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The Effect of Serum on the Crystallization of Calcium Oxalate in Whole Human Urine: Inhibition Disguised as Apparent Promotion

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Summary. The effect of serum on calcium oxalate crystallization was studied in whole human urine. At concentrations between 0.005% to 0.10% (v/v), serum had no effect on the metastable limit of urine as detected by the Coulter Counter. However, serum caused a marked increase in the number and volume of calcium oxalate crystals deposited in response to an oxalate load. That this increase in volume reflected true deposition of calcium oxalate was confirmed by determining calcium concentration and by including control urines containing added serum but no exogenous oxalate. Analysis of particle size distributions showed that the crystals deposited in the presence of serum were smaller than those occurring in the control. Since serum did not affect the limit of metastability, the enhancement of crystal volume is unlikely to be a result of heterogeneous nucleation. We hypothesize that the apparent promotion effect of serum is due to its inhibition of crystal aggregation which increases the crystal surface area available for calcium oxalate deposition.

Key words: Urolithiasis, Calcium oxalate, Crystal growth and aggregation, Serum, Whole urine.

Introduction

It has been proposed by some workers that a deficiency or lack of urinary inhibitors could be the main factor that distinguishes stone formers from normal individuals. Conversely, others have maintained that the predisposition to stone formation is caused by an abundance of promotory substances.

Many inhibitors of crystallization have been identified, but few molecules have been shown to act as promoters. The inevitable occurrence of an organic matrix in urinary calculi [1] and its mucoportein properties [2] were proposed as support for the theory that proteins promote urolithiasis by binding inorganic ions and inducing epitactic nucleation of stone salts. However, evidence has also been obtained [25] which indicates that the presence of matrix in stones can just as easily be explained by the incorporation of urinary protein into the crystalline phase by adventitious adsorption on to the growing crystal surface. The lack of conclusive evidence for either mechanism of matrix inclusion has, in recent years, prompted an examination of the effect of urinary proteins on calcium oxalate crystallization. Uromucoid [9], Tamm-Horsfall mucoprotein [18] and an unidentified protein [15] have been reported to promote the precipitation of calcium oxalate from homogeneous solutions. However, results have been conflicting: others have been unable to detect any promoting effect of uromucoid [6] or Tamm-Horsfall mucoprotein [7, 11, 17, 23] or indeed of any urinary macromolecules [8] on the spontaneous precipitation and growth of calcium oxalate. On the contrary, these authors and others have reported that Tamm-Horsfall mucoprotein and protein fractions [10, 12] are efficient inhibitors of these processes. Following observations [4, 13] that haematuria influences the measurement of inhibitory activity, we examined the effect of human serum albumin and globulins on calcium oxalate crystal growth and aggregation in a dilute seeded crystal system [5]. All inhibited growth, but had a more potent effect on crystal aggregation. The aim of this study was to evaluate the influence of human serum on calcium oxalate crystallization in whole human urine, with particular emphasis on its possible ability to promote crystal nucleation.

Materials and Methods

Collection and Preparation of Urine

24 h urine samples were collected without preservative from 6 healthy men during the same 24 h period. Samples were refrigerated during the collection period and during storage prior to use. Each sample was checked for microscopic haematuria using Multistix test strips (Miles Laboratories, Australia): none showed any evidence of blood. They were then pooled. Two additional pooled specimens

