Calc. Tiss. Res. 13, 295—303 (1973)

The Influence of Multidentate Organic Phosphonates on the Crystal Growth of Hydroxyapatite

John L. Meyer and George H. Nancollas
Department of Chemistry, State University of New York at Buffalo

Received February 13, accepted May 19, 1973

The rate of crystal growth of hydroxyapatite seed crystals in stable supersaturated solutions of calcium phosphate has been studied under reproducible conditions at 25° and at a constant pH of 7.40 in the presence of the organic phosphonates HEDP, 1-hydroxyethylidine 1,1-diphosphonic acid, NTMP, nitrilotri (methylene phosphonic acid); ENTMP, N,N,N'N' ethylenediamine-tetra (methylene phosphonic acid); TENTMP, triethylenediamine tetra (methylene phosphonic acid). It is suggested that the marked inhibitory influence of the additives upon the rate of crystal growth is due to the formation of strong, substitution inert chelate bonds with the calcium ions present at kinks and dislocations on the crystal surface of HAP. The results of this study show that the potentially hexadentate ligand ENTMP is more effective as a crystal growth inhibitor than the tetradentate NTMP or the tridentate HEDP. The general ineffectiveness of the monophosphonates as crystal growth inhibitors supports the conclusion that the calcium ions are chelated at the surface thereby preventing further deposition of calcium phosphate at that growth site. The relatively low concentration of added phosphonate as compared with the calcium ion concentration rules out calcium chelation in the bulk of the solution as a significant factor in the observed crystal growth inhibition.

Key words: Hydroxyapatite — Crystallization — Kinetics — Phosphonates — Inhibition.

Le taux de croissance cristalline de l'hydroxyapatite dans des solutions supersaturées stables de phosphate de calcium a été étudié dans des conditions reproductibles à 25° et à un pH constant de 7,4, en présence de phosphonates organiques HEDP, 1-hydroxyethylidine 1, d'acide 1-diphosphonique, NTMP, nitrilo-tri (méthylène acide phosphonique), ENTMP N,N,N'N' éthylène diamine-tetra (méthylène acide phosphonique), TENTMP triethylène-diamine tetra-(méthylène acide phosphonique). Il semble que l'action nette d'inhibition des produits surajoutés sur le taux de croissance cristalline est en rapport avec des liaisons de chélation fortes et de substitution inerte avec les ions calcium situés au niveau des noeuds et zones de dislocations de la surface cristalline d'HAP. Les résultats de cette étude montre que le ligand ENTMP potentiellement hexadenté est plus efficace comme inhibition de croissance cristalline que le NTMP tétradenté ou le HED tridenté. L'inéfficacité générale des monophosphonates comme inhibiteurs de croissance cristalline confirme la conclusion que les ions calcium sont complexés à la surface, empêchant par suite un dépôt ultérieur de phosphate de calcium au niveau de cette zone. La concentration relativement faible de phosphonate surajouté, comparée à la concentration en ion calcium, exclut la chélation du calcium dans la solution comme facteur important de l'inhibition de croissance cristalline observée.

Die Geschwindigkeit des Kristallwachstums von Hydroxyapatit-Kristallkeimen in stabilen übersättigten Calciumphosphatlösungen wurde unter reproduzierbaren Bedingungen studiert und zwar: bei 25°, bei einem konstanten pH von 7,4 und in Anwesenheit der organischen

For reprints: Dr. George H. Nancollas, Department of Chemistry, State University of New York at Buffalo, Buffalo, New York 14214, U.S.A.
This investigation was supported in part by grant DE 3223-01 from the National Institute of Health.

