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Procedure for the Study of Acidic Calcium Phosphate Precursor Phases in Enamel Mineral Formation

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Summary. Considerable evidence suggests that an acidic calcium phosphate, such as octacalcium phosphate (OCP) or brushite, is involved as a precursor in enamel and other hard tissue formation. Additionally, there is *in vitro* evidence suggesting that fluoride accelerates and magnesium inhibits the hydrolysis of OCP to hydroxyapatite (OHAp). As the amount of OCP or brushite in enamel cannot be measured directly in the presence of an excess of hydroxyapatite, a procedure was developed that allows for their indirect *in vivo* quantification as pyrophosphate. This permits study of the effects of fluoride and magnesium ions on enamel mineral synthesis. Rat incisor calcium phosphate was labeled by intraperitoneal injection of $NaH_2{}^{32}PO_4$. The rats were then subjected to various fluoride and magnesium treatments with subcutaneous implanted osmotic pumps. They were then killed at predetermined intervals; the nascent sections of the incisors were collected, cleaned, and pyrolyzed at 500°C for 48 hours to convert acidic calcium phosphates to calcium pyrophosphate; the pyrophosphate was separated from orthophosphate by anion-exchange chromatography; and the resulting fractions were counted by liquid scintillation spectrometry. The activities of the pyro- and orthophosphate fractions were used to calculate the amount of acidic calcium phosphate present in the nascent mineral. The results demonstrated that the percentage of radioactive pyrophosphate in nascent incisors decreased with time, with increasing serum F^- concentration, and with decreasing serum magnesium content. The technique described here should prove to be a powerful new tool for studying the effects of various agents on biological mineral formation.

Key words: Enamel – Fluoride – Hydroxyapatite – Magnesium – Octacalcium phosphate – ^{32}Phosphate.

The formation of tooth enamel involves a complex of physiological and biochemical events that interact with, and to a large extent control, the process of mineralization. The formation and hydrolysis of acidic calcium phosphate precursors that participate in the growth of enamel mineral play an important role in the crystal growth mechanism which, in turn, relates in basic ways to physicochemical properties of tooth enamel crystals such as solubility, rates of dissolution, morphology, impurity and defect content, and the ways in which CO_3^{2-} is incorporated into the structure. Considerable *in vitro* [1–8] and *in vivo* [9–13] evidence points to the possible involvement of octacalcium phosphate (OCP), $Ca_8H_2(PO_4)_6 \cdot 5H_2O$, in hydroxyapatite (OHAp) biosynthesis. It has also been suggested [3] that fluoride plays a specific role in the mineralization process by facilitating conversion of an acidic calcium phosphate precursor (e.g., brushite, $CaHPO_4 \cdot 2H_2O$, or OCP) into apatite during growth of mineral crystals.

This report describes *in vivo* procedure for measuring, as a function of time, the amount of acidic calcium phosphates present in the portion of the mineral that was formed within a specific time interval. The results were interpreted in terms of the formation and hydrolysis of acidic precursors that may be involved in the formation of enamel mineral.

Materials and Methods

The procedure utilized ^{32}P and was based on a method developed by Francois [14] in which acidic calcium phosphates are converted by pyrolysis to pyrophosphate, and then the ortho- and pyrophosphates are separated in an ion exchange column. We describe here improvements in his procedure, giving it a sensitivity such that it can reliably detect effects of other components on the formation and hydrolysis of the precursor. The procedure is outlined in Figure 1.

Fluoride Effect

Initially, we attempted to administer fluoride by adding 100 μg/ml to drinking water. However, because the rat is nocturnal and tends to drink mostly at night, and because the half-life of blood fluoride is only 45–60 minutes [15], we encountered wildly fluctuating blood fluoride levels which depended on the time of day the sample was collected. Therefore, to maintain optimal, constant blood levels of fluoride, osmotic pumps were used.