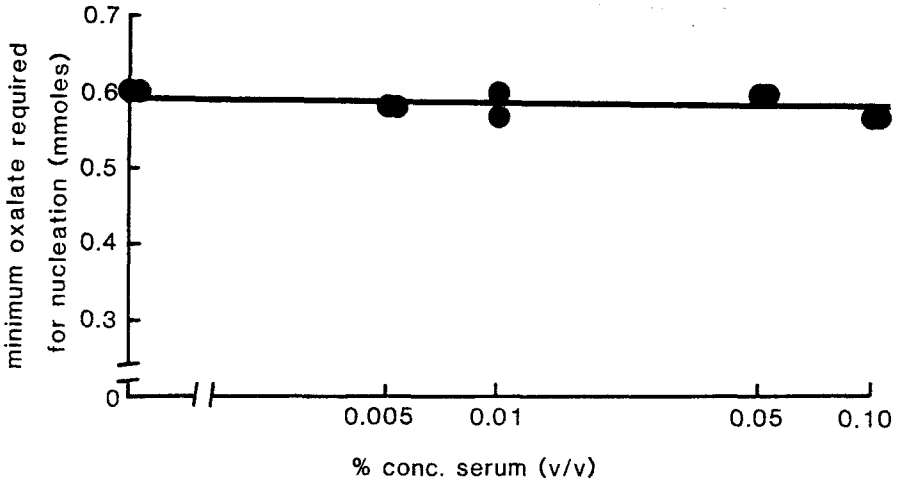

Fig. 1. The effect of serum on the metastable limit. Each data point denotes one titration

were obtained in the same manner. The urine was then centrifuged at 500 x *g* for 10 min. Examination of the sediment under the light microscope revealed only cellular debris. The supernatant was filtered through a 0.22 μm filter. During the period of study the urine was stored at 4 °C and prior to each experiment was warmed to 37 °C and refiltered. At each step the absence of crystals was confirmed microscopically.

Fresh samples of filtered serum (0.22 μm) were obtained from 3 normal individuals and added to aliquots of the pooled urines prior to each experiment.

Experimental System

The experimental system for measuring the parameters of crystal growth and aggregation and the metastable limit in whole urine is described in detail elsewhere [21]. In outline, the system is divided into two sections: the first part of the technique involves the empirical determination of the metastable limit of the urine. This is defined as the minimum amount of oxalate required to produce crystals detectable by the Coulter Counter and is determined by titrating 20 ml aliquots of urine with sodium oxalate followed by incubation at 37 °C for 30 min in a shaking water bath. The number of crystals > 2 μm in each sample is then determined using a Coulter Counter. Crystal number initially rises linearly with increasing oxalate concentration. The point at which this line intercepts the abscissa is taken as the minimum amount of oxalate necessary to induce detectable crystal formation and is defined as the empirical metastable limit of the urine. The second part of the technique involves examining crystal growth after nucleation has occurred. Once the minimum amount of oxalate required to produce nucleation in 20 ml of urine is determined, five times this amount plus an additional 30 μmoles is then added dropwise to flasks containing 100 ml of urine and the growth of precipitated crystals is followed for 90 min using the Coulter Counter to measure the crystal size distribution. In this study, each experiment was repeated to give a total of 4 replicates for each pooled urine sample. To take account of the possibility of precipitated protein interfering with the interpretation of the data, controls consisting of incubations omitting exogenous oxalate were routinely included.

At the end of each experiment the precipitated crystals were removed by filtration and examined under the light microscope.

Measurement of Crystal Growth Using Calcium Depletion

During the 90 min incubation period, 2 ml aliquots of urine were removed from the flasks at 10 min intervals and filtered through a 0.22 μm filter. 50 μl of conc. HCl was then added to each aliquot to dissolve any calcium oxalate crystals formed after filtration and the calcium concentrations were determined using a Varian AA875 Atomic Absorption Spectrophotometer (Varian Techtron, Melbourne, Australia). These measurements were performed on 2 separate urine samples processed identically to those used in the crystallization experiments.

Statistical Methods. For the sake of clarity, and in order to show the variation in the data obtained in each set of experiments, results are presented as the mean ± 1 standard deviation. Statistical analysis of results, however, was made using non-parametric methods: the Wilcoxon test for matched pairs was used for comparison of curves.

Results

The Effect of Serum on the Metastable Limit

The minimum amount of sodium oxalate required to produce detectable crystals in urine containing increasing amounts of serum is shown in Fig. 1. It can be seen that this amount was unaltered in the presence of serum: even at a serum concentration of 0.1%, which would be equivalent to macroscopic haematuria, there was no change in the metastable limit. Similar results were obtained with the additional pooled urines.

