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Urolithiasis inhibitors and calculus nucleation

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Summary. The possible inhibitors of heterogeneous nucleation were investigated. The effects of magnesium, pyrophosphate, citrate and Chondroitin Sulphate on calcium phosphate or uric acid heterogeneous nucleation of calcium oxalate were studied. It was found that whereas magnesium, pyrophosphate and citrate acted as effective inhibitors in the presence of calcium phosphate as heterogeneous nucleant, only chondroitin sulphate manifested important inhibitory effects when uric acid was the heterogeneous nucleant.

Key words: Urine – Chondroitin sulphate – Inhibition – Crystal nucleation

Introduction

Inhibition is a phenomena that clearly can affect crystalline growth and aggregation and is related to the adsorption of a particular substance on a crystal surface. Controversy has been voiced about the so-called inhibitors of the nucleation. Two types of inhibitors have been described: inhibitors of the homogeneous nucleation and inhibitors of the initial heterogeneous nucleus growth. Studies comparing the inhibitors of homogeneous nucleation in the urine of normal subjects and stone-formers have produced conflicting results. Thus, according to Robertson et al. [10, 11], there was no difference in the empirical formation product between normals and stone-formers. Pak, however, claims that urine from stone-formers precipitates at a lower level of supersaturation than normal urine [9]. Less controversy seems to surround the existence of inhibitors of the heterogeneous nucleation [12], although this has not get been clearly demonstrated. In this paper, we studied the effect of several products (generally described as inhibitors) [12], on the heterogeneous nucleation of calcium oxalate; to distinguish inhibitors of the calcium oxalate growth and aggregation of inhibitors of the initial heterogeneous nucleus growth, to establish the existence of the inhibitors of heterogeneous nucleation.

Material and methods

Reagents and solutions

Stock solutions include aqueous solutions of sodium oxalate ($7.5\ 10^{-3}$ M of anhydrous salt, prepared and renewed daily), calcium chloride aqueous solution ($2.5\ 10^{-2}$ M and $2.5\ 10^{-1}$ M of anhydrous salt), monosodium phosphate aqueous solution ($6.4\ 10^{-2}$ M of the dihydrate salt), uric acid aqueous solution ($1.2\ 10^{-2}$ M of the acid), 7.0 and 8.0 "Trizma" buffer solutions prepared by Sigma (0.1 M), and a 10 g/l aqueos solution of the inhibitor assayed.

Measurements of the inhibitory capacity of heterogeneous nucleation in calcium oxalate crystallization

The inhibitory capacity of heterogeneous nucleation for each substance in calcium oxalate crystallisation, was evaluated in urine, measuring the number of crystals obtained (by use of a Brand counting chamber and optical microscopy: 400×) in presence and absence of a particular heterogeneous nucleant and using different quantities of the assayed inhibitor, in the following procedures

Experiment 1. Studies on inhibition of calcium phosphate heterogeneous nucleation in calcium oxalate crystallization. – In a 1.5×16 cm test tube were placed an appropriate volume of urine (to give a final volume of 10 ml), 0.3 ml of $6.4\ 10^{-2}$ M mono sodium phosphate dihydrate and 0.1 ml of $2.5\ 10^{-1}$ M anhydrous calcium chloridre. After mixing 4 ml of pH 8.0 "Trizma" buffer was added. The system was left to stand for 2 min and then 0.5 ml of $7.5\ 10^{-3}$ M sodium oxalate and 0.1 ml of a solution containing the amount of the inhibitor assayed, were added. The final mixture was thermostatted at 20°C for 60 min and the results evaluated as above.

Experiment 2. Studies on inhibition of uric acid heterogeneous nucleation in calcium oxalate crystallization. – In a 1.5×16 cm test tube were placed an adequate volume of urine (to achieve a final

