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A Method for Studying Inhibitory Activity in Whole Urine

R. L. Ryall, C. M. Hibberd, and V. R. Marshall

Urology Unit, Department of Surgery, Flinders Medical Centre, Bedford Park, Australia

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Summary. A method has been developed for inducing and quantifying calcium oxalate crystallisation in whole human urine. The propensity of a given urine to induce crystal formation was described in two ways: 1) its ability to resist spontaneous nucleation of calcium oxalate crystals was assessed by titrating 20 mls of the urine with increasing quantities of sodium oxalate (0–150 μmol) to determine its practical metastable limit. This limit was inversely related to the endogenous calcium concentration. 2) its capacity to inhibit crystal growth was quantified by determining the rate of growth of calcium oxalate crystals precipitated in response to a fixed oxalate load (30 μmol) above its metastable limit. The crystals produced were predominantly calcium oxalate dihydrate and were morphologically identical to those occurring naturally in urine. Citrate had no effect on the metastable limits of 3 urines examined, but markedly inhibited crystal growth. Pyrophosphate had a similar effect on crystal growth, and in addition, raised the metastable limit of one of the urine samples.

Key words: Urolithiasis, Calcium oxalate, Whole urine, Crystal growth, Metastable limit, Inhibitory activity.

Introduction

It has been generally acknowledged that urine from both renal stone formers and normal individuals is commonly supersaturated with respect to calcium oxalate [10, 9]. The first prerequisite for calcium oxalate stone formation is thus satisfied in both groups of subjects, and thereby raises the question why stones form in one group and not in the other. The ability of normal people to resist stone formation has been presumed for many years to be due to the presence in urine of inhibitors. Most information regarding inhibitors has been obtained from model aqueous crystallisation systems which have used dilute urine or urine fractions. However, urine contains a number of substances which are known to affect the crystallisation of calcium oxalate, and their relative effects in dilute urine are unlikely to reflect their influence on calcium oxalate crystallisation in whole urine under physiological conditions [3]. Thus there is a recognised need for methods which permit the measurement of the inhibitory capacity of whole urine. In this paper we describe a technique for inducing and measuring calcium oxalate crystallisation in undiluted human urine.

Materials and Methods

Determination of Metastable Limit

Twenty four hour urine specimens were collected without preservative from 32 male volunteers with no history of kidney stone disease. The samples were refrigerated for the duration of the collection and any showing visible signs of precipitation were discarded. The urine was brought to 37 °C and centrifuged at 37 °C for 15 min at 8,000 x g. The urinary sediments were examined microscopically for the presence of crystals: only those in which no crystals were observed were processed further. The urine was then filtered through 0.22 μm Millipore filters. 20 ml aliquots of each urine were then treated with 200 μl samples of sodium oxalate to give final exogenous oxalate concentrations of 0–1.5 mM. Rarely, with very dilute urines, this concentration range had to be doubled. The urine samples were incubated in a shaking water bath at 37 °C for 30 min and the number of crystals larger than 2 μm in each sample was determined using a Coulter Counter Model TAII fitted with a Population Count Accessory. In those aliquots in which crystallisation was detected, crystal number initially rose linearly in response to increasing oxalate concentration (Fig. 1). The presence of crystals was confirmed by microscopic examination. At the higher oxalate concentrations this linear relationship was not always observed, presumably because of significant crystal aggregation, and particle coincidence caused by the large number of crystals produced in these urine samples. The minimum amount of oxalate necessary to induce nucleation detectable by the Coulter Counter, i.e. crystals above 2 μm in diameter, was taken to be the measured limit of metastability of each urine and was determined by interpolation of the line to the abscissa as shown in Fig. 1. This measured limit of metastability will depend to varying degrees upon the urinary ionic concentrations of calcium and oxalate and upon the concentrations of inhibitors and promoters. In one urine crystallisation occurred in

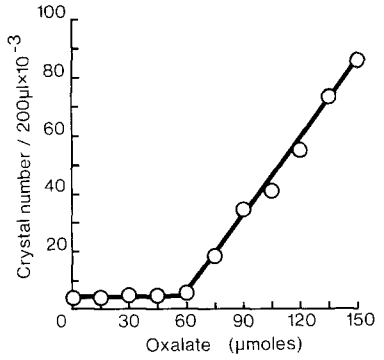

Fig. 1. The determination of the practical metastable limit of a urine. Crystal number after a 30 min incubation period is plotted in relation to the amount of oxalate added to 20 ml aliquots of the urine

the absence of any added oxalalte, indicating pre-existing supersaturation of the urine with calcium oxalate. In this case the line was extrapolated to give a negative value of oxalate.