Phosphate HEDP = 1-Hydroxy-äthylidin-1,1-Diphosphonsäure; NTMP = Nitrilo-trimethylen-Phosphonsäure; ENTMP = N,N,N′N′-Äthylendiamin-tetramethylen-Phosphonsäure; TENTMP = Triäthylendiamin-tetramethylen-Phosphonsäure. Es wird vorgeschlagen, daß die deutliche Hemmwirkung dieser zugesetzten Substanzen auf die Geschwindigkeit des Kristallwachstums durch die Bildung von starken, Substitutions-unempfindlichen Chelat-Bindungen mit den Calciumionen bedingt ist, welche an Knick- und Verschiebungsstellen auf der Kristalloberfläche von HAP vorkommen. Die Ergebnisse dieser Untersuchung zeigen, daß das potentiell sechswertige Anion ENTMP ein wirksamerer Hemmer des Kristallwachstums ist als das vierwertige NTMP oder das dreiwertige HEDP. Die allgemeine Unwirksamkeit der Monophosphonate als Kristallwachstums-Hemmer bekräftigt den Schluß, daß die Calciumionen an der Oberfläche als Chelatkomplexe gebunden sind, wodurch eine weitere Ablagerung von Calciumphosphat an dieser Wachstumsstelle verhindert wird. Die relativ niedrige Konzentration des beigefügten Phosphonats, im Vergleich mit der Konzentration der Calciumionen, schließt die Bildung von Calciumchelatkomplexen in der Lösung als wichtigen Faktor in der beobachteten Kristallwachstums-Hemmung aus.

Introduction

Many different substances have been proposed as the natural inhibitor in serum and urine which prevents the formation of undesirable solid concretions. One of the most attractive has been the pyrophosphate ion (Fleisch, 1964) which can be formed from the orthophosphate ions present in any biological fluid. The existence of pyrophosphate has been demonstrated in many biological systems (Fleisch and Bisaz, 1962; Russell *et al.*, 1971; Hausmann *et al.*, 1970). In addition, it has been shown independently that the presence of pyrophosphate in spontaneous calcification (Francis, 1969) as well as in more controllable crystal growth systems (Marshall and Nancollas, 1969) will prevent or greatly inhibit the precipitation of calcium phosphate from its supersaturated solution. The direct test of pyrophosphate as an inhibitor for undesirable biological calcification in *in vivo* situations has met with limited success, possibly because of the hydrolytic instability of the P-O-P linkage. Both enzymatic and simple acid-base hydrolysis have been proposed to explain the much lower inhibitory influence of the condensed phosphates (Schibler, Russell and Fleisch, 1968).

Another class of additives of a similar type to the polyphosphates, has proven to be most effective in inhibiting calcification (Francis, 1969). In these compounds, referred to as organic phosphonates, the relatively inert P-C-P linkage is substituted for the hydrolyzable P-O bond of the polyphosphates. The phosphonates have, in addition, been shown to be unaffected by exposure to a biological medium (Francis, 1969). Some of the phosphonates tested have been shown to be extremely effective, when present at concentrations as low as 10^{-6} M, in preventing the precipitation and the dissolution of calcium phosphate both *in vivo* and *in vitro* (Russell *et al.*, 1970; Jethi and Wadkins, 1971; Fleisch, Russell and Francis, 1969; Francis *et al.*, 1969). They have also been shown to interfere with the post-eruptive maturation of dental enamel in rats (Briner *et al.*, 1971). These results, coupled with the observation that small quantities of a diphosphonate, methylene diphosphonate, can prevent $^{45}Ca^{2+}$ and $H^{32}PO_4^{2-}$ exchange from a calcified organic matrix (Jethi and Wadkins, 1971), indicate that an unusually stable association is taking place between the inhibitor and the calcium and/or phosphate ions present at the surface of the calcified material.

