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Inhibition of Calcium Oxalate Monohydrate (COM) Crystal Growth by Pyrophosphate, Citrate and Rat Urine

H. Sidhu, R. Gupta, S. K. Thind and R. Nath

Department of Biochemistry, Postgraduate Institute of Medical Education and Research, Chandigarh, India

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Summary. An assay system for the measurement of the rate of Calcium Oxalate Monohydrate (COM) seed crystal growth in a metastable solution of calcium chloride and sodium oxalate containing traces of ^{14}C-oxalic acid was used to assess the inhibitory activity of pyrophosphate (10^{-5} M–10^{-4} M), citrate (10^{-4} M–10^{-3} M) and urines of normal and pyridoxine deficient rats. Both pyrophosphate and citrate were strong inhibitors of COM crystal growth and caused a 50% decrease in crystal growth rate at 1.50×10^{-5} M and 2.85×10^{-4} M respectively. Normal rat urine strongly inhibited the COM crystal growth, while pyridoxine deficient animals showed a significant ($p < 0.01$) decrease in mean inhibitory activity as compared to pair-fed controls. A lowered urinary inhibitory potential accompanied with hyperoxaluria and hypercalciuria, which is known to be associated with pyridoxine deficiency, may be a contributory risk of calcium oxalate crystallization and stone formation.

Key words: COM crystal growth, Pyrophosphate, Citrate, Rat urine.

Introduction

Calcium oxalate, the most common crystalline constituent of urinary stones, exists in urine as two different phases viz. calcium oxalate monohydrate (COM) and as the dihydrate (COD). Normal urine is supersaturated with respect to COM but supersaturation is higher in patients with urolithiasis than in normals [14]. To explain the existence of metastable zone of saturation in urine, presence of organic and inorganic inhibitor substances has been postulated [5]. Most urinary inhibitory activity can be attributed either to low molecular weight urinary constituents like pyrophosphate (PPi) and citrate or to high molecular weight constituents like acid-mucopolysaccharides, RNA like material and glycoproteins [15]. Stone formation has been proposed to be caused by an imbalance between urinary oversaturation with stone constituent ions and the concentration of protective inhibitors [2]. There is now strong evidence that the urine of stone formers has a low concentration of crystal growth inhibitors compared to the urine of healthy controls [3, 17]. Reports from several laboratories have confirmed a higher risk of calcium oxalate lithiasis in vitamin B_6 deficient animals and man [7, 10], which equally could be due to supersaturation of urine with calcium and oxalate and to a lack of the normal inhibitory potential of urine.

Thus in the present study, the assay to measure COM crystal growth in metastable solution of calcium chloride and sodium oxalate containing traces of ^{14}C-oxalic acid [12], was established and it was used to quantitate inhibitory activity of PPi, citrate and rat urine. Alterations in the inhibitory potential of rat urine have been examined in vitamin B_6 deficient conditions, to completely assess the risk of calcium-oxalate lithiasis.

Materials and Methods

Preparation of COM Seed Crystals

Seed crystals were prepared by mixing equal volumes of 0.01 M $CaCl_2 \cdot 2H_2O$ and 0.01 M sodium oxalate, dropwise with constant stirring at 4 °C by the method of Pak et al. [13]. The crystals were collected by centrifugation, washed with water, with methanol and then dried. Seed crystal slurries were prepared by suspending 1.5 mg/ml of crystals in 50 mM sodium acetate buffer (pH 5.7) containing 96 mM NaCl. The crystals were identified as COM by observing under a high power microscope. A single batch of seed crystals was used throughout the present study.

Crystal Growth Assay

The COM seed crystal growth was determined in a metastable solution of calcium chloride and ^{14}C-sodium oxalate, by the method of Nakagawa et al. [12]. One ml of the test sample (containing desired amount of inhibitor) or buffer (control) was added to 10 ml of assay system containing 1×10^{-3} M calcium chloride and 0.2×10^{-3} M

sodium oxalate containing traces of ^{14}C-oxalate (10^{-2} μCi/ml) in 50 mM sodium acetate buffer (pH 5.7) containing 96 mM NaCl. The reaction was started by addition of 1 ml of seed crystal slurry. Final 12.0 ml assay system thus contains 0.833 mM calcium, 0.167 mM oxalate and 0.125 mg/ml of COM seed crystals. Incubation was carried out at 37 °C with constant shaking (~ 30 cycles/min) and 1 ml aliquots were removed every 10 min, filtered through millipore filter (0.22 μ) and ^{14}C-radioactivity determined in the filtrate in a Packard Scintillation counter (Model C2425) using Bray's scintillation fluid.