Where the effects of citrate and pyrophosphate were being tested, 1.1 ml of solution was added to 220 mls of the urine before division into the 20 ml aliquots. Pyrophosphate was tested at a final exogenous concentration of 5×10^{-5} M and citrate at a final concentration of 4.4×10^{-3} M, to produce an approximate doubling of the mean reported endogenous concentrations of these compounds [3, 15]. The endogenous concentrations of pyrophosphate and citrate in the urine samples were not measured.

Response to a Fixed Oxalate Load Above the Metastable Limit

Once the minimum amount of oxalate necessary to induce precipitation in 20 mls of urine was determined, 5 times that quantity of oxalate, plus an additional load of 30 µmol in 1 ml was added dropwise to triplicate 100 ml samples of the urine. Citrate and pyrophosphate were added in a volume of 0.5 ml.

The urines were then incubated in a shaking water bath for 90 min at 37 °C and the Coulter Counter was used to determine the number and volume of precipitated crystals at 10 min intervals.

Calcium was measured by atomic absorption spectroscopy.

Results

The coefficient of variation for the determination of the minimum amount of oxalate necessary to induce precipitation in 10 samples of the same urine was 5.6%.

Three of the 32 urine samples precipitated predominantly "coffin"-shaped calcium oxalate monohydrate (COM) crystals (Fig. 2a), while the crystals produced in the remaining urines consisted principally or entirely of classical "envelope" calcium oxalate dihydrate (COD) crystals, either singly (Fig. 2b) or in varying sized aggregates (Fig. 2c) identical to those occurring naturally in urine, but unlike those occurring in hyperoxaluria where the monohydrate predominates.

Figure 3 shows the relationship between the minimum amount of oxalate necessary to induce spontaneous nucleation of calcium oxalate and the endogenous urinary calcium concentration. There was a significant ($n = 32$, $r = -0.68$, $p < 0.0005$) inverse relationship between the amount of oxalate and the corresponding calcium concentration.

The time course of crystal growth in response to the 30 µmol oxalate load above the metastable limit is shown in Fig. 4. These results represent the mean of 10 experiments on the same urine sample, but are typical of those obtained