One 8.5 μl/hour pumping rate osmotic pump (ALZET®, Model #2ML1, Alza Corp., Palo Alto, CA 94304) containing 0.95 mol/liter NaF was implanted subcutaneously into the dorsal cervical region of each test rat 24 hours prior to ^{32}P injection. This permitted constant serum fluoride levels with maximum delivery of 819 μg F/kg/hour for 7 days (Table 1). To obtain dose/response data, combinations of the smaller 0.87 μl/hour pumping rate minipumps (model #2001, Alza Corp.) were used in animals receiving lower doses. The animals were maintained on standard pelleted rat chow (Ralston Purina Co., St. Louis, MO 63188) and distilled water *ad libitum* up to the time of the animal's death.

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Certain commercial materials and equipment are identified in this paper to specify the experimental procedure. In no instance does such identification imply recommendation or endorsement by the National Institute of Standards and Technology or the ADA Health Foundation or that the material or equipment identified is necessarily the best available for the purpose.

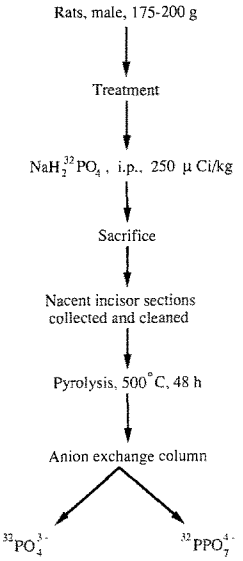

Fig. 1. Outline of the experimental procedure.

Table 1. Serum fluoride (μmol/liter) at a pumping rate of 819 μg F/kg/hour

Hours post-$^{32}PO_4{}^{3-}$ injection	Serum fluoride[a] (n = 6)
24	53.2 ± 2.9
48	42.1 ± 5.2
72	57.4 ± 9.8
96	52.6 ± 7.7

[a] ± SD; the 48-hour F level is significantly lower than others at $P = 0.01$.

Magnesium Effect

Rats were maintained on a pelleted magnesium-deficient diet (Bio-Serv, Frenchtown, NY 08825) for 20 days prior to initiation of the experiment, along with distilled water *ad libitum*, up to the time of death. Physiological signs of magnesium deficiency became evident: skin reddening with scratching, hyperactivity, and diarrhea. Three rat serum samples were assayed at random for magnesium just prior to injection of ^{32}P to confirm magnesium deficiency. In another study, magnesium was supplemented by implanting one model #2ML1 osmotic pump containing 3.87 mol/liter $MgCl_2$. This permitted a maximum release rate of 4,212 μg Mg/kg/hour and resulted in a steady serum level. When both fluoride and magnesium effects were tested in this same group, only magnesium-deficient rats were used.

To increase the range of serum magnesium concentration, magnesium-deficient and magnesium-supplemented rats were used, thereby giving a range of serum magnesium concentrations of about 7–35 μg/ml. Magnesium is very tightly controlled homeostatically and large excesses are rapidly eliminated. Furthermore, very high doses of magnesium are lethal. In short, we tried to maximize the range of both fluoride and magnesium treatments within the limits imposed by the lethal dose, solubility and/or elimination rate.

^{32}P Incorporation into Incisors

As the rodent incisor grows continuously throughout life, it is a particularly useful model for examining tooth development. $NaH_2{}^{32}PO_4$ (250 μCi/kg) (New England Nuclear, Boston, MA 02118) was injected intraperitoneally as a marker to label newly synthesized tooth mineral [16]. The rats were killed by asphyxiation in ether at various time intervals postinjection, and the upper and lower incisors were immediately extracted. All pulp, blood, and adhering tissues were removed. The basal opaque region of nascent incisor containing primarily enamel (>90%) was then snipped off and pyrolyzed at 500°C for 48 hours to convert acidic calcium phosphates to pyrophosphate. A basic calcium phosphate, such as OHAp, remains virtually unchanged during pyrolysis and contributes only to the orthophosphate fraction. When pyrolyzed, OCP yields between 33 and 50% of its phosphate as pyrophosphate; brushite yields all of its phosphate as pyrophosphate [17, 18].

