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Effects of phytate and pyrophosphate on brushite and hydroxyapatite crystallization

Comparison with the action of other polyphosphates

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Abstract This is a comparative study of the effects of phytate and pyrophosphate and other polyphosphates on the crystallization of hydroxyapatite and brushite, the most frequent calcium phosphates involved in calcium oxalate urolithiasis. Brushite and hydroxyapatite crystal formation was studied in synthetic urine, through kinetic-turbidimetric measurements that allowed evaluation of the inhibitory effects on crystallization of insoluble salts. The effectiveness in preventing brushite crystallization decreases in the sequence phytate > polyphosphate > EDTPO > etidronate > pyrophosphate > triphosphate > medronate; whereas the order of effectiveness in preventing hydroxyapatite crystallization was EDTPO > etidronate = pyrophosphate > triphosphate > medronate > polyphosphate > phytate. Phytate, a natural inhibitor in urine, most effectively blocked brushite precipitation ($1.21 \cdot 10^{-5}$ M prevented crystallization during time periods of at least 1 h), and pyrophosphate was the natural inhibitor that most effectively blocked hydroxyapatite precipitation ($2.87 \cdot 10^{-6}$ M prevented crystallization during time periods of at least 1 h). This demonstrates that low excretion of these substances would pose a risk of renal lithiasis.

Key words Phytic acid · Pyrophosphate · Diphosphonates · Crystallization inhibitors · Hydroxyapatite · Brushite

Introduction

The role that polyphosphates play in regulating biological calcification is an interesting matter related to both pathological and non-pathological processes. Urinary lithiasis is a clear example of a renal pathology in which polyphosphates can exert important effects as crystallization inhibitors. One of the first urinary substances identified as having a clear capacity to inhibit the crystallization of insoluble calcium salts (oxalate and phosphate) was pyrophosphate [6, 7, 26]. Since then, a variety of substances contained in urine have been described as crystallization-inhibitor molecules, some of them of low molecular weight such as magnesium and citrate [3, 16, 25, 30, 32] and others with high molecular weight such as glycosaminoglycans, Tamm-Horsfall glycoprotein [4, 17, 18, 36], nephrocalcin [27, 28], and osteopontin [20]. It is very interesting to observe the evolution of ideas about the importance of crystallization inhibitors in urine during the last decades. Thus, in the 1970s, after identification of some urinary inhibitors such as pyrophosphate and the study of their in vitro effects, it was accepted that these substances could play an important role in preventing renal lithiasis. At the end of that decade and during the 1980s, the idea of the existence of a potent inhibitor of calcification as a substance present in normal urine, an acid of relatively low molecular weight, was supported. The substance was originally thought to be a small peptide, but this work was later disproved [2]. Phosphocitrate was also cited as a potent inhibitor [33], but no conclusive proof has been supplied subsequently. In the last decade, it seems that the tendency has been to consider that protein inhibitors of stone formation play the major role in the natural defence against nephrocalcinosis [5, 29]. Nevertheless, in this case the in vitro experiments seem to be inconclusive regarding the inhibitory capacity of these macromolecular substances, some authors defending their inhibitory capacity, others demonstrating crystallization-promoter properties [31]. In fact, there is no unequivocal evidence that any single macromolecular substance or group of macromolecular substances are directly involved in preventing stone formation as crystallization inhibitors, although their action as antiadherent substances (lubricants) in preventing the adherence of solid particles on the

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uroepithelium seems clear [12, 35]. However, this is a different action, which must not be mistaken for the crystallization inhibition.

Recently, it has been demonstrated that phytate, a substance of relatively low molecular weight present in normal urine [8], exerts a potent action as crystallization inhibitor of calcium salts (oxalate and phosphate) in urine [11, 13, 15]. The aim of this paper was to perform a comparative study of the effects of phytate and pyrophosphate on the crystallization of hydroxyapatite and brushite, the most frequent calcium phosphates involved in calcium oxalate urolithiasis as heterogeneous nuclei [10, 21]. On the other hand, a comparative study with the effects caused by other polyphosphates is also included.

Materials and methods

Reagents and solutions

Synthetic urine [9] was prepared immediately before use by mixing equal volumes of solutions A and B. Solution A contained 11.02 g/l $Na_2SO_4{\cdot}10H_2O$, 1.46 g/l $MgSO_4{\cdot}7H_2O$, 4.64 g/l NH_4Cl and 12.13 g/l KCl. Solution B contained 2.65 g/l $NaH_2PO_4{\cdot}2H_2O$, 18.82 g/l $Na_2HPO_4{\cdot}12H_2O$, 13.05 g/l NaCl and 0.08 g/l $C_2O_4Na_2$. The pH of solutions A and B had been adjusted previously to the working value.