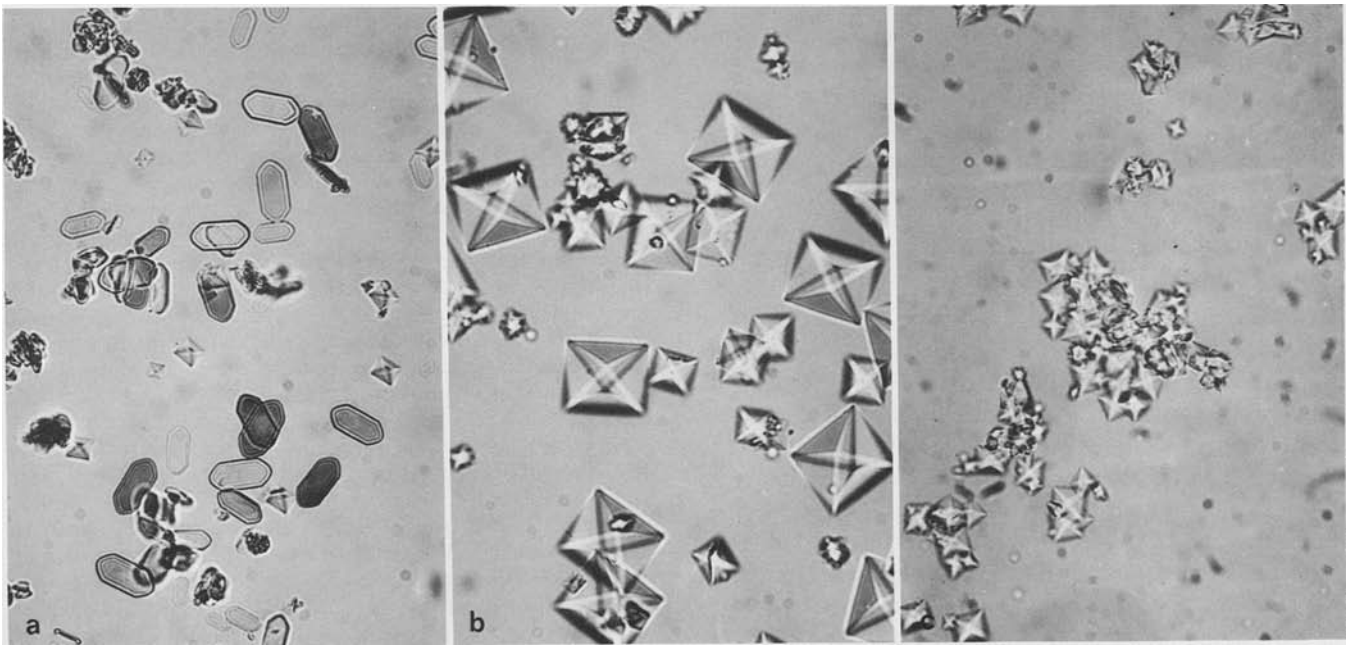

Fig. 2. a "Coffin" calcium oxalate monohydrate crystals precipitated from a normal urine sample. A few "envelope" calcium oxalate dihydrate crystals can also be seen (×400). b Single crystals and small aggregates of calcium oxalate dihydrate (×400). c Large aggregate of calcium oxalate dihydrate (×400)

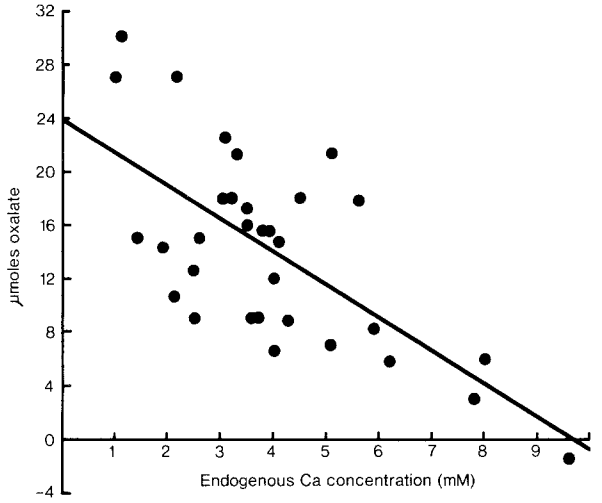

Fig. 3. The relationship between the minimum amount of oxalate required to induce spontaneous nucleation of calcium oxalate in 100 mls of urine and the endogenous concentration of calcium ($n = 32, r = -0.68, p < 0.0005$, least squares analysis)

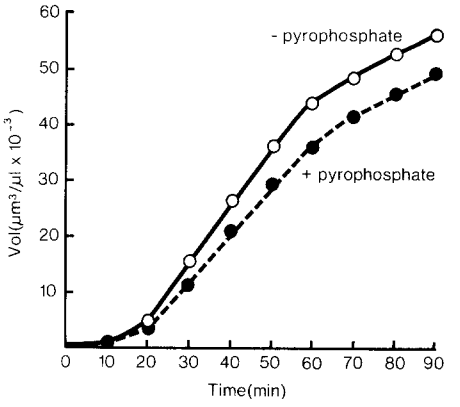

Fig. 6. The effect of pyrophosphate on crystal growth in a urine sample from a normal male. The rate of crystal growth as designated by the slope of the linear portion of the curve is reduced by 20% in the presence of pyrophosphate

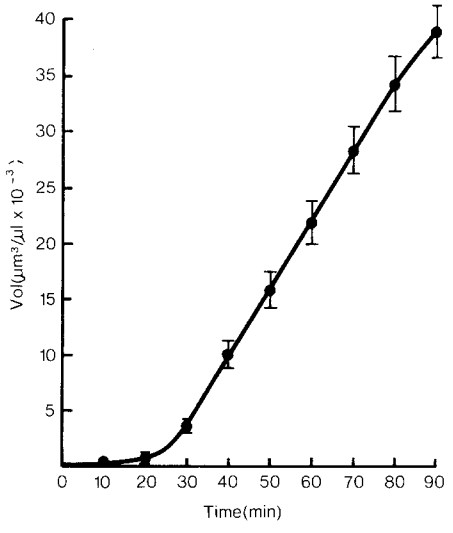

Fig. 4. The increase in crystal volume with time in a urine sample. The points represent the mean of 10 determinations and the bars denote 1 standard deviation