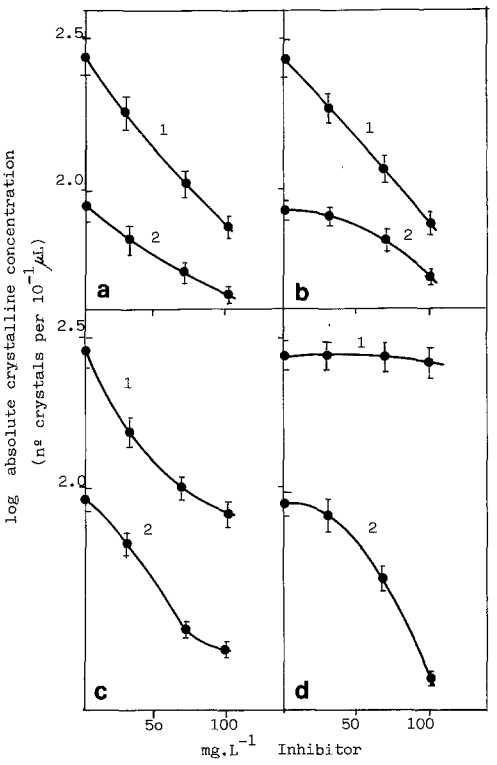

Fig. 1a–d. Inhibitory effects of diverse substances in calcium oxalate crystallization. Line 1: In the presence of calcium phosphate as heterogeneous nucleant (Experiment 1). Line 2: Conditions identical to 1 but in absence of calcium phosphate. **a** Magnesium (II), **b** pyrophosphate, **c** citrate, **d** chondroitin sulphate

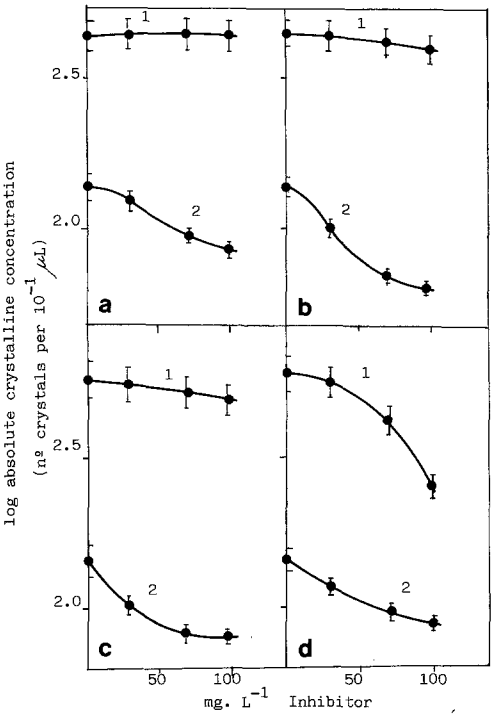

Fig. 2a–d. Inhibitory effects of diverse substances in calcium oxalate crystallization. Line 1: In the presence of uric acid as heterogeneous nucleant (Experiment 2). Line 2: Conditions identical to 1 but in absence of uric acid. **a** Magnesium (II), **b** pyrophosphate, **c** citrate, **d** chondroitin sulphate

volume of 10 ml), 5 ml of 1.2 10^{-2} M uric acid and a 0.1 M hydrochloric acid solution to give a final pH of 4. The mixture was shaken and left to stand for 2 min. Then 0.1 ml of 2.5 10^{-2} M anhydrous calcium chloridre was added. The system was again mixed and 0.5 ml of 7.5 10^{-3} M sodium oxalate and 0.1 ml of a solution containing the amount of the inhibitor assayed, were added. The final mixture was thermostatted at 20°C for 60 min and the results evaluated as above.

The results obtained for each heterogeneous nucleant in each experiment, were plotted as the absolute concentration of the crystals evaluated in each tube vs the concentration of the inhibitor assayed. The size of the resulting calcium oxalate particles was of the order of 10–15 µm. Controls where oxalate was not added were also obtained. Thus, when calcium phosphate, was present no crystals were detected, whereas in the presence of uric acid, some typical uric crystals were found and the uncoated ones were not evaluated. The values shown in the graphs are averages of three replicates for each measurement.

Results

The inhibitory effects of magnesium (II), pyrophosphate, citrate and chondroitin sulphate on the heterogeneous nucleation of calcium oxalate by calcium phosphate or uric acid are shown in Figs. 1 and 2. The results obtained were compared with those resulting from identical conditions but in absence of the particular heterogeneous nucleant. Several effects were noted. In the presence of calcium phosphate as the heterogeneous nucleant (Fig. 1), magnesium, diphosphate and citrate manifested important inhibitory effects, whereas chondroitin sulphate practically exhibited no effects. However, in absence of such heterogeneous nucleant (calcium phosphate), the four substances caused important inhibitory effects.