Calcium stock solution was prepared by dissolving 100.09 g of calcium carbonate in 1 l of water with addition of HCl. When assayed, tested admixtures were dissolved in the synthetic urine solution.

Etidronic acid [(1-hydroxyethylidene)diphosphonic acid], medronic acid (methylenediphosphonic acid) and sodium triphosphate were obtained from Fluka (Buchs, Switzerland), phytic acid dodecasodium salt from Sigma (St. Louis, USA), ethylenediamine-*N*,*N*,*N*,*N*-tetrakis-(methylenephosphonic acid) (EDTPO) from ICN Biomedicals (Aurora, Ohio, USA) and sodium pyrophosphate (sodium diphosphate) and sodium polyphosphate from Merck (Darmstadt, Germany). Chemicals of reagent-grade purity and deionized-redistilled water were used.

Brushite crystallization studies

To study the brushite crystal formation in synthetic urine and the effects of potential crystallization inhibitor substances (phytate, pyrophosphate, triphosphate, polyphosphate, medronate, etidronate and EDTPO), kinetic-turbidimetric measurements were performed by means of a photometer (Metrohm 662) equipped with a fibre-optic light-guide measuring cell with attached light path 2×10 mm reflector, and using monochromatic light (550 nm). The crystallization processes were carried out in a thermostated ($T = 37$ °C) and magnetically stirred cylindrical glass flask (height 12 cm, diameter 6 cm). A schematic diagram of the experimental device used is shown in Fig. 1. One hundred millilitres of A solution and 100 ml of B solution were added to the crystallization flask, the measuring cell was immersed in the solution and the magnetic stirrer was switched on. Then 0.61 ml of 1 M calcium stock solution was added to achieve the desired supersaturation, and the chart-recorder was immediately switched on in order to register the absorbance-time curve, for a maximum time of 1 h, when no precipitation took place. The pH of the final solution after mixing was 6.5. The pH was continuously controlled during the experiment duration using a glass electrode introduced in the crystallizing solution. It had been proved previously that brushite crystals were formed under such conditions [14].

Hydroxyapatite crystallization studies

The study of the hydroxyapatite crystal formation in synthetic urine and the effects of potential crystallization inhibitor substances (phytate, pyrophosphate, triphosphate, polyphosphate, medronate, etidronate and EDTPO) was performed using the same experimental device used for brushite crystallization. The synthetic urine solution A did not contain $MgSO_4{\cdot}7H_2O$ because magnesium inhibits hydroxyapatite crystallization. The volume of 1 M calcium stock solution used to initiate the crystallization process was 0.30 ml, recording the absorbance-time curve for a maximum time of 1 h, when no precipitation took place. The pH of the final solution after mixing was 7.0. It had been proved previously that hydroxyapatite was obtained under these conditions [14].

Study of the formed solid phase

Solid samples of the different experiments were studied by X-ray diffraction techniques (Siemens D-5000) and by scanning electron microscopy (Hitachi S-530), demonstrating the formation of brushite and hydroxyapatite crystals in the above-mentioned crystallization conditions.

Results

The effects of the diverse phosphate derivatives assayed on brushite and hydroxyapatite crystallization are shown in Figs. 2 and 3 and are summarized in Table 1. As can be observed, the effectiveness in preventing brushite crystallization decreases in the sequence: phytate > polyphosphate > EDTPO > etidronate > pyrophosphate > triphosphate > medronate. As little as $1.21{\cdot}10^{-5}$ mol/l of phytate caused a complete inhibition of brushite crystallization during time periods of at least 1 h (see Table 1).

The order of effectiveness in preventing hydroxyapatite crystallization was: EDTPO > etidronate = pyrophosphate > triphosphate > medronate > polyphosphate > phytate. As little as $2.87{\cdot}10^{-6}$ mol/l of pyrophosphate caused a total inhibition of hydroxyapatite crystallization during time periods of at least 1 h (see Table 1).

