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THE INHIBITORY EFFECT OF POLYMERIC CARBOXYLIC AMINO-ACIDS AND URINE ON CALCIUM OXALATE CRYSTALLIZATION

by

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Summary: A new method has been established to follow the inhibitory effect of some polymeric-dicarboxylic-amino-acids and other poly-anionic derivatives. The technique is based on using calcium specific electrode to measure continuously free calcium ion activity in solution to assess the formation of calcium oxalate precipitate.

The retardation effect of Poly-L-glutamic and aspartic acids has been established in 5-100 ppm range and compared to other monomeric and polymeric compounds with inactivated functional groups. The retardation effect is in agreement with previous reports on inhibition effect on crystal growth rate of seed crystals, with the same retardants.

The inhibitory effect was also determined with normal urine and compared to pathological urine.

INTRODUCTION

Several investigators have reported, in the past, that the presence of some crystal growth inhibitors in the human urine are responsible for the retardation effect of the calcium oxalate precipitation.

In some recent works an inhibitory effect, in aqueous seeded solutions, by polymeric peptides and proteins (1-6) as well as polyglutanic and aspartic acids (7), has been reported. From numerous studies it has been concluded that small urinary macromolecules such as glucosaminoglycans and RNA-like fragments, are responsible, to some extent, for the supersaturation stabilization in human urine with respect to calcium oxalate precipitation and the control of growth and aggregation processes (8,9).

The present study evaluates the effect of polymeric carboxylic-amino-acids such as polyglutamic and polyaspartic acids together with other polymers on the retardation of precipitation of calcium oxalate and compares it to the effects of normal and pathological urine.

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The main idea behind this work is to evaluate, by using a new technique, the retardation effect of several polymeric amino-acids and derivatives on the nucleation and crystallization processes of calcium oxalate in mimic urine and to compare them to the retardation effect, noticed previously (5), on the crystal growth of calcium oxalate.

The study has been carried out using a procedure that has been recently developed by our group (10). The technique is based on the use of calcium specific electrode which measures in solution the rate and extent of calcium oxalate precipitation with time.

MATERIALS AND METHODS

Mimic urine was prepared on the basis of compounds present in normal urine according to the Ciba Geigy Scientific Tables Documenta (11) and Doremus's previous prescription (12). One liter of solution contains 20 g urea, 2.227 g Na_2SO_4 (anhy.), 4.84 g KCl, 5.00 g NaCl, 2.18 g NaH_2PO_4 and 0.55 g $CaCl_2$. All the materials were BDH A.R. The water triple distilled. Creatinine, hyppuric acid, ammonium chloride were not included since in preliminary studies it was found that they have no significant effect on calcium oxalate precipitation. Magnesium ions were excluded to prevent any potential interference to the electrode. Each sample of mimic urine was diluted 5 times to obtain the required 10^{-3} M in $CaCl_2$ concentration and thus the ionic strength of the mimic urine was $6.3 \cdot 10^{-2}$ at the beginning of each experiment. pH of the mimic urine systems was measured prior to each experiment and was adjusted to be 5.7. The pH of the systems in which human urine was added was in the range of 5.1 to 6.8. No further adjustments were made.

Calcium chloride solutions (BDH A.R.) were used to adjust ionic strength and calcium concentrations. Sodium oxalate solutions were prepared prior to every measurement (Baker's C.P.) in $1 \cdot 10^{-3}$ M concentration.

A series of additives were used: Poly-L-glutamic acid, sodium salt, type I (PGA-I) (MW 7000, degree of polymerization, DP 40), Poly-L-glutamic acid, sodium salt, type III-B (PGA-III) (MW 7000 DP 400); Poly-L-aspartic acid, sodium salt, type II-A (PAsA-II) (MW 9000 DP 60); Poly-β-benzyl-L-aspartate, type I-A (MW 14000 DP 68). Polyoxyethylene (MW 300000), all pruchased from Sigma Chemicals Company. Sodium heparin - Teva Pharmaceuticals Industries, Poly-acrylic acid (Paa) (MW 90000), Poly-(2- acrylamide-2-methyl-1-propane) sulfonic acid (PAS), and carboxymethyl cellulose (CMC), Aldrich chemicals.