The results obtained when uric acid acted as heterogeneous nucleant are shown in Fig. 2. As can be seen, in this circumstance magnesium, pyrophosphate and citrate exhibited very week effects, while the chondroitin sulphate manifested important inhibitory effects. In absence of uric acid as heterogeneous nucleant again the four substances assayed caused a notable inhibitory action.

Discussion

A factor of fundamental importance in calcium oxalate lithogenesis is heterogeneous nucleation, but because of the supersaturation usually found in urine, forma-

Table 1. Diminution of the calcium oxalate relative supersaturation due to the presence of citrate (Mol. $l^{-1} * 10^{-4}$). RS values are evaluated taking in consideration all equilibria in which calcium oxalate ions are involved [2]. The calcium oxalate solubility product, calcium-oxalate ion association constant, and the formation constant of the calcium-citrate complex, were obtained from the bibliography [5, 6, 14]

$[Ca]_{total}$	$[Ca^{2+}]$	$[Ox]_{total}$	$[Ox^{2-}]$	$[Citrate]_{total}$	Relative sobresaturation $\Omega=[((Ca^{2+})(Ox^{2-}))^{1/2}Ps^{1/2}]$
Calcium phosphate as *heterogeneous* nucleant					
29.1	27.4	4.49	2.79	0.00	2.78
29.1	26.9	4.49	2.81	1.42	2.76
29.1	26.2	4.49	2.83	3.33	2.74
29.1	25.7	4.49	2.85	4.76	2.72
Uric acid as *heterogeneous* nucleant					
13.4	12.5	4.27	3.34	0.00	1.93
13.4	12.2	4.27	3.35	1.42	1.91
13.4	11.8	4.27	3.38	3.33	1.89
13.4	11.6	4.27	3.40	4.76	1.87

tion of calcium oxalate stones is very improbable [1]. On the other hand, crystals of uric acid or calcium phosphate are not very active nucleators of calcium oxalate crystallization [4, 8]. We have shown that when a metastable supersaturated calcium oxalate solution which rapidly precipitated when seeded with calcium oxalate crystals, remained practically stable during a long period of time when seeded with calcium phosphate or uric acid crystals (unpublished results). Nevertheless, by following procedures based on the use of a counting chamber and optical microscopy, to study heterogeneous nucleation in calcium oxalate precipitation, it was demonstrated that calcium phosphate and uric acid induced calcium oxalate precipitation in some cases [3]. In these procedures crystals of calcium phosphate, mainly as an amorphous precipitate of hydroxylapatite [13], or uric acid were obtained by precipitation "in situ" in a colloidal form. The above apparent contradictory facts can be explained because the capacity to induce heterogeneous nucleation of a given substance not only depends on its nature but also is notably affected by the size and morphology of its crystalline particles.

The methodology employed in this paper to induce the heterogeneous nucleation by calcium phosphate or uric acid, has been presented and discussed in detail in a previous paper [3]. The aim of this paper is to study the possible inhibitory effects of several substances on the heterogeneous nucleation of calcium oxalate by calcium phosphate or uric acid. Magnesium, pyrophosphate, citrate and chondroitin sulphate, manifested considerable effects, in absence of the particular heterogeneous nucleant and in all the conditions assayed. In the presence of calcium phosphate as heterogeneous nucleant, magnesium, pyrophosphate and citrate acted as effective inhibitors, whereas in the presencse of uric acid as heterogeneous nucleant only the chondroitin sulphate exhibited important inhibitory effects. The relative contribution of the citrate to the solution depletion (diminution of the calcium oxalate relative supersaturation) is given in Table 1. As can be seen, solution depletion was not important in any of the cases, and consequently the negative slope graphs in Figs. 1 and 2 must be assigned mainly to the inhibitory effects. In the case of the other substances, formation of complex species with calcium ions is less active than in front of citrate and consequently its contribution to solution depletion was even less important. On the other hand, the affinity of calcium phosphate for the chondroitin sulphate is inferior to the affinity that the magnesium, pyrophosphate or citrate manifest, and consequently the failure of inhibition that this substances exhibited can not be attributed to its dissapearance owing to the adsorption on the calcium phosphate. Moreover, it can be observed in Fig. 2d that in presence of uric acid, only the chondroitin sulphate manifested important inhibitory effects and precisely this substance shows an important affinity for the uric crystals, demonstrating that the relatively flat line 1 in Fig. 2a–c, can not be assigned to the affinity of the uric acid for such inhibitors. Thus it can be concluded that magnesium, phyrophosphate, citrate and chondroitin sulphate showed important inhibitory effects on the calcium oxalate crystallization, owing to its adsorption on the calcium oxalate crystalline surface. However, when in the presence of calcium phosphate heterogeneous nuclei, only magnesium, pyrophosphate and citrate suffered important adsorption on the calcium phosphate crystalline surface, causing important inhibitory effects, whereas in the presence of uric acid as heterogeneous nucleant, only in the chondroitin sulphate experiments was there a notable adsorption on