Separation of Ortho- and Pyrophosphates

^{32}P-labeled ortho- and pyrophosphates were separated by anion-exchange chromatography. A 0.7 × 50 cm glass column was packed with anion-exchange resin (Bio Rad AGI-Z8 Cl, 100–200 mesh), equilibrated with a buffer solution containing 0.05 mol/liter Tris-HCl buffer (Sigma Chemical, St. Louis, MO 63178), 0.12 mol/liter NaCl, and 5.0 mmol/liter EDTA at pH 6.5–7.0. Up to 10.0 mg of pyrolyzed tooth fragments was dissolved in 1.0 ml of 1.0 mol/liter $HClO_4$. Then 3.0 ml of 0.15 mol/liter Tris base was added to neutralize as much of the acid as possible (final pH 2.5–3.0) without precipitating calcium phosphate; 2.0 ml of this solution was then immediately layered on the column, and the orthophosphates eluted in two 20 ml fractions with the starting buffer. Subsequently, pyrophosphate was similarly eluted with the starting buffer containing 0.32 mol/liter NaCl; this is a modification of Nakamura et al.'s method [19]. Tris buffer was added to this system to minimize hydrolysis of pyrophosphate in such an acidic environment. The half-life of pyrophosphate, at a concentration of 0.23 mmol/liter, was found to be approximately 13.5 hours in 1.0 mol/liter $HClO_4$ at room temperature. Clean separation of the ortho- and pyrophosphate fractions were confirmed by thin layer chromatography.

Counting of Ortho- and Pyrophosphate

A 5.0 ml aliquot from each elution fraction (after thorough mixing) was added in duplicate to 10 ml of Aquasol (New England Nuclear) liquid scintillation cocktail. Each sample was counted three times, 10 minutes each on a Packard 3385 Tri-Carb Liquid Scintillation Spectrometer. The activities of the ortho- and pyrophosphate fractions were used to calculate percent ^{32}pyrophosphate:

$$\frac{^{32}PPO_7{}^{4-}\text{ (CPM)}}{^{32}PO_4{}^{3-}\text{ (CPM)} + {}^{32}PPO_7{}^{4-}\text{ (CPM)}} \times 100 = \%\ {}^{32}PPO_7{}^{4-}$$

Measurement of Serum Fluoride and Magnesium

Blood was collected (5 ml) by cardiac puncture just before death of the animal and allowed to clot in polyethylene tubes. The tubes were then centrifuged at 2,000 *g* for 10 minutes to separate the serum from the blood cells. To 1.0 ml of serum, 0.1 ml of "total ionic strength adjusting buffer" (TISAB III, Orion Research, Cambridge, MA 02139) was added and fluoride was measured with a specific-ion combination electrode (Orion). The remaining serum was appropriately diluted with distilled water and assayed for magnesium by atomic absorption spectrometry [20].

Measurement of Incisor Fluoride Accumulation

Up to 2.5 mg of incisor from the nascent portion was dissolved in 0.5 ml of 0.5 mol/liter $HClO_4$ and brought to a volume of 1.0 ml with distilled water; 0.1 ml of TISAB III (final pH 4.5–5.0) was added and fluoride was measured as previously described. A lower than optimum pH for fluoride measurement was used to prevent precipitation of calcium phosphate which was present in relatively high concentrations.