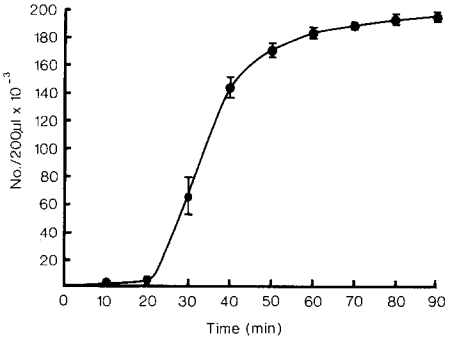

Fig. 5. The change in crystal number with time, corresponding to the volume data shown in Fig. 4. The bars denote 1 standard deviation

with all the urine specimens i.e. an initial time lag followed by a linear portion. The slope of the linear section of the curve was used as an index of the rate of crystal growth. The coefficient of variation for the determination of the rate of crystal growth in this series of 10 experiments was 6.7%.

Figure 5 shows the change in crystal number with time after addition of the oxalate load. The crystal numbers correspond to the volume results shown in Fig. 4, but again, are typical of those obtained with all the urine samples, with an initial time lag followed by a period of rapid increase in crystal number, the rate of which decreased with time. In many urine samples a decrease in crystal number in the latter part of the curve was observed, indicating that crystal aggregation was outweighing the increase in crystal number caused by crystals less than 2 µm in diameter growing and aggregating into the detection range of the instrument.

Pyrophosphate was found to have a variable influence on the metastable limit of the three urine samples tested: in two instances it had no effect, while in the third, it raised the amount of oxalate required to produce nucleation from 11.6 mol to 17.4 mol. At the same concentration, pyrophosphate also retarded the rate of crystal growth which occurred after addition of the oxalate load (Fig. 6) in all cases, the degree of retardation being a function of each individual urine sample. In the example shown in Fig. 6 the rate of crystal growth calculated from the linear portion of the curve in the presence of pyrophosphate was 80% of that occurring in the control, which contained no added pyrophosphate.

Citrate at the concentration tested did not affect the metastable limit of any of the three urines examined. In contrast, it had a marked inhibitory effect on the rate of crystal growth occurring in response to the oxalate load,

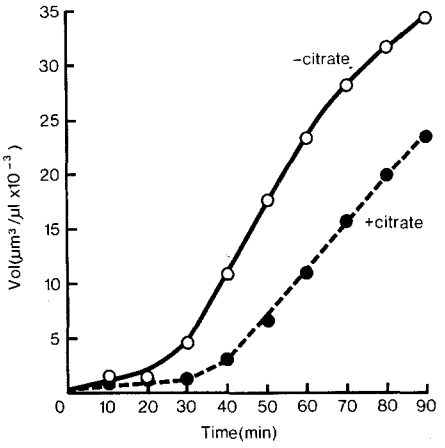

Fig. 7. The effect of citrate on crystal growth in a urine sample from a normal male. The rate of crystal growth calculated from the linear portion of the curve in the presence of added citrate is 49% of that in the control

the degree of inhibition again varying from one urine specimen to another. Fig. 7 shows its effect on one urine sample where the rate of growth in the presence of added citrate was only 49% of that occurring in the control.

Discussion

The need for experimental systems which permit the assessment of the effects of inhibitors in whole urine has been acknowledged for some time [3]. In this paper we describe a method for inducing and quantifying calcium oxalate crystallisation in whole urine. The crystals produced are morphologically identical to those occurring naturally in urine from normal subjects and stone-formers [11]. Using the method, the resistance of a urine to calcium oxalate crystal formation may be described in two ways: 1) its capacity to tolerate increasing quantities of oxalate before the formation product of calcium oxalate is exceeded, and 2) once that point has been reached, its subsequent response to a fixed oxalate load. The inhibitory activity of urine, pyrophosphate, chondroitin sulphate and citrate [14] and the solubility of calcium oxalate are dependent upon pH. Therefore, no attempt was made in this study to standardise or control the pH, since we regarded the natural pH of each urine sample as an integral determinant feature of its measured metastable limit and total inhibitory activity.

The practical limit of metastability of each urine with respect to calcium oxalate is determined by titrating the urine with increasing amounts of sodium oxalate and estimating the minimum amount of oxalate that is required to induce detectable precipitation. The technique is therefore similar in principle to those described by Pak et al. [8] and Baumann and Wacker [1]. It differs from these methods in that the metastable limit is determined after a 30 min incubation period rather than 24 h [1] or 48 h [8] and a Coulter Counter is used to detect precipitation. In the two earlier techniques the occurrence of precipitation was confirmed by a fall in the calcium concentration. This empirical determination of supersaturation in terms of a measured concentration product of calcium and oxalate obviates the tedious estimation of a number of urinary constituents and the computer analysis of more than 20 ionic complexes [10] on which the theoretical calculation of supersaturation based on activity products relies. Furthermore the computer calculation does not take into account the binding of calcium and other ions to urinary macromolecular components such as proteins [2] or glycosaminoglycans [6] and therefore any effect that these macromolecules may have on the supersaturation.