The Effect of Serum on the Deposition of Calcium Oxalate

Figure 2 shows the measured crystal volume in urine and in urine containing 0.01% (v/v) serum during the 90 min incubation period. The total crystal volumes shown here were derived from the crystal numbers measured by the Coulter Counter. It can be seen that when the oxalate load was added there was an initial lag phase in the two growth curves. This lag phase is characteristic of the method and is followed by a period of linear growth. When 0.01% serum was added to urine there was a small, but significant increase ($P < 0.01$) in the volume of calcium oxalate crystals precipitated in comparison with the control urine, which was sustained up to 60 min. After this time the volumes were indistinguishable.

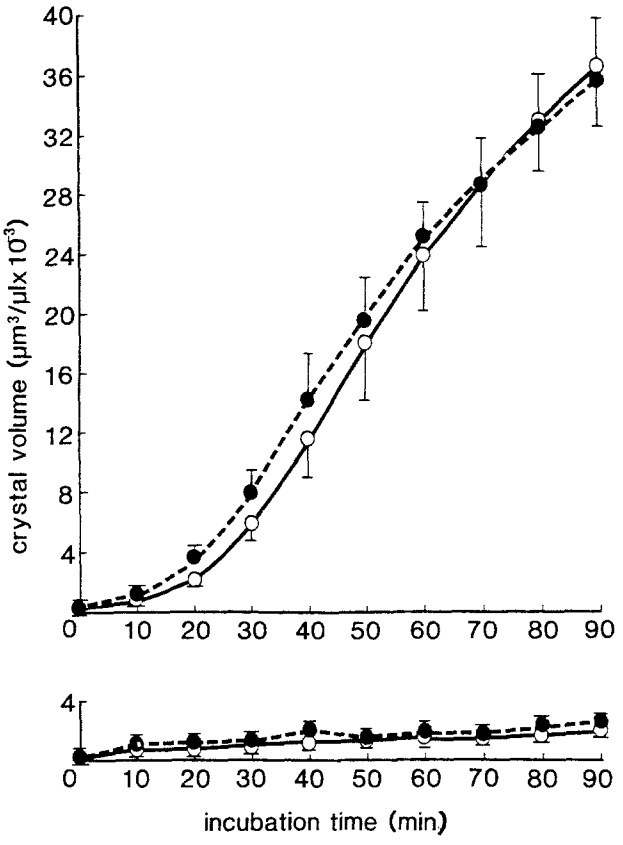

Fig. 2. The change in crystal volume in whole urine (○) and in the presence of 0.01% serum (●), after a given load of oxalate above the metastable limit. Each *point* denotes the mean (± 1 S.D.) of 6 replicates. For the sake of clarity the *lower axis* shows the change in particle volume in the absence of an oxalate load, in urine (○) and in the presence of 0.01% serum (●), where each *point* represents the mean of 3 replicates

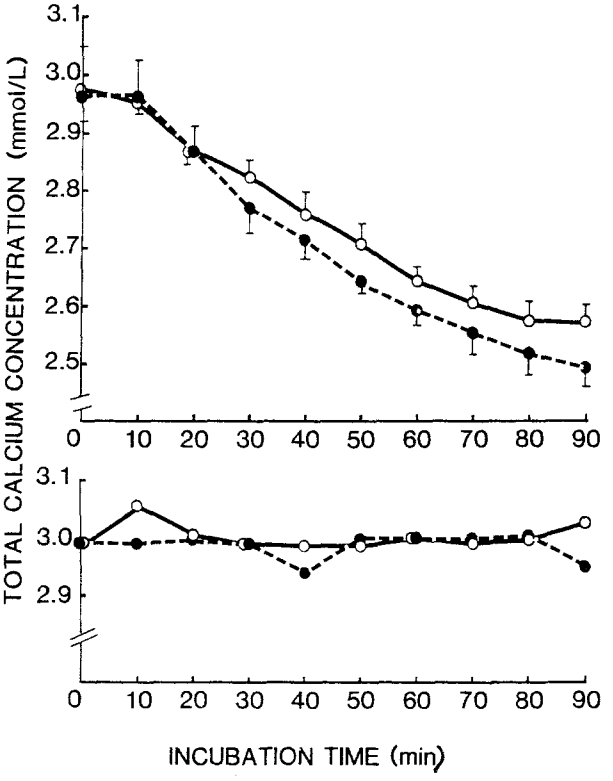

Fig. 3. The change in calcium concentration in whole urine (○) and in the presence of 0.01% serum (●), after a given load of oxalate above the metastable limit. Each *point* denotes the mean (± 1 S.D.) of 4 replicates. The *lower axis* shows the change in calcium concentration in the absence of an oxalate load in urine (○) and in the presence of 0.01% serum (●), where each *point* represents the mean of 2 replicates