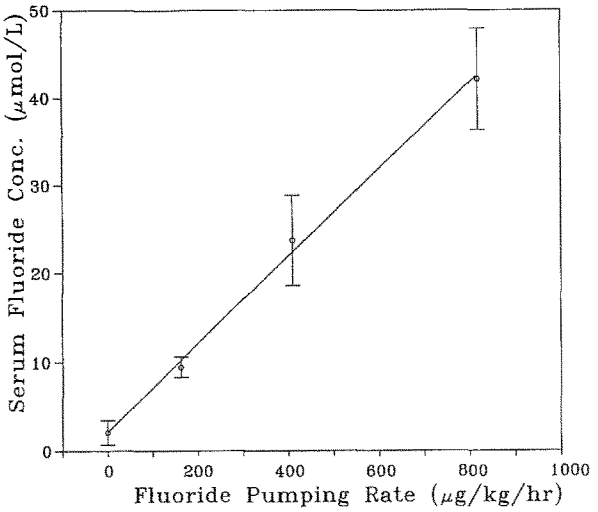

Fig. 2. Serum fluoride concentration (mean ± SD) as a function of the rate of fluoride supplemented by osmotic pumps. n = 7, 5, 6, and 5 in the order of increasing pumping rate.

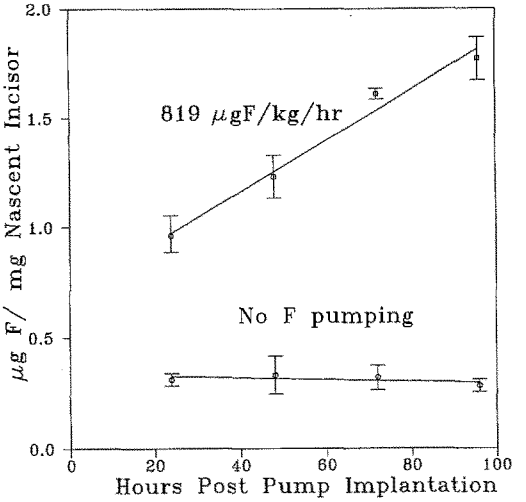

Fig. 3. Fluoride (F) content (mean ± SD) of the nascent portion of the rat incisor at various times after implantation of fluoride pumps. n = 8 and 6 for the control and F supplemented groups, respectively.

Results

Table 1 shows the fluoride levels in serum at various times. These data reveal that the fluoride concentration in the serum had reached an elevated level within 24 hours after ^{32}P injection and remained quite constant thereafter for 96 hours. For reasons that remained unclear, the fluoride level at 48 hours (42.1 μmol/liter) was lower than those at other times (average 54.5), but it was much higher than that (3 μmol/liter) measured in animals without fluoride supplementation.

Figure 2 shows steady-state fluoride levels in serum at various pumping rates. The serum fluoride levels were nearly linearly related to the pumping rate.

Figure 3 shows that during the 4-day experimental period the fluoride content of the nascent portion of the incisor increased roughly linearly with time after implantation of the pump, whereas the fluoride content in the absence of pumping remained constant.

If the hydrolysis of the acidic calcium phosphate precursor is a first order reaction, then the logarithm of the percent of the ^{32}P in the form of pyrophosphate would decrease linearly with time. Figure 4 shows that such a relationship may exist for the control, the fluoride supplemented (at the rate of 819 μg F/kg/hour), and the magnesium-deficient animals. Both fluoride supplementation and magnesium deficiency reduced the pyrophosphate content at each sampling time. The data points in each experiment appear to fall on a straight line. The percent pyrophosphate content decreased linearly with the log of the fluoride pumping rate (Fig. 5), which in turn is linearly related to the serum F level (Fig. 2). The logarithmic relationship is reasonable because log F concentration is proportional to the F^- chemical potential which is the driving force of the F^- reaction with the precursor. The percent pyrophosphate content increased monotonically when the magnesium deficiency was corrected by magnesium supplementation (Table 2).