The calcium ion concentration was measured continuously by 93200 P Orion Calcium specific electrode mounted in PBS-710 El-Hama modified digital pH/mv meter, using double junction reference electrode (Orion Model 90-02). The electrodes were immersed in the mimic urine solution, kept at 25°C by water thermostat and constantly stirred. The output was also connected to a recorder. Calibration of the electrodes was done prior to every experiment by stock calcium chloride solutions. The additives (poly amino acids or urine) were added to the mimic urine and equilibrated to a constant reading of concentration prior to the addition of the oxalate solution.

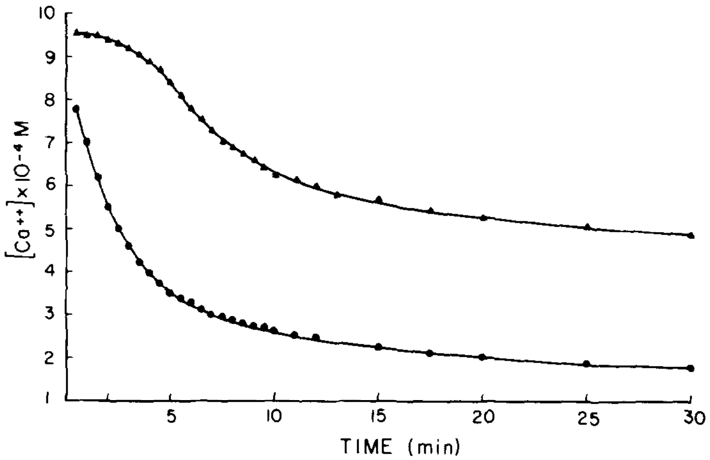

Figure 1: The decrease in calcium ion concentration with time in mimic urine containing no inhibitor (o) and 100 ppm PGA (Δ).

The electrodes measured the decrease in the calcium concentration immediately after the simultaneous addition of the oxalate solution and the mimic urine into the reaction vessel.

The calcium oxalate precipitate was filtered and centrifuged out (17000 rpm) and analyzed by X-ray technique (Philips diffractometer with Cu radiation and Ni filter); differential thermal analysis (Mettler Thermoanalyzer) and scanning electronic microscope (Joel SEM Model JSM 35).

Possible poisoning effect of the electrodes by PGA has been ruled out in a control experiment, in which the calcium ions were mixed with the retardant for several hours, there was no change in the calcium concentration measurements

RESULTS

1. Use of polymeric-amino-carboxylic-acids

Mimic urine solution of 10^{-3} M Ca^{++} concentration was mixed with equimolar oxalate solution and the calcium ion concentration (activity) was followed using specific calcium electrode. Different methods of addition and mixing the reactants have been tried and several types of reaction vessels and volumes have been tried and several types of reaction vessels and volumes have been tested to increase the reproducibility of the results. An accuracy of ±5% has been achieved. From Fig. 1 it can be seen that there is no delay time (except for up to 45 sec. due to electrode equilibration) and after 30 minutes the calcium ion concentration reaches a plateau ($1.8 \cdot 10^{-4}$ M) corresponding to calculated K_{sp} value for the given ionic strength.

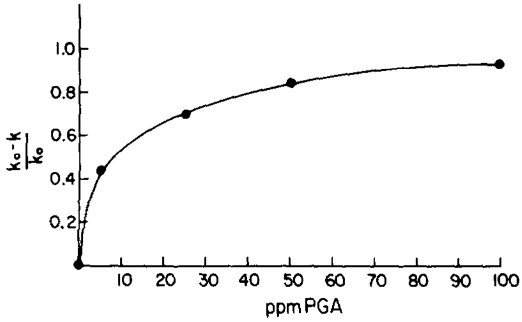

Figure 2: The retardation effect of PGA-I added in several concentrations.

In a similar experiment 100 ppm of PGA-I or PAsA-I were added to the mimic urine. From Fig. 1 it is clearly noticed that the drop in the calcium ion concentration is retarded markedly and the curve is practically composed of two parts. In the first minutes there is only moderate or no decrease in the calcium concentration related to induction time which is in good agreement with Crawford's (4) results. The second part of the curve represents faster decrease but still slow in comparison to the control reaction. Same types of curves are obtained with 100 ppm of PAsA. It is important to notice that the curves representing free calcium activity measured at the solution when no additives exist or when PGA & PAsA are present reach a plateau and the same concentration after 22-26 hours.