It is noteworthy that in the serum and control urines which had no added oxalate there was no increase in crystal volume during the 90 min incubation, indicating that the increased volume in the presence of serum was not caused by precipitated protein. This experiment was repeated in the two additional pooled urines, with similar results, but with the magnitude of the promotion varying with each sample. Addition of the serum did not alter the morphology of the precipitated crystals, which consisted almost exclusively of calcium oxalate dihydrate.

Measurement of Calcium Oxalate Deposition Using Calcium Depletion

In order to confirm that the increase in crystal volume detected by the Coulter Counter in the presence of serum reflected true calcium oxalate deposition, measurements of calcium depletion were made in a separate urine sample. The effect of serum on calcium oxalate deposition, expressed as decreasing calcium concentration is shown in Fig. 3. The decrease in urinary calcium during the incubation was found to be significantly more rapid ($P < 0.01$) in the presence of 0.01% serum, clearly indicating an increase in the rate of calcium oxalate crystal deposition. The two controls showed no change in calcium concentration during the experiment. Similar results were obtained with a second urine sample.

The Effect of Serum on Crystal Number

Figure 4 shows the increase in crystal number for the same incubations shown in Fig. 2. There was a significant increase ($P < 0.01$) in the number of crystals in the urine containing 0.01% serum above that occurring in the control urine. The two controls which had no added oxalate showed no increase in crystal number over the incubation period. Similar results were obtained with the additional pooled urines.

Figure 5 shows the crystal volume distributions recorded by the Coulter Counter 90 min after addition of the oxalate load, in the presence and absence of serum. Although the total crystal volumes deposited in the presence and absence of serum were similar, as designated by the areas under the curves, the modes of the curves were different. In the pres-

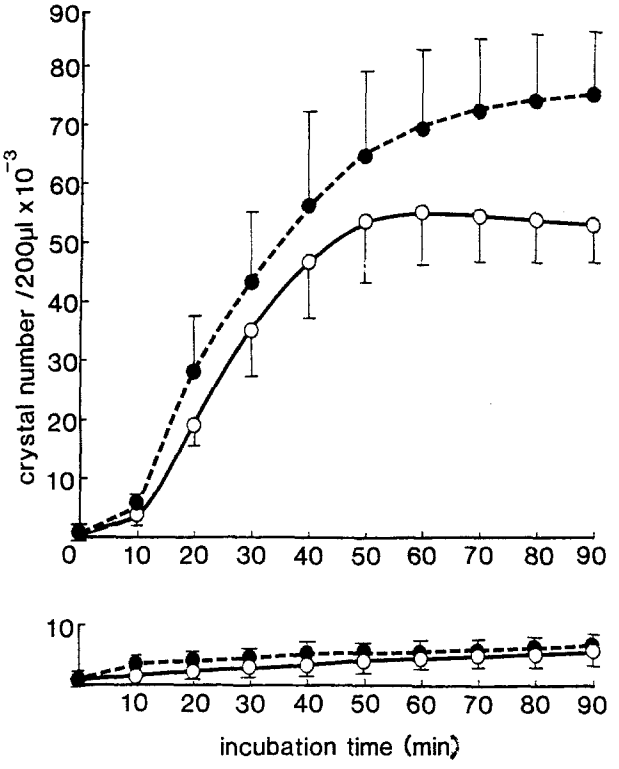

Fig. 4. The change in particle number in whole urine (○) and in the presence of 0.01% serum (●), after a given load of oxalate above the metastable limit. Each *point* denotes the mean (± 1 S.D.) of 6 replicates. The *lower axis* shows the change in particle number in the absence of an oxalate load, in urine (○) and in the presence of 0.01% serum (●), where each *point* represents the mean of 3 replicates