Effect of Pyrophosphate and Citrate

Growth kinetics of COM seed crystals was examined in the presence of 1×10^{-5} M to 1×10^{-4} M PPi and 1×10^{-4} M to 1×10^{-3} M citrate in the assay system described above.

Effect of B_6-Deficient and Control Rat Urine

Nutritional deficiency of vitamin B_6 was produced in male weanling rats of Wistar strain (body weight 40–50 g) as described in an earlier publication [10]. Pair-fed controls were maintained and after 45 days the pyridoxine deficiency was confirmed biochemically by erythrocyte alanine transaminase assay [10]. The 24 h urine samples were collected (in thymol) on two consecutive days by placing the rats individually in metabolic cages with free access to diet and water. Total volumes were recorded and samples stored at −20 °C till further analysis. Aliquots of 24 h urine samples (at 37 °C) were filtered through 0.22 μ millipore filter to remove any suspending material or preformed crystals, and creatinine was estimated in the filtrate. One ml of urine sample (1.0 μmole creatinine/ml) was added to 10.0 ml of metastable assay system described before. The reaction was initiated by 1.0 ml of seed crystal slurry and incubation performed with shaking at 37 °C. An aliquot (1.0 ml) was withdrawn at 40 min, filtered through 0.22 μ millipore filter and ^{14}C-oxalate measured (C_t). Also the initial counts at zero time (C_i), equilibrium counts after 24 h of seeding (C_α) and control counts (C_w), in the absence of urine were determined. The inhibitory activity of various urine samples was then calculated using the mathematical expression [3]:

$$\text{Inhibitory Activity (I.A.)} = \frac{C_t - C_w}{C_i - C_t} \times \frac{C_i - C_\alpha}{C_w - C_\alpha}$$

Results and Discussion

Growth Rate of COM Seed Crystals

The rate of COM seed crystal growth in a metastable solution of calcium chloride and sodium oxalate, in terms of decrease in ^{14}C-oxalate in solution at different time intervals after addition of seed crystals is shown in Fig. 1. A number of time course experiments were performed to validate this assay system and reproducible results were obtained. The kinetics of COM crystal growth in this system is of second order [8] and thus loss of calcium or oxalate from the medium follows the equation:

$$\frac{dCa}{dt} = K[(Ca) - (Ca)_\alpha]^2 = K[(Ox) - (Ox)_\alpha]^2$$

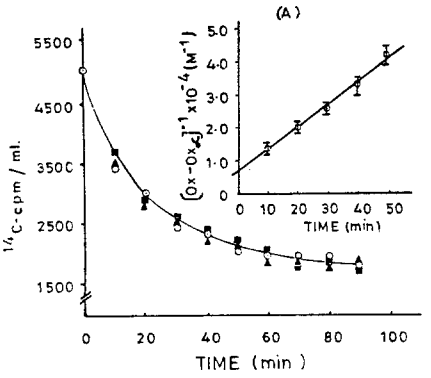

Fig. 1. Time course of COM seed crystal growth. (*A*): Growth kinetics of COM seed crystals; Growth rate constant K = 55.81 ± 2.42 M^{-1} min^{-1}/mg seed crystals/100 ml. ○, △ and □ represent three different time course studies

where (Ca) and (Ox) are calcium and oxalate concentrations and $(Ca)_\alpha$ and $(Ox)_\alpha$ are values of each at equilibrium and K is the rate constant. The above equation can be integrated into

$$[(Ox) - (Ox)_\alpha]^{-1} = Kt + [(Ox)_0 - (Ox)_\alpha]^{-1}$$

where $(Ox)_0$ is initial oxalate concentration and the plot of $[(Ox) - (Ox)_\alpha]^{-1}$ vs "t" gives a straight line (Fig. 1A). The empirical COM seed crystal growth rate constant obtained from the slope of this line is 55.81 ± 2.42 M^{-1} min^{-1}/mg seed crysatals/100 ml. In all the calculations C_α/C_i was taken as 0.297, which was the mean value obtained in 30 time-course studies.