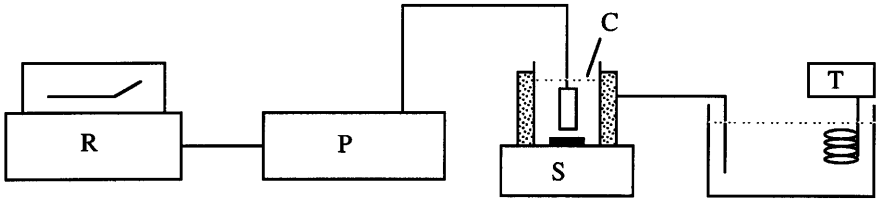

Fig. 1 Schematic of the experimental setup. (*T* thermostatic bath, *C* thermostated crystallization chamber, *S* magnetic stirrer, *P* photometer equipped with a fibre-optic light-guide measuring cell, *R* recorder)

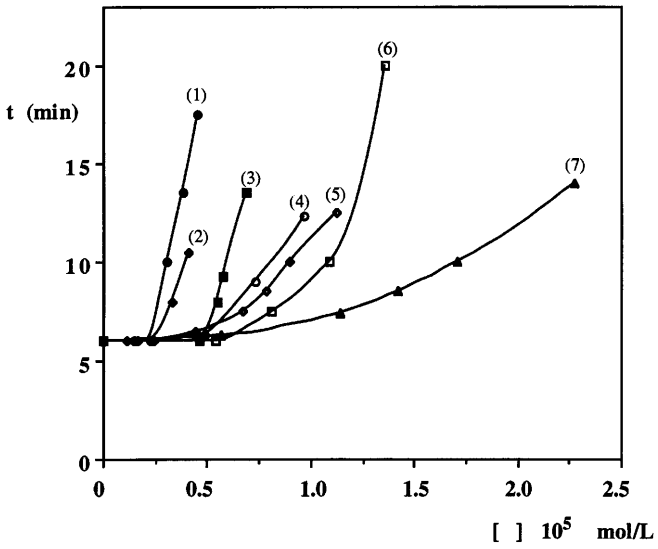

Fig. 2 Induction time (minutes) for brushite crystallization in synthetic urine (pH 6.5, $[Ca^{2+}] = 3.05 \times 10^{-3}$ M, $[PO_4^{3-}] = 78.5 \times 10^{-3}$ M, $T = 37$ °C) in the presence of different concentrations of *(1)* phytate, *(2)* polyphosphate, *(3)* EDTPO, *(4)* etidronate, *(5)* pyrophosphate, *(6)* triphosphate, *(7)* medronate

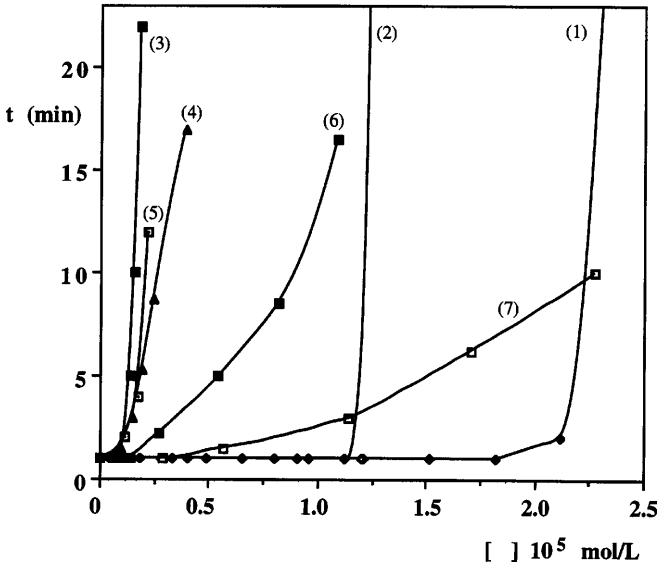

Fig. 3 Induction time (minutes) for hydroxyapatite crystallization in synthetic urine (pH7.0, $[Ca^{2+}] = 1.50 \times 10^{-3}$ M, $[PO_4^{3-}] = 78.5 \times 10^{-3}$ M, $T = 37$ °C) in the presence of different concentrations of *(1)* phytate, *(2)* polyphosphate, *(3)* EDTPO, *(4)* etidronate, *(5)* pyrophosphate, *(6)* triphosphate, *(7)* medronate

Discussion

From our results and considering previously reported data [1], it can be deduced that the effects of the diverse phosphate derivatives assayed on brushite or hydroxyapatite crystallization clearly depend on both the nature of the insoluble calcium phosphate formed and the type of crystallization step inhibited (nucleation or crystal growth). This demonstrates the specificity of the crystallization-inhibiting processes, since they depend on the interactions of the concrete substance (inhibitor) with the nuclei of the solid formed or on the different rates of adsorption/desorption of the inhibitor on the various calcium phosphate surfaces (brushite or hydroxyapatite).