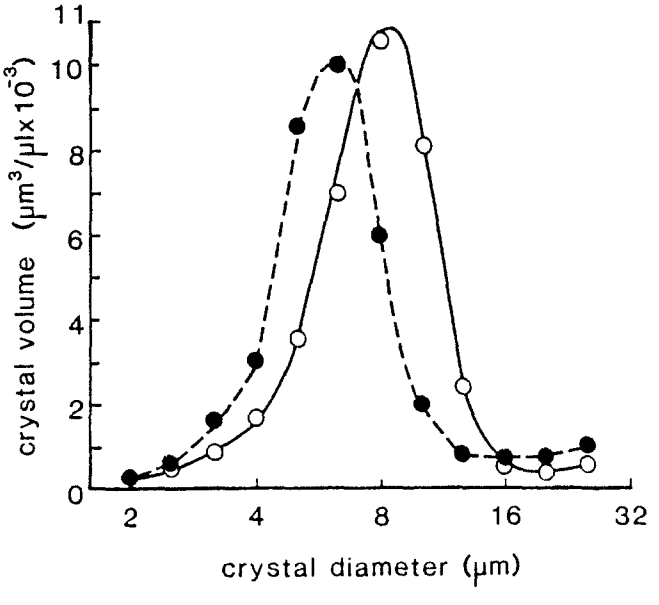

Fig. 5. The particle volume distribution in whole urine (○) and in the presence of 0.01% serum (●), at 90 min after the addition of an oxalate load above the metastable limit. Each *point* represents the mean of 6 determinations

ence of serum the mode of the volume distribution curve corresponded to a diameter of 6.4 µm, while that in the control was equivalent to a diameter of 8 µm.

Discussion

Evidence for a determinant role for proteins in calcium oxalate urolithiasis has been contradictory: proteins have been reported both to inhibit [7, 10–12, 17, 23] and to enhance [3, 9, 15, 18] calcium oxalate crystallization. Our own results have been similarly conflicting. We have shown, using a dilute seeded crystallization system [5] that human serum inhibits crystal aggregation, and to a lesser extent, crystal growth, and that most of this activity can be accounted for by the protein content of the serum. However, in the present investigation we found that the addition of serum significantly increased the volume and number of calcium oxalate crystals precipitated from whole urine. That this increased volume reflected true deposition of calcium oxalate, and not precipitated protein, was confirmed by determining the calcium concentration and by including control urines containing added protein but no exogenous oxalate. The usual explanation offered for this apparent promotory effect of protein is that protein molecules in solution act as a microsubstrate for the deposition of calcium and oxalate ions and thereby induce heterogeneous nucleation of calcium oxalate crystals.

It would therefore appear that the same protein preparation can act as a promoter of calcium oxalate crystallization under certain circumstances and as an inhibitor under others. It is unlikely that these paradoxical effects are simply a consequence of different experimental conditions, with inhibition occurring in the dilute seeded system and promotion in whole urine, since Drach et al. [3] and Paternain et al. [15] reported that mucoprotein enhanced the precipitation of calcium oxalate from inorganic solutions. Furthermore, we have previously reported similarly conflicting findings with a number of inhibitors under the same experimental conditions [19]. In these experiments heparin, chondroitin sulphate, magnesium and citrate inhibited both the growth and aggregation of calcium oxalate seed crystals added to a metastable solution of this salt, over a wide range of concentrations. However, at very low concentrations in their respective dose-response curves all of these substances caused a relatively small, but reproducible enhancement of crystal growth, but not of crystal aggregation. These experiments were later repeated with chondroitin sulphate and the findings confirmed [20]. We proposed in the later study that the dual effect of chondroitin sulphate occurred at very low concentrations because the degree of coverage of the crystal surface was sufficient to interfere with crystal aggregation, while having little effect on crystal growth. This would increase the area of crystal surface exposed to the solution and thus enable a more rapid deposition of calcium oxalate which would be reflected in a larger

total crystal volume relative to that occurring in a control which contained no chondroitin sulphate. By using a computer model [20] we confirmed that the effect of chondroitin sulphate at these very low concentrations, is in fact slightly inhibitory, despite its apparent ability to promote crystal deposition.