Effect of PPi and Citrate

Figure 2 shows the effect of increasing concentration of PPi on the time course of COM crystal growth. Pyrophosphate was found to be an inhibitor of COM crystal growth in the concentration range, 10 μM-100 μM and the crystal growth was completely inhibited at 8×10^{-5} M. To determine the effect of increasing PPi concentration on crystal growth rate constant "K", the data of time course study has been plotted as shown in Fig. 3. The plot of rate constant data in the form of Langmuir adsorption isotherm (Fig. 3A) i.e. $\frac{K_0}{K_0 - K_{exp}}$ vs $\frac{1}{(PPi)}$, where K_0 and K_{exp} are the rate constants in the absence and presence of PPi respectively, gave a straight line, thereby confirming the earlier reports that inhibition by PPi is by surface adsorption phenomenon [9]. A 50% decrease in rate of COM crystal growth (broken line, Fig. 3A) was produced by 1.5×10^{-5} M PPi. Pyrophosphate has been reported to be an important natural urinary inhibitor of calcium oxalate crystallization [8, 9]. The normal urinary PPi concentration range is $1–7 \times 10^{-5}$ M, which is high enough to inhibit COM crystal growth. A decreased

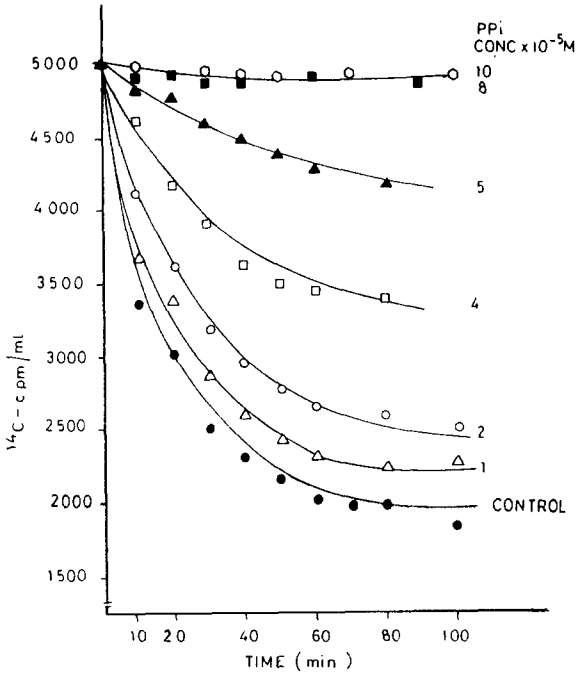

Fig. 2. Time course of COM crystal growth with increasing concentration of pyrophosphate (*PPi*)

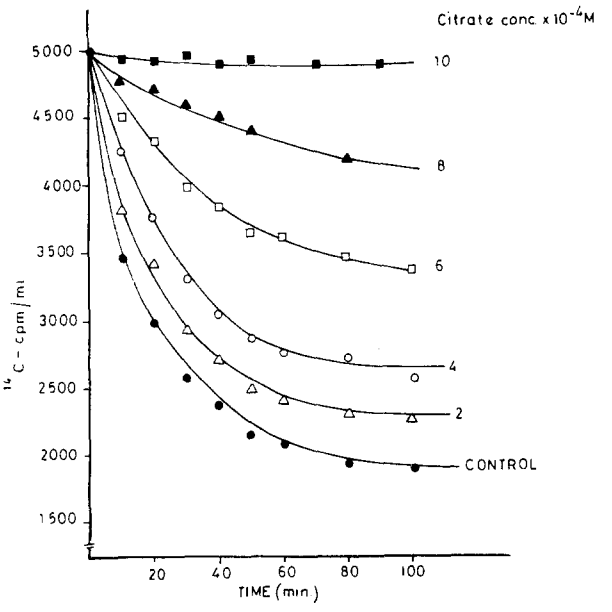

Fig. 4. Time course of COM crystal growth with increasing concentration of citrate