It is interesting to observe how the nucleation of hydroxyapatite crystals is more easily inhibited than the nucleation of brushite. Thus, whereas around 1×10^{-6} M of EDTPO or pyrophosphate caused inhibitory effects on hydroxyapatite crystalline nucleation, the minimum concentrations that caused inhibitory effects on brushite crystalline nucleation corresponded to 3×10^{-6} M of phytate. Etidronate caused crystallization-inhibitory effects clearly superior to those produced by medronate in both hydroxyapatite and brushite formation, thus demonstrating the influence that a hydroxyl group tied to the carbon atom adjacent to the phosphate group can exert on calcium phosphate crystallization processes. It is interesting to consider that disodium etidronate belongs to the bisphosphonate class of drugs which are generally targeted for the treatment of a variety of bone diseases such as hypercalcaemia of malignancy, Paget's disease and osteoporosis [19, 37].

Some recent papers [22] seem to demonstrate that calcium phosphate crystallization can occur within the nephron and consequently within minutes. In such cases the increase, by the action of the inhibitor, of several minutes in the crystallization time would be enough to prevent such crystallization. The evaluation of the minimum concentration of crystallization inhibitors that prevents calcium phosphate precipitation during long periods of time ($t > 1$ h) is also useful in examining their capacity to avoid the development of calcium phosphate deposits in cavities of low urodynamic efficacy. If precipitation was produced over a time period of more than 1 h due to inhibitory effects, it would take place in the bladder, and tiny crystals would be eliminated without any further complications. As can be deduced from the data in Table 1, whereas phytate was the natural inhibitor contained in urine that most effectively blocked brushite precipitation, pyrophosphate was the natural inhibitor that more effectively blocked hydroxyapatite crystallization. In both cases, it is interesting to observe how the concentration that totally blocks calcium phosphate precipitation corresponds to values that can be found in human urine and consequently low excretion of these substances could imply renal lithiasis risk. It must be taken into account that calcium phosphates are recognized to be one of the most effective and common heterogeneous nucleus for calcium oxalate crystallization [10, 21]. On the other hand, it must also be considered that our present studies have been performed in synthetic urine, and this might not necessarily reflect accurately the physiological events in human urine.

The role that phytate plays in regulating biological calcifications is uncertain at the present. Thus, apart from the important active quantities found in urine [8],

Table 1 Structure and effects of the assayed substances on brushite and hydroxyapatite crystallization in the conditions indicated in the Materials and methods section

Substance	Chemical structure	$[n]^a$ mol/l	$[n]^b$ mol/l	$[n]^c$ mol/l	$[n]^d$ mol/l
Phytic acid	picture	3.03×10^{-6}	12.12×10^{-6}	1.36×10^{-6e}	24.24×10^{-6}
Pyrophosphate	picture	5.75×10^{-6}	46.0×10^{-6}	1.15×10^{-6}	2.87×10^{-6}
Triphosphate	picture	7.91×10^{-6}	23.72×10^{-6}	2.77×10^{-6}	15.82×10^{-6}
Polyphosphate	picture $OH_2(HPO_3)_{12-13}$	3.17×10^{-6}	14.77×10^{-6}	3.17×10^{-6f}	14.77×10^{-6}
Medronic acid	picture	11.49×10^{-6}	63.22×10^{-6}	11.49×10^{-6}	80.46×10^{-6}
Etidronic acid	picture	7.35×10^{-6}	39.21×10^{-6}	1.47×10^{-6}	12.25×10^{-6}
EDTPO	picture	5.55×10^{-6}	16.20×10^{-6}	1.39×10^{-6}	2.31×10^{-6}

[a] Lowest concentration with inhibitory effects on brushite crystallization
[b] Extrapolated concentration with total inhibition of brushite crystallization for time periods of at least 1 h
[c] Lowest concentration with inhibitory effects on hydroxyapatite crystallization
[d] Extrapolated concentration with total inhibition of hydroxyapatite crystallization for time periods of at least 1 h
[e] At concentrations within the range 1.36×10^{-6} to 24.24×10^{-6} mol/l, only the final precipitated amount decreased, but the induction period was not modified
[f] At concentrations within the range 3.17×10^{-6} to 12.66×10^{-6} mol/l, only the final precipitated amount decreased, but the induction period was not modified

its intracellular concentrations are relatively high, within the range 10^{-4} to 10^{-5} M, depending on the type of biological tissue [34]. The intracellular calcium concentration is normally low, and it is now well established that under certain conditions mitochondria can accumulate large net amounts of calcium and phosphate in vivo [23, 24]. Thus, phytate may influence intracellular calcification processes preventing or controlling such calcium phosphate deposits, and would then participate in calcium or phosphate homeostasis. Obviously, as can be deduced from the data of the present study, the intracellular concentrations of phytate would be high enough for this purpose.

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