kidney Int 13:361
11. Grover PK, Ryall RL, Marshall VR (1990) Does Tamm-Horsfall mucoprotein inhibit or promote calcium oxalate crystallization in human urine? Clin Chim Acta 190:223
12. Hallson PC, Rose GA (1979) Uromucoids and urinary stone formation. Lancet 1:1000
13. Hess B, Nakagawa Y, Coe FL (1989) Nephrocalcin isolated from human kidney stones is a defective calcium-oxalate-monohydrate crystal-aggregation inhibitor. In: Walker VR, Sutton RAL, Cameron ECB, Pak CYC, Robertson WG (eds) Urolithiasis. Plenum Press, New York, p 137
14. Hoyer JR (1994) Role of uropontin in calcium stone formation. In: Ryall RL, Bais R, Marshall VR, Rofe AM, Smith LH, Walker VR (eds) Urolithiasis II. Plenum Press, New York, p 253
15. Iwata H, Kamei O, Abe Y, Nishio S, Wakatsuki A, Ochi K, Takeuchi M (1988) The organic matrix of urinary uric acid crystals. J Urol 139:607
16. 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
17. Koide T, Yoshioka T, Yamaguchi S, Hosokawa S, Utsunomiya M, Sonoda T (1990) Urinary crystal surface binding substances on calcium oxalate crystals. Urol Res 18:387
18. Lian JB, Prien EL Jr, Glimcher MJ, Gallop PM (1977) The presence of protein-bound γ-carboxyglutamic acid in calcium-containing renal calculi. J Clin Invest 59:1151
19. Morse RM, Resnick MI (1988) A new approach to the study of urinary macromolecules as participant in calcium oxalate crystallization. J Urol 139:869
20. Morse RM, Resnick MI (1989) A study of the incorporation of urinary macromolecules into crystals of different mineral composition. J Urol 141:641
21. Nakagawa Y, Abram F, Kézdy J, Kaiser ET, Coe FL (1983) Purification and characterization of the principal inhibitor of calcium oxalate monohydrate crystal growth in human urines. J Biol Chem 258:12594
22. Nakagawa Y, Netzer M, Coe FL (1990) Immunohistochemical localization of nephrocalcin (NC) to proximal tubule and thick ascending limb of Henle's loop (TAHL) of human and mouse kidney: resolution of a conflict. Kidney Int 37:A474
23. Nishio S, Wakatsuki A, Iwata H, Matsumoto A, Takeuchi M (1989) Urinary macromolecular-inhibitory-substances of calcium-oxalate-monohydrate crystal growth. In: Walker VR, Sutton RAL, Cameron ECB, Pak CYC, Robertson WG (eds) Urolithiasis. Plenum Press, New York, p 235
24. Petersen TE, Thøgerson I, Petersen SE (1989) Identification of hemoglobin and two serine proteases in acid extracts of calcium containing kidney stones. J Urol 142:176
25. Resnick MI, Gammon CW, Sorrell MB, Boyce WH (1980) Calcium-binding proteins and renal lithiasis. Surgery 88:239
26. Resnick MI, Sorrell ME, Bailey JA, Boyce WH (1982) Inhibitory effects of urinary calcium-binding substances and calcium oxalate crystallization. J Urol 127:568
27. Robertson WG, Peacock M (1972) Calcium oxalate crystalluria and inhibitors of crystallisation in recurrent renal stone-formers. Clin Sci 43:499
28. Robertson WG, Scurr DS (1986) Modifiers of calcium oxalate crystallization found in urine. I. Studies with a continuous crystallizer using an artificial urine. J Urol 135:1322
29. Robertson WG, Peacock M, Nordin BEC (1968) Activity products in stone-forming and non-stone-forming urine. Clin Sci 34:579
30. Romberg RW, Werness PG, Riggs BL, Mann KG (1986) Inhibition of hydroxyapatite crystal growth by bone-specific and other calcium-binding proteins. Biochemistry 25:1176
31. Ryall RL, Bagley C, Marshall VR (1981) Independent assessment of the growth and aggregation of calcium oxalate crystals using the Coulter Counter. Invest Urol 18:401
32. Ryall RL, Harnett RM, Marshall VR (1981) The effect of urine, pyrophoshate, citrate, magnesium and glycosaminoglycans on the growth and aggregation of calcium oxalate crystals in vitro. Clin Chim Acta 112:349

33. Ryall RL, Hibberd CM, Marshall VR (1985) A method for studying inhibitiory activity in whole urine. Urol Res 13:285
34. Ryall RG, Ryall RL, Marshall VR (1986) A computer model for the determination of extents of growth and aggregation of crystals from changes in their size distribution. J Cryst Growth 76:290
35. 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 urine. Urol Res 19:181
36. Ryall RL, Ryall RG, Doyle IR, Marshall VR (1993) The dependence of crystal growth on the state of crystal aggregation: Implications for urolithiasis research. J Crystal Growth 133:87
37. Scurr DS, Robertson WG (1986) Modifiers of calcium oxalate crystallization in urine. II. Studies on their mode of action in an artificial urine. J Urol 136:128
38. Shiraga H, Min W, VanDusen WJ, Clayman MD, Miner D, Terrell CH, Sherbotie JR, Foreman JW, Przysiecki C, Neilson EG, Hoyer JR (1992) Inhibition of calcium oxalate crystal growth in vitro by uropontin: Another member of the aspartic acid-rich protein superfamily. Proc Natl Acad Sci USA 89:426
39. Sørenson S, Hansen K, Bak S, Justesen SJ (1990) An unidentified macromolecular inhibitory constituent of calcium oxalate crystal growth in human urine. Urol Res 18:373
40. Stapleton AMF, Ryall (in press) Blood coagulation proteins and urolithiasis are linked: Crystal matrix protein is the F1 activation peptide of human prothrombin. Br J Urol
41. Stapleton AMF, Seymour AE, Brennan JS, Doyle IR, Marshall VR, Ryall RL (1993) The immunohistochemical distribution and quantification of crystal matrix protein. Kidney Int 44:817
42. Stapleton AMF, Simpson RJ, Ryall RL (1993) Crystal matrix protein is related to human prothrombin. Biochem Biophys Res Commun 195:1199