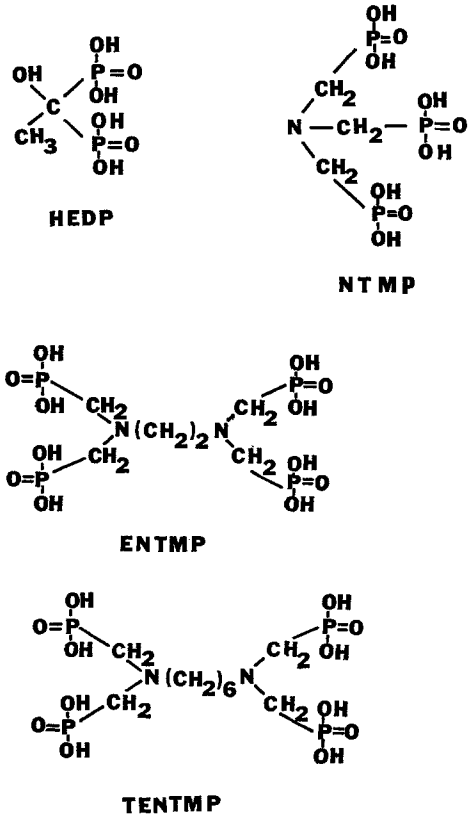

Fig. 1. Molecular structures of organic phosphonates. HEDP: 1-hydroxyethylidine 1,1-diphosphonic acid; NTMP: nitrilotri (methylene phosphonic acid); ENTMP: N,N,N′,N′-ethylenediaminetetra (methylene phosphonic acid); TENTMP: tri ethylenediamine tetra (methylene phosphonic acid)

Because of the recent interest in this class of compounds, it was decided to test their effectiveness as inhibitors of the crystal growth of synthetic hydroxyapatite (HAP) under the carefully controlled conditions of seeded growth from stable supersaturated solution which have been developed in our laboratory. The use of a pure synthetic system avoids the complications introduced by the impurities present in a biological system and therefore will yield more direct information concerning ion-ion and ion-crystal interaction. The usefulness of the reproducible procedure of seeded crystal growth in contrast to the spontaneous precipitation experiments has already been emphasized (Nancollas and Mohan, 1970).

A series of polyfunctional phosphonates, some of which have not been previously investigated as calcification inhibitors, were selected for study. The structures are presented in Fig. 1, from which it can be seen that the additives vary appreciably in both the number and spatial distribution of the chelating phos-

phonate groups. It was hoped that the study of such a series would throw light on the mechanism whereby these additives could so effectively prevent calcification *in vivo*.

Materials and Methods

Reagent grade chemicals were used without further purification, with the exception of calcium chloride, which was recrystallized twice before use. The organic phosphonates were kindly donated by Dr. T. M. King of the Monsanto Corporation. All solutions were prepared with triple-distilled, carbonate-free water in grade A glassware. The HAP seed crystals, prepared and characterized as previously described (Nancollas and Mohan, 1970), were aged for at least six months before use.

The experimental procedure was similar to that previously described (Meyer and Nancollas, 1972). The inhibitors were added to the stable supersaturated solutions immediately before the addition of seed crystals (1 ml slurry containing about 20 mg of crystals). The pH of the crystal growth solution was maintained at 7.40 ± 0.01 by means of pH stat-controlled addition of carbonate-free potassium hydroxide solution. The temperature was maintained constant at $25.0 \pm 0.05°$.

Results

Each of the inhibitors in Fig. 1 is capable of completely inhibiting the initial stages of the crystal growth of HAP when present at concentration levels as low as 1×10^{-6} M. The results of experiments carried out at this concentration are

Table 1. Induction periods for nucleation and crystal growth of calcium phosphate on HAP seed from supersaturated solution in the presence of 10^{-6} M of various organic phosphonates

Inhibitor	Number of phosphonate groups	Induction period
ENTMP	4	24 h
TENTMP	4	12 h
NTMP	3	4 h
HEDEP	2	4 h

summarized in Table 1. No appreciable deposition of calcium phosphate occurred during induction or delay periods of at least four hours after the addition of seed material. Indeed, the most effective of the inhibitors, ENTMP, prevented the deposition of any material from the supersaturated solution for a period in excess of 24-h whereas TENTMP allowed a slow rate of crystal growth only after a 12 h induction period. A measurable rate of crystal growth in the presence of 10^{-6} M of the other two ligands NTMP and HEDP was observed after about 4 h induction although the initial stages of crystal growth were completely inhibited.