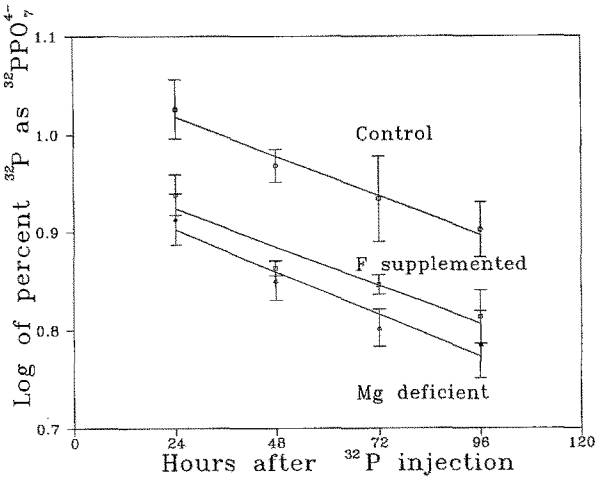

Fig. 4. Percent ^{32}P (mean ± SD) in the form of pyrophosphate in the nascent portion of rat incisors as a function of time for the control (n = 7), the fluoride-supplemented (n = 6), and the magnesium-deficient animals (n = 8).

Discussion

The results given above clearly show that the ^{32}P-pyrolysis technique has been refined to the point where the newly formed ortho- and pyrophosphate contents of mineralized tissue can be quantified with sufficient sensitivity to detect the effects of various components on acidic calcium phosphate formation and on its hydrolysis with time. Through the use of the methods described in this paper, we have shown that (1) an acidic calcium phosphate acts as a precursor to the formation of tooth mineral *in vivo*, (2) the precursor content falls off with time in a manner that approximates a first order reaction, (3) fluoride accelerates the hydrolysis of the precursor, (4) the fluoride effect is dose responsive, (5) magnesium deficiency decreases precursor content, and (6) magnesium supplementation increases precursor content. Thus, the acidic calcium phosphate content is responsive to the magnesium level in the blood. The effects of fluoride and magnesium are consistent with the *in vitro* observations [21–23] that magnesium ions inhibit and fluoride ions accelerate the hydrolysis of OCP and brushite.

As there was no practical method for the separation and quantification of acidic calcium phosphate in tooth mineral,

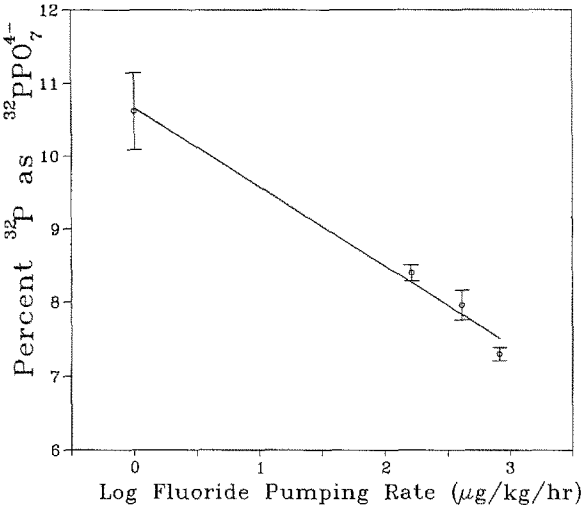

Fig. 5. Percent ^{32}P (mean ± SD) in the form of pyrophosphate in the nascent portion of rat incisors 48 hours after ^{32}P injection: effect of F supplementation rate. Numbers of animals in the various groups are the same as those in Figure 2.

Table 2. Forty-eight-hour incisor pyrophosphate content at different serum magnesium levels

Serum Mg^{+2} (μmol/liter)	% $^{32}PPO_7^{4-}$	N
361.9 ± 57.2[a]	6.64 ± 0.52*	12
530.6 ± 209.7	7.85 ± 0.24	9
2043.7 ± 130.0	8.39 ± 0.13	10

[a] ± SD; all differences between two values are statistically significant at $P = 0.01$.

the present indirect pyrolysis method was used. Pyrophosphate is a major product in the pyrolysis of acidic calcium phosphates whereas OHAp is unaffected [17]. Thus, our basis for distinguishing between OCP (or brushite) and OHAp lies in anion-exchange separation of the more negatively charged pyrophosphate from the less negatively charged orthophosphate. To minimize pyrophosphate hydrolysis, care was taken to ensure that dissolved incisor sections did not remain in an acidic environment any longer than was required to perform the chromatographic separation.