It has been shown that crystal growth may still occur under conditions of near maximum adsorption by protein. This phenomenon, which is steric in nature, is related to the inability of the protein to block all of the crystal growth sites on the crystal surface [22]. Under these circumstances one might expect that crystal aggregation would be markedly inhibited, but that crystal growth would be only slightly reduced. Serum protein is a potent inhibitor of calcium oxalate crystal aggregation, but a relatively weak inhibitor of crystal growth [5]. If the amount of protein present in a reaction solution is sufficient to retard the aggregation of seed, or newly nucleated crystals, but not enough to inhibit crystal growth significantly, then we would expect to see a higher crystal number and an increased volume (or mass) of deposited material. Moreover, since aggregation is reduced, the mean diameter of the crystals would also be less in the presence of protein. In the present study the addition of serum resulted in a large increase in the crystal number, a significant increase in the total volume of calcium oxalate precipitated in the initial stages of the reaction, and a marked reduction in the mean crystal size. It might be argued that similar results would occur if the protein were acting as a heterogeneous nucleator of calcium oxalate. However, if this were the case, then increasing concentrations of protein would almost certainly cause a corresponding reduction in the urinary metastable limit. We could not detect such an effect in this study, nor has it been previously reported. On the contrary, Kitamura and Pak [11] found that mucoprotein slightly raised the metastable limit.

We therefore propose that the observed effects of protein on the crystallization of calcium oxalate that we have observed in this study can be explained on the basis of its strong inhibitory influence on crystal aggregation, combined with a relatively weak inhibitory effect on crystal growth. Furthermore, the observations of other workers, including reports of promotory effects, are consistent with this hypothesis. Thus, the higher crystal number and volume observed by Drach et al. [3], and the increased precipitation of calcium oxalate reported by Hallson and Rose [9], Paternain et al. [15], and Rose and Sulaiman [18], which these authors interpreted as promotion of crystallization, could have been secondary to the inhibition of crystal aggregation by protein. None of these authors attempted to measure crystal aggregation and they therefore did not consider the possible effects that the state of crystal aggregation would inevitably have had on the measurement and interpretation of crystal growth when expressed as mass of material deposited [9, 15, 18] or increasing crystal diameter [3]. Thus, by taking into consideration the influence of the state of aggregation on the rate of deposition of solute from solution, it is possible to reconcile the apparently discrepant reports of the effects of protein on calcium oxalate crystallization.

Recent findings suggest that the observations of Hallson and Rose [9] and Rose and Sulaiman [18] may be a consequence, at least in part, of their use of concentrated urine samples. Scurr and Robertson [23] showed that at normal ionic strengths found in urine Tamm-Horsfall mucoprotein is an effective inhibitor of crystal agglomeration, but that at high ionic strengths this macromolecule polymerizes readily and in so doing, interferes with the inhibitory actions of other urinary constituents so that crystal agglomeration is promoted. If, in the concentrated urine system used by Rose and co-workers, the ionic strength of the urine was sufficient to cause the polymerization of Tamm-Horsfall mucoprotein this might provide an alternative or additional explanation for their observed promotory effects of Tamm-Horsfall mucoprotein and the macromolecular fraction greater than 10,000 daltons.

The results of this investigation emphasise other important considerations which must be borne in mind in future studies. Firstly, there is no doubt that serum, and human serum albumin and globulins exert a very potent effect on calcium oxalate crystallization in whole urine. It is therefore vital in any examination of the inhibitory effects of whole or dilute human urine to exclude the possibility of even microscopic haematuria, particularly since this condition is virtually a characteristic feature of the disease. Secondly, since we have shown that the inhibitory effect of serum can masquerade as a promotory influence, it is possible that other urinary components, such as Tamm-Horsfall mucoprotein and glycosaminoglycans may exhibit similar effects at specific concentrations. It is clear that the influence of the state of crystal aggregation on the measured rate of crystal growth should always be taken into account, regardless of the experimental system used. And provided cognizance is also taken of the gross effect of crystal aggregation, crystal growth should be expressed in terms of increasing crystal diameter as has been previously advocated [16, 20, 22].

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