Fig. 2 illustrates the retardation effect with various amounts of PGA-I when a plot of $(k_o-k)/k_o$ versus time is obtained $k_o=(t_{\frac{1}{2}})^{-1}$ for 0 ppm; $k=(t_{\frac{1}{2}})^{-1}$ in the presence of additives. Retardation effect is more pronounced with higher concentrations of polymeric compounds but it seems to reach again a plateau after a few hours. It is worth noticing that the delay time becomes insignificant when the PGA concentration is decreased to 25 or 5 ppm range. All curves reach plateau after 20-30 hours.

The effect of the chain-length and number of repeatable glutamic acid units has been examined and is summarized in Table 1. It can be seen that the PGA with the lower molecular weight has a stronger retardation effect in comparison

Table 1: Effects of various additives on calcium oxalate precipitation

(Calcium concentration 10^{-3}M and oxalate 10^{-3}M)

Additive	Mol. Wt (Avg.)	Conc. (ppm)	$t_{½}$ (a) (min.)	$\frac{C_o}{C_t}$ (Ca^{++}) (b) (at 15 min.)
none (control)	--	--	3.0	1
PGA (Type I)	7000	100	25.0	0.379
		50	17.0	0.440
		25	11.0	0.449
		5	5.5	0.524
PGA (Type III)	70 000	100	20.0	0.423
PAsA (Type I)	9000	100	19.5	0.431
		50	17.0	0.440
		25	11.0	0.478
glutamic acid (monomer)	147	100	--	0.916
glutamyl-glutamic acid	276	100	--	0.980
poly-Benzyl-aspartate	14 000	100	--	0.970
PAS(c)	∿50 000	100	--	0.379
PAA(c)	90 000	100	--	0.354
CMC	--	100	15.0	0.379
Sodium heparin	--	100	--	0.390
Albumin	--	500	--	1.050
Polyoxyethylene	300 000	500	--	0.990

a) $t_{½}$ - The time required to reduce the Calcium ion activity to half of its starting value.

b) C_t - Calcium ion concentration after 15 min. in the presence of an additive; C_o - calcium ion concentration in a control experiment.

c) Calcium ion concentration $2 \cdot 10^{-3}$.

to PGA with higher molecular weight. It is important to indicate (Fig. 3) that glutamic or aspartic acid (monomer) and glutamyl-glutamic acid (dimer) had no retardation effect when added in higher concentrations (on monomer concentration

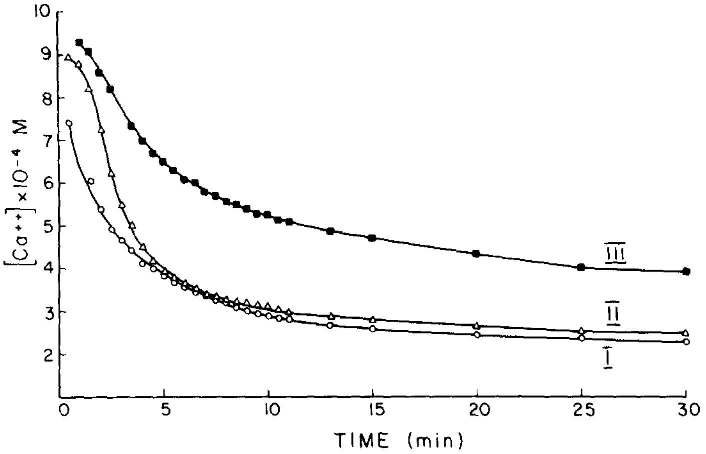

Figure 3: Comparison of the retardation effect of aspartic acid-monomer in 100 ppm (■), poly-β-benzyl-aspartate in 100 ppm (Δ) and aspartic acid monomer (o).

basis). When the free carboxylic group on the polymer had been blocked by benzyl ester radical the inhibitory effect disappeared completely (Fig. 3).