Differences in the ability of the four additives to inhibit crystal growth are shown more clearly in Fig. 2 in which the relative rates of calcium and phosphate deposition in the presence of 5×10^{-7} M of each of the inhibitors present initially in the supersaturated solution are shown. The seed crystals were added to solution at time zero. Here the potentially hexadentate ligand, ENTMP was the most effective while TENTMP and HEDP had nearly the same inhibitory effect at all

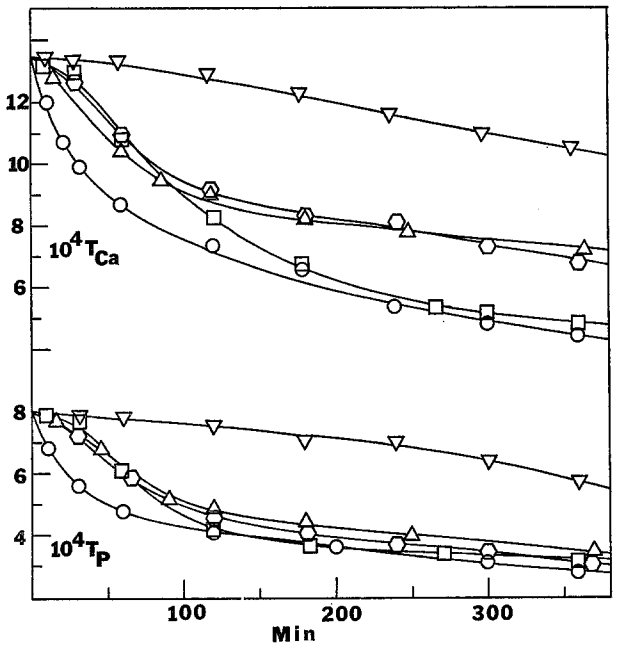

Fig. 2. The concentrations of total calcium, T_{Ca}, and total phosphate, T_p, plotted as a function of time. ○ No additive; □ NTMP; △ HEDP; ⬡ TENTMP; ▽ ENTMP. All additives 5×10^{-7} M

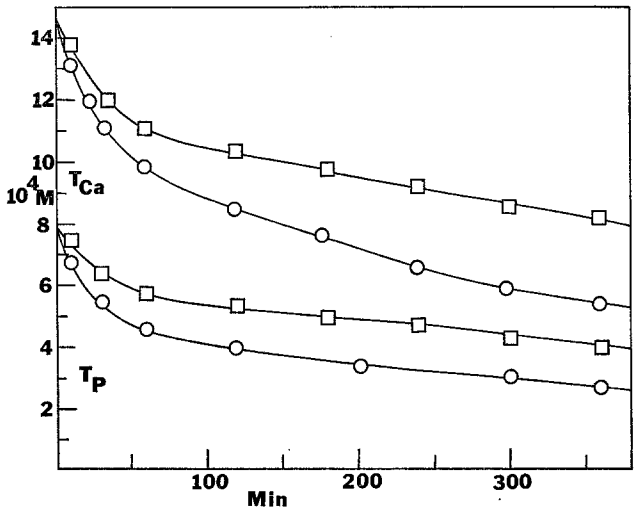

Fig. 3. The concentrations of total calcium, T_{Ca}, and total phosphate, T_p, plotted as a function of time in the presence (□) and absence (○) of 1×10^{-7} M ENTMP

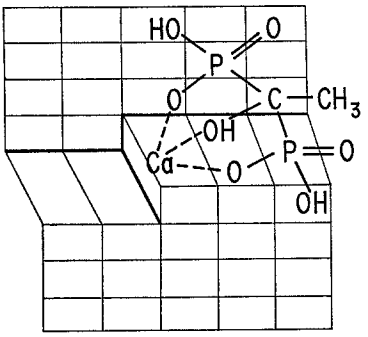

Fig. 4. Possible mode of bonding of HEDP to a calcium ion at an active growth site on the surface of a HAP crystal

stages of crystal growth although much less than that observed for ENTMP. NTMP behaved similarly to TENTMP and HEDP in the early stages (about 60 min), but its effect decreased rapidly as crystal growth proceeded. It can be seen in Fig. 3 that even when present at a trace level of 1×10^{-7} M, ENTMP exhibited a marked effect on the rate of crystal growth on HAP. The growth curves in the presence of the other inhibitors at this concentration are nearly superimposable with the control (no additive) and therefore are omitted from Fig. 3 for clarity.