The second aspect of urinary inhibitory activity which may be measured using the method described, is the response of a urine to a fixed oxalate load above its formation product. A load of 30 μmol of oxalate was chosen because trial and error experiments had shown that this quantity always resulted in measurable crystal growth. It is meaningless to test the response of urines to a given oxalate load without pre-defining their relative states of supersaturation with respect to calcium oxalate. One advantage of assessing inhibitory activity in terms of the response of a urine to a fixed load above its limit of metastability is that it allows a direct comparison of all urines tested in this way, i.e. when the oxalate load is added, all urines are equivalently saturated with respect to calcium oxalate.

The reaction of a urine to the oxalate load can be assessed in a number of ways. These include the growth rate of the crystals produced, their rate of aggregation, and their size at the end of the 90 min incubation period. Crystal size can be easily estimated using the Coulter Counter to ascertain the mean crystal diameter. The rate of crystal growth can be expressed as the rate of increase in crystal volume [12, 7]. Although microscopic examination of the crystals at the end of the incubation period showed quite clearly that extensive crystal aggregation had occurred in many urine samples, the rate of crystal aggregation could not be determined from a consideration of total crystal number as has been previously advocated [12]. Because in the method described the crystals nucleate spontaneously from the urine, they will not be detected by the Coulter Counter until they attain a diameter of 2 μm (the lower size threshold used in the method) and total crystal number will rise until the rate of entry of crystals into the visible range is exceeded by the reduction in crystal number caused by crystal aggregation.

To illustrate the effects of different inhibitor concentrations on the limit of metastability and the rate of crystal growth in whole urine, citrate and pyrophosphate were added to three urine specimens at concentrations intended to double the known mean endogenous concentrations of these substances in urine [15, 3]. In one instance pyrophosphate was an effective inhibitor of nucleation and/or early crystal growth, raising the metastable limit of the

urine so that an additional 6 μmoles of oxalate was necessary to induce detectable crystallisation. However, the same concentration exerted a small, but nonetheless significant inhibitory effect on the growth of crystals larger than 2 μm in all three urine samples tested. In contrast, Hallson et al. [4] found that the crystals precipitated from a given urine sample did not differ in response to altered pyrophosphate concentrations. However, the method used by these authors involved concentration of the urine to a fixed osmolarity. A major objection to the use of dilute urine [3] is that the effective concentration ranges of different inhibitors are disparate and that diluting the urine may selectively favour or disadvantage one or more inhibitors in relation to others. Although the same is true when urine is concentrated, it is important to note that crystals in vivo form in concentrated urine and not in dilute urine. It is possible that Hallson et al. [4] were unable to observe an effect of pyrophosphate because by concentrating the urine they were also concentrating other, perhaps more potent inhibitors which effectively overrode any effects that pyrophosphate may otherwise have shown.

In the present investigation, raising the concentration of citrate did not alter the metastable limit of any of the urine specimens examined, despite the fact that citrate is an effective chelator of calcium and might have been expected to increase the amount of oxalate necessary to induce precipitation. It did, however, reduce the growth rate of the crystals precipitated from the urines after addition of the oxalate load. Hallson et al. [5] also found that citrate depressed the amount of calcium oxalate precipitated from whole, concentrated urine.

The propensity of a given urine specimen to form kidney stones will depend on its degree of supersaturation, the concentration of any promoters, and on its total inhibitory activity. A combination of these factors will affect the formation product of the urine and the size and state of aggregation of the crystals produced from it [9]. The method we have described presents a means of quantitatively determining the practical limit of metastability of a urine with respect to calcium oxalate and the size and rate of growth of crystals precipitated from the urine in response to an oxalate load. The method enables the measurement of these parameters without otherwise altering the chemical status of the urine or changing the relative concentration of other urinary components, including inhibitors or promoters, and has the potential of enabling the quantitative determination of linear crystal growth rates and valid crystal aggregation rates in whole urine [13].

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Dr. Rosemary L. Ryall
Urology Unit
Department of Surgery
Flinders Medical Centre
Bedford Park
South Australia, 5042