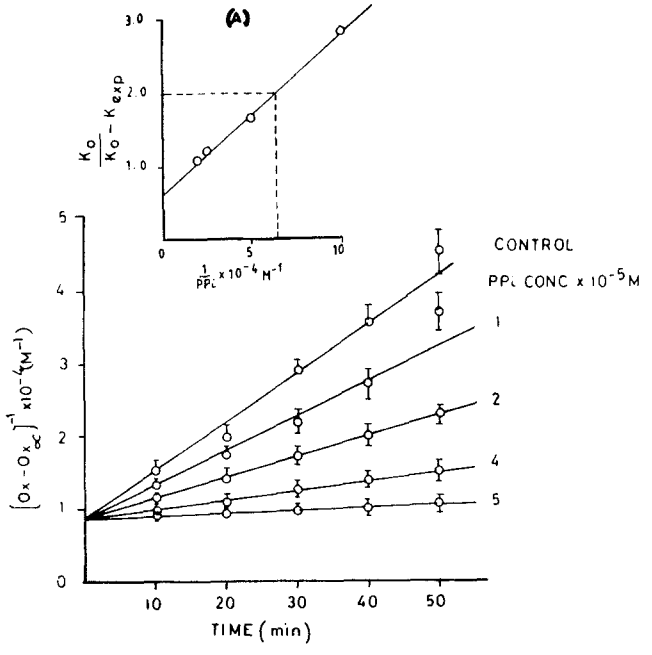

Fig. 3. Effect of increasing concentration of PPi on COM crystal growth rate constant. (*A*): Langmuir adsorption isotherm type plot; K_0 and K_{exp} are rate constants in the absence and presence of PPi respectively

urinary PPi excretion in calcium oxalate stone formers has been reported [16] and orthophosphate therapy of recurrent calcium oxalate stoneformers has been shown to significantly increase the urinary PPi levels, thereby preventing oxalate lithiasis.

Citrate, is also known to inhibit calcium oxalate crystal growth, both due to its ion-pairing effect with calcium and surface adsorption on COM seed crystals [19]. In the present study citrate was found to inhibit COM crystal growth in the concentration range, 0.1 mM–1.0 mM (Fig. 4). Kinetic analysis of time course data was performed as detailed for PPi and a 50% decrease in COM growth rate was obtained at 2.85×10^{-4} M citrate (data not shown). The exact fit of the rate constant data into the Langmuir Adsorption isotherm, confirmed adsorption of citrate on the crystal growth sites.

Effect of Normal and Pyridoxine Deficient Rat Urine

Effect of three different normal rat urine samples (1 μmol creatinine/assay), on the time course of COM crystal growth is shown in Fig. 5. The difference between the two curves represents the inhibition produced by rat urine. Based on the assumption that various inhibitors in the urine effect COM crystal growth by reversibly combining with the growth sites on the crystal, a mathematical expression for inhibitory activity (I.A.) has been derived by Coe et al. [3] and this was used to calculate I.A. of rat urine in the present study. The normal rat urine samples exhibited strong inhibitory activity towards COM crystal growth (I.A. = 3.53 ± 0.18/μmol creatinine), corroborating with report of Nakagawa et al. [11], who have reported strong inhibitory activity of rat and rabbit urine and their kidney homogenates. The inhibitory activity of rat urine was also found to have a linear correlation with the increasing concentration of urine in the assay system (Fig. 5A).

Twenty-four hour urine samples of pyridoxine deficient rats and their pair-fed controls, were tested for their I.A.