Discussion

The crystal growth of HAP has previously been shown to occur in at least two distinct stages (Nancollas and Mohan, 1970). An initial rapid period of growth in which the depositing phase maintains a calcium:phosphate mole ratio of $\sim$1.45 accounts for $\sim$90 min of the crystal growth process. This initial period is followed by a much slower stage (about 1 week) during which the crystals mature until the calcium:phosphate mole ratio reaches the value 1.67 required for pure HAP. The presence of at least two distinct phases suggested by the kinetic results has been supported by scanning electron microscope observation of the HAP crystals at their various stages of growth (Meyer *et al.*, 1972). Results of an earlier study involving stannous and fluoride ions are also consistent with this view (Meyer and Nancollas, 1972). Fluoride ion exhibited a slight inhibitory effect on the initial stages of growth but was seen to actually accelerate subsequent deposition of calcium and phosphate, suggesting that different crystalline phases of calcium phosphate were involved. All stages of crystal growth were affected by stannous ions when present at concentrations more than 10^{-5} M. The actual nature of the inhibiting species was much in doubt, however, since stannous ion is appreciably hydrolyzed at near neutral pH. Adsorption of the stannous species on the surface of the crystal was proposed since similar work involving dicalcium phosphate dihydrate crystal growth has shown that the inhibition by stannous ion can be adequately described by a Langmuir adsorption isotherm (Marshall, 1970). In the light of these findings, it is reasonable to interpret the

results of the present investigation in terms of an adsorption phenomenon. The possibility of prevention of crystal growth by effective removal following chelation of calcium ions in solution is excluded, since less than 0.1% of the calcium can be complexed even at the highest concentration of phosphonates used in this study. The estimation, described below, of the amount of surface of the seed crystals that could be covered by the inhibitor, yields somewhat surprising results. The surface area of the seed crystals as determined using a dynamic surface area instrument (Monosorb, manufactured by Quantachrome Co., L. I., N. Y.) is 40 m^2/g. The maximum area covered by a single molecule of the most effective inhibitor in this study, ENTMP, calculated on the basis of normal reported bond distances, is about 44 A^2. This assumes that the molecule lies flat on the surface of the crystal without regard to the actual mode of bonding at the surface. If all the ENTMP present in solution at a concentration sufficient to completely inhibit crystal growth (10^{-6} M), were absorbed on the surface of the crystals, a maximum total surface area of 1.2×10^3 cm^2 of the crystal could be covered which represents approximately 15% of the total surface area of the crystals. It should be pointed out that these calculations represent the maximum surface coverage, but the actual value is probably less since it is likely that not all the ENTMP is adsorbed on the crystals. This simple calculation clearly shows that prevention of crystal growth by monolayer coverage of the crystal surfaces by phosphonate is impossible. The only mechanism whereby complete inhibition can be achieved by such a small concentration of additive is the adsorption at active growth sites on the crystal faces. Crystal growth at active sites, generally envisioned as dislocations or kinks on the crystal surface, has been generally accepted, since the introduction of the classical BCF theory (Burton *et al.*, 1951), as the only mechanism which would explain the observed crystal growth of many salts at low supersaturation (Walton, 1967). This fact, coupled with the probability that only a few dislocations are present on each surface can, therefore, explain the inhibitory effect of low concentrations of additives.