the uric acid crystalline surface, manifesting, as a consequence, great inhibitory effects. Hence, it must be clearly recognized that we must distinguish inhibitors of the calcium oxalate growth and aggregation from inhibitors of the initial heterogeneous nucleus growth (inhibitors of the heterogeneous nucleation). In this respect, the inhibition of the homogeneous nucleation can be explained as a consequence of the adsortion of the inhibitor on the embryos, which are formed in the supersaturated solution of the precipitating substance. This fact increases the difficulty of growth of such embryos and they are destroyed by collisions with other components of the solution; as a result the rate of nucleation decreases and the crystallization is strikingly retarded [7]. Thus, whereas inhibitors of the calcium oxalate heterogeneous nucleation can be different from the inhibitors of the calcium oxalate growth and aggregation, the inhibitors of the calcium oxalate homogeneous nucleation must also act as inhibitors of the calcium oxalate growth and aggregation.

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References

1. Finlayson B, Reid F (1978) The expectation of free and fixed paticles in urinary stone disease. Invest Urol 15:442–447
2. Grases F, Millan, Garcia Raso A (1988) Polyhidroxycarboxylic acids as inhibitors of calcium oxalate crystal growth; relation between inhibitory capacity and chemical structure. J Crystal Growth 89:496–500
3. Grases F, Gil JJ, Conte A (1988) Simple model for the heterogeneous nucleation study in calcium oxalate urolithiasis by use of optical microscopy. Br J Urol 61:468–473
4. Hallson PC, Rose GA, Sulaiman S (1982) Urate does not influence the formation of calcium oxalate crystals in whole human urine at pH 5.3. Clin Sci 62:421–425
5. Meites L (1963) Formation constants of metal complexes. In: Meites L (ed) Handbook of analytical chemistry. Mc Graw Hill, New York, pp 1–39
6. Nancollas GH, Gardner GL (1974) Kinetics of crystal growth of calcium oxalate monohydrate. J Cryst Growth 21:267
7. Naono H (1967) The effect of triphosphate on the crystallization of strontium sulphate. Bull Chem Soc Jpn 40:1104–1110
8. Pak CYC, Arnold LH (1975) Heterogeneous nucleation of calcium oxalate by seeds of monosodium urate. Proc Soc Exp Biol Med 149:930–932
9. Pak CYC, Galosy RA (1980) Propensity for spontaneous nucleation of calcium oxalate. Am J Med 69:681–689
10. Robertson WG, Peacock M, Nordin BEC (1972) Measurements of activity products in urine from stone-formers and normal subjets. In: Finlayson B, Hench LL, Smith LH (eds) Urolithiasis – physical aspects. National Academy of Sciences, Washington, pp 243–254
11. Robertson WG, Peacock M, Nordin BEC (1972) Crystalluria. In: Finlayson B, Hench LL, Smith LH (eds) Urolithiasis – physical aspects. National Academy of Sciences, Washington, pp 243–254
12. Robertson WG, Peacock M (1985) Pathogenesis of urolithiasis. In: Scheneider HJ (eds) Urolithiasis: etiology diagnosis. Springer, Berlin Heidelberg New York, pp 185–334
13. Termine JD, Percival RA (1970) Arch Biochem Biophys 140:307–317
14. Tomazic B, Nancollas FH (1979) The kinetics of dissolution of calcium oxalate hydrates. J Cryst Growth 46:355

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