Three other polymers with free carboxylic or sulfonic groups were examined and found to inhibit the calcium precipitation in a similar way to the PGA (Table 1). On the other hand, when regular protein such as albumin or polymers such as polyethylenglycol or polyoxyethylene were used no retardation effect was observed (Table 1).

2. Effect of normal and pathological urine

The effect of normal and pathological urine on the precipitation of calcium oxalate from mimic urine has been studied by adding 5-10% of fresh urine to the examined solution. It is clearly noticed that while the normal urine retards the calcium precipitation (5% significantly less than 10%), the addition of 10% of pathological urine does not show any significant retardation effect (Fig. 4). The experiments have been carried out on 15 pathological urines. In experiments carried out before surgery predictions were made about patients' conditions with respect to the type of stone formed in their kidneys. Good agreements with stone content extracted from patients in surgery was obtained. More than 25 normal urines have been examined in the same manner. It

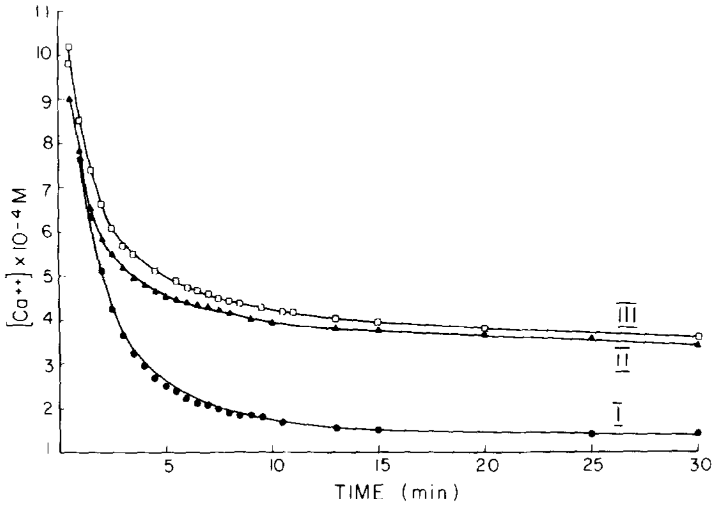

Figure 4: The retardation effect of 10% normal urine (□ and Δ) in comparison to 10% pathological urine (o).

is important to notice that no observable induction time phenomenon was seen in the normal as well as in the pathological urines.

In order to prove that microcrystals of calcium oxalate probably present in urine were not responsible for the immediate induction of precipitation, a sample of pathological urine was filtered through 0.2 μ millipore filter. The retarding effect for both filtered and untreated pathological urine was absent in both cases.

DISCUSSION

Previous studies have established that many compounds can possibly retard the growth of calcium oxalate seed crystals. Several anions and polyanions have been demonstrated to have a powerful inhibitory effect on the formation of calcium oxalate even when added in a molar concentration of 10^{-4} to 10^{-6}. Ito and Coe (5) have shown that acidic peptide and polyribonucleotide fragments can inhibit seed crystal growth at $5 \cdot 10^{-6}$M concentration whereas the monomer nucleotides are inactive at the same concentration. Poly-L-aspartic or glutamic acid were found also inhibitory at $5 \cdot 10^{-6}$M of amino acid, whereas the monomeric amino acids were inert. Ito and Coe used mimic urine solutions with 0.06 mg/ml of preformed seed crystals of calcium oxalate monogydrate and measured at various time intervals the content of removed aliquots from the solution by filtration. This method allowed estimation of the fraction of oxalate that had been transferred from the liquid phase and had entered the solid phase by combining with calcium during growth of the seed crystals. Our results are based on a continuous measurement of the calcium ion concentration remaining in the solution during the nucleation and crystal growth of the calcium precipitate and thus permits possible estimation of the effect of the reatardants on the nucleation process as well as on the crystal growth. It can be seen clearly that in our system, the PGA and PAsA have similar effect of inhibition as previously shown by Ito's method. The actual molar concentration of polyanionic chains, required for retardation is 10^{-6} to 10^{-8}, corresponds well to that observed for heparin.

There was no significant difference between PGA and PAsA and other synthetic poly-anionic compounds. By blocking the anionic functions with ester-groups the polymer becomes completely inactive confirming the requirement for free anionic functions to intereact with calcium ions or calcium oxalate nuclei.

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