The differences observed in the inhibitory action of the phosphonates can be discussed in terms of the structural arrangement of the chelating phosphonate groups. The most effective of the additives, ENTMP, is structurally similar to the powerful sequesterant EDTA (ethylenediamine tetraacetic acid) and would also be expected to be a good chelating agent for calcium ions situated at crystal surface sites. In TENTMP, another potential hexadentate ligand, the introduction of four additional methylene groups between the two diphosphonate groupings would be expected to decrease its chelating ability by introducing additional strain to the chelate rings formed with the calcium ion. The interaction of all four functional groups of TENTMP with a single cation at the surface of the crystal is also unlikely from entropy considerations, and this may explain its relative ineffectiveness as an inhibitor.

Although to a lesser extent than ENTMP, both NTMP and HEDP are very effective inhibitors of the crystal growth of HAP. Based on the number of chelating groups, it would be expected that NTMP would exert the greater influence. However, it is seen in Fig. 2 that HEDP is more effective than NTMP at least for the later stages of crystal growth, even though NTMP appears to have a slightly greater effect on the early stages (Fig. 2). It is clear that factors

other than simply the number of chelating groups must also be taken into account in discussing the stability of the calcium complexes with the ligands. The geometrical arrangement of the chelating groups may be a dominant factor, especially since methylene phosphonate functional groups are present in NTMP, whereas simple phosphonate groups are bonded to the central tetrahedral carbon in HEDP. HEDP can form a stable six membered chelate ring, with calcium bonded to the two phosphonate groups. On the other hand, NTMP forms five-membered chelate rings (also stable for metal-ligand complexes) with both the phosphonate groups and the central nitrogen participating in the calcium bonds. The question remains, however, as to whether the small, relatively non-polarizable cation, calcium, can for a strong bond with the polorizable nitrogen atom of NTMP. It is well known that a "hard" cation such as calcium forms an electrostatic bond with a similar atom such as the oxygen of the phosphonates in preference to an amino nitrogen (Nancollas, 1966). If a bond is not formed with the nitrogen of NTMP, a much less stable eight-membered chelate ring would result and this could explain the apparent greater inhibitory power of the diphosphonate over the triphosphonate. A ^{1}H and ^{31}P study, by nuclear magnetic resonance, of the association of Ca^{2+} with NTMP and its N-oxide in solution revealed similar binding energetics, indicating that the tertiary nitrogen was not participating in the coordination to calcium (Carter *et al.*, 1967). However, a similar study with the structurally analogous NTA (nitrilotriacetic acid) showed a marked decrease in affinity for Ca^{2+} in solution upon oxidation of the nitrogen (Carter *et al.*, 1967). Calcium bonding with the hydroxyl group of HEDP is also possible since a stable six membered chelate ring would result. Previous studies have indicated that in hydroxyethylimino-diacetic acid the hydroxyl group participates in calcium bonding (Nancollas and Park, 1967). A study is currently underway to relate the thermodynamic functions for the formation of calcium phosphonate ion-pairs in solution with their ability to prevent crystal growth from stable supersaturated solution.

In summary, the mechanism proposed for the inhibition of crystal growth by the phosphonates is the formation of strong, substitution inert chelate bonds with calcium ions present at kinks and dislocations on the crystal surface of HAP (active growth sites). This model is represented in Fig. 4. The observation that monophosphonates are generally not effective inhibitors (Russell *et al.*, 1970) supports the conclusion that the calcium ions are chelated at the surface, as do the results of this study which show that the potentially hexadentate ligand ENTMP is more effective than the tetradentate NTMP or tridentate HEDP. It is likely that the inhibitor acts by bonding to a cation already present at a dislocation on the surface of the crystal, thereby preventing further deposition of calcium phosphate at that growth site. An ion already complexed by a phosphonate in solution may also be adsorbed at the dislocation, however, since a large excess of "free" calcium is present in solution, the small amount of complexed cation would not be expected to compete favorably with the bulk calcium for the growth sites. The fact that growth is stopped immediately upon addition of seed crystals also supports this conclusion.