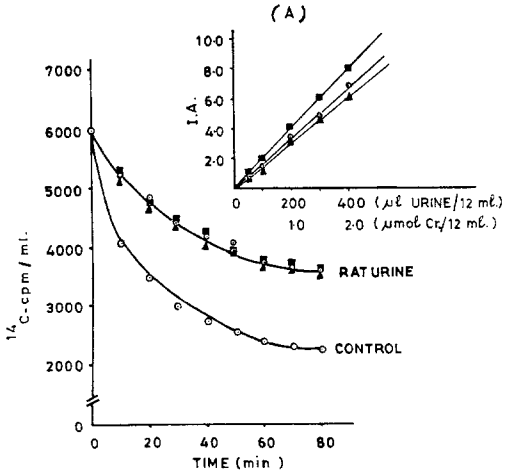

Fig. 5. Time course of COM crystal growth in the presence of rat urine (1 μmol Cr/assay). □, △ and • represent three different urine samples. (*A*): Effect of increasing amount of rat urine on the inhibitory activity (*I.A.*), calculated as described in materials and methods

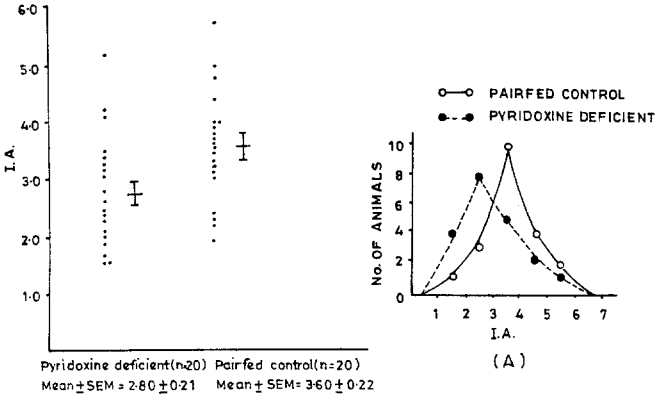

Fig. 6. Effect of pyridoxine deficiency on the inhibitory activity of rat urine towards COM crystal growth. (*A*): Frequency distribution curves representing the two groups

towards COM crystal growth (Fig. 6). The urine samples tested showed a marked variation in inhibitory activity. Although some of the values were overlapping there was a statistically significant ($p < 0.01$) decrease in the mean I.A. of urine of pyridoxine deficient group as compared to the pair-fed control animals (Fig. 6). The shift of I.A. towards lower values in vitamin B_6 deficient animals is clearer from the frequency distribution curves shown in Fig. 6A. The inhibitory activity of urine is due to the additive effect of various inhibitory constituents like PPi, citrate, glycosamioglycans, acidic glycoproteins etc. [20]. The decreased inhibitory activity observed in pyridoxine deficiency could be due to alterations in some of these physiological urinary constituents. The pyridoxine deficient rats exhibited a significant ($p < 0.01$) hypocitraturia (citrate excretion: pair fed control = 21.52 ± 1.42 μmol/day/rat; vit. B_6 deficient = 15.68 ± 0.84 μmol/day/rat), which has also been reported by Gershoff [6]. Recent studies [1] have shown that the urinary levels of glutamate pyruvate transaminase (GPT) and glutamate oxaloacetate transaminase (GOT) are three times higher in normals as compared to stone formers and addition of GOT to the urine of stone formers retarded the stone growth via formation of glutamic acid. The urinary activity of these pyridoxal phosphate dependent enzymes is expected to be decreased in pyridoxine deficiency, thereby decreasing the overall inhibitory activity. A decreased concentration of glycosaminoglycans (GAGS) has also been reported in vitamin B_6 deficient rat tissues [18] and since urinary GAGS are derived from the visceral pool, their excretion may also be decreased in pyridoxine deficiency.

Earlier reports from our laboratory have demonstrated a significant hyperoxaluria and hypercalciuria in vitamin B_6 deficient rats [4, 10]. Urinary supersaturation with calcium oxalate along with lower levels of inhibitory activity can significantly enhance the risk of calcium oxalate crystallization leading to a higher risk of calcium oxalate lithiasis in vitamin B_6 deficient population. The above COM crystal growth assay is being used to assess inhibitory activity of recurrent calcium oxalate stone formers in this hospital. Also attempts are being made to isolate and characterize some of the important inhibitors present in normal urine and to study their alterations in recurrent stone formers.

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Dr. S. K. Thind
Department of Biochemistry
Postgraduate Institute of
Medical Education and Research
Chandigarh – 160012
India