References

Briner, W. W., Francis, M. D., Widder, J. S.: Factors affecting the rate of post-eruptive maturation of dental enamel. Calc. Tiss. Res. **7**, 249–256 (1971)

Burton, W. K., Cabrera, N., Frank, F. C.: The growth of crystals and the equilibrium structure of their surfaces. Phil. Trans. A **243**, 299–358 (1951)

Carter, R. P., Crutchfield, M. D., Irani, R. R.: Nitrilotri (methylenephosphonic acid) N-oxide and nitrilotriacetic acid N-oxide: Acidity and complexing of calcium and magnesium ions. Inorg. Chem. **6**, 943–946 (1967)

Fleisch, H.: Role of nucleation and inhibition in calcification. Clin. Orthop. **32**, 170—180 (1964)

Fleisch, H., Bisaz, S.: Isolation from urine of pyrophosphate, a calcification inhibitor. Amer. J. Physiol. **203**, 671–675 (1962)

Fleisch, H., Russell, R. G. G., Francis, M. D.: Diphosphonates inhibit hydroxyapatite dissolution in vitro and bone resorption in tissue culture in vivo. Science **165**, 1262–1264 (1969)

Francis, M. D.: The inhibition of calcium hydroxyapatite crystal growth by polyphosphonates and polyphosphates. Calc. Tiss. Res. **3**, 151–162 (1969)

Francis, M. D., Russell, R. G. G., Fleisch, H.: Diphosphonates inhibit formation of calcium phosphate crystals in vitro and pathological calcification in vivo. Science **165**, 1264–1266 (1969)

Hausmann, E., Bisaz, S., Russell, R. G. G., Fleisch, H.: The concentration of inorganic pyrophosphate in human saliva and dental calculus. Arch. oral Biol. **15**, 1389–1392 (1970)

Jethi, R. K., Wadkins, C. L.: Studies of the mechanism of biological calcification. II. Evidence for a multi-step mechanism of calcification by tendon matrix. Calc. Tiss. Res. **7**, 277–289 (1971)

Marshall, R. W.: Complex formation, crystal growth and dissolution in calcium phosphate solutions, Ph. D. Thesis, SUNYAB 1970

Marshall, R. W., Nancollas, G. H.: The kinetics of crystal growth of dicalcium phosphate dihydrate. J. Phys. Chem. **73**, 3838–3844 (1969)

Meyer, J. L., Eick, J. D., Nancollas, G. H., Johnson, L. N.: A scanning electron microscope study of the growth of hydroxyapatite crystals. Calc. Tiss. Res. **10**, 91–102 (1972)

Meyer, J. L., Nancollas, G. H.: The effect of stannous and fluoride ions on the rate of crystal growth of hydroxyapatite. J. dent. Res. **51**, 1443–1450 (1972)

Nancollas, G. H.: Interactions in electrolyte solutions. Amsterdam: Elsevier 1966

Nancollas, G. H., Mohan, M. S.: The growth of hydroxyapatite crystals. Arch. oral Biol. **15**, 731–745 (1970)

Nancollas, G. H., Park, A. C.: Proton magnetic resonance studies on some metal complexes of methyliminodiacetic acid and hydroxyethyliminodiacetic acid. J. Phys. Chem. **71**, 3678–3681 (1967)

Russell, R. G. G., Bisaz, S., Donath, A., Morgan, D. B., Fleisch, H.: Inorganic pyrophosphate in plasma in normal persons and in patients with hypophosphatasia, osteogenesis imperfecta, and other disorders of bone. J. clin. Invest. **50**, 961–969 (1971)

Russell, R. G. G., Mühlbauer, R. C., Bisaz, S., Williams, D. A., Fleisch, H.: The influence of pyrophosphate, condensed phosphates, phosphonates and other phosphate compounds on the dissolution of hydroxyapatite in vitro and on bone resorption induced by parathyroid hormone in tissue culture and in thyroparathyroidectomised rats. Calc. Tiss. Res. **6**, 183–197 (1970)

Schibler, D., Russell, R. G. G., Fleisch, H.: Inhibition by pyrophosphate and polyphosphate of aortic calcification induced by vitamin D_3 in rats. Clin. Sci. **35**, 363–372 (1968)

Walton, A. G.: The formation and properties of precipitates, p. 46–50. New York: Interscience 1967