This pyrolysis method is simple, but not entirely free of artifacts. For example, if the pyrolysis temperature is too high, an acidic orthophosphate in enamel can convert to pyrophosphate quantitatively, but it may subsequently react with OHAp of the enamel to form $Ca_3(PO_4)_2$ [17], thereby reducing the yield of pyrophosphate to give a low estimate of acidic calcium phosphate. On the other hand, if the pyrolysis temperature is too low, pyrophosphate formation may be incomplete [18]. The pyrolysis conditions used here were chosen to give the maximum pyrophosphate formation from OCP. Also, components such as carbonate are known to reduce the pyrophosphate yield [24]. Combustion of organic material may locally elevate pyrolysis temperature, thus reducing pyrophosphate yield. OCP can give a range of values from 33 to 50% of PO_4 as pyrophosphate according to reactions I and II, respectively:

$$\text{(I)}\quad Ca_8H_2(PO_4)_6 \cdot 5H_2O = 2Ca_3(PO_4)_2 + Ca_2P_2O_7 + 6H_2O$$

$$\text{(II)}\quad 2Ca_8H_2(PO_4)_6 \cdot 5H_2O = 2Ca_5(PO_4)_3OH + 3Ca_2P_2O_7 + 11H_2O$$

In view of these difficulties, the method depends on treating all samples identically so that even though absolute quantities may not be measured, the relative percent yields of pyrophosphate are comparable.

References are sometimes made to the presence of HPO_4^{2-} in nonstoichiometric apatites which, when pyrolyzed, would also yield $P_2O_7^{4-}$, and this could be a partial cause for the $P_2O_7^{4-}$ in the samples in the present study. However, the maturation rate of nonstoichiometric apatites is generally much slower than the processes studied here which occurred within a 4-day period. Another possible source of $P_2O_7^{4-}$ would be adsorbed HPO_4^{2-} groups. However, it seems difficult to explain why the adsorbed HPO_4^{2-} groups would decrease with time, and why the decrease is affected by fluoride and magnesium. Thus, though the measured $P_2O_7^{4-}$ may contain contributions from the HPO_4^{2-} groups in nonstoichiometric apatites and on the crystal surfaces, the decrease of $P_2O_7^{4-}$ with time observed in the present study most likely reflected the disappearance of the acidic precursors which were converted to the more stable apatitic phase.

The nearly straight lines in Figure 4 suggest that the hydrolysis of the precursor during the first 72 hours is a first-order reaction, i.e., its rate is proportional to the precursor content. T-tests performed on the parameters associated with the three least squares lines indicate that there were no significant differences in the slopes, and the intercept of the control group was larger than those of the treated groups. These observations suggest that (1) at a given precursor content, the apparent hydrolysis rate is relatively independent of the fluoride and magnesium concentrations; and (2) that the displacements between the curves and their intercepts relate to the amounts of precursor that are formed initially. However, this question requires further experimental investigation, because there are contraindications to the validity of extrapolating these lines to zero time of the $^{32}PO_4$ injection: The extrapolation is for a relatively large increment of time (24 hours), and the half-life of $^{32}PO_4$ in the blood, after it is injected intraperitoneally, is relatively long (about 6 hours) so that the $^{32}PO_4$ content in enamel mineral during the first 24 hours may be atypical of subsequent variations. Intravenous injection of $^{32}PO_4$ and a shorter sampling time might overcome this difficulty. Magnesium ions probably affect both the rate of formation and the rate of hydrolysis of the precursor; the effect of fluoride is probably mostly on the hydrolysis rate. Such differences in the modes of action make direct comparisons of F^- and Mg^{2+} effects problematical.

The result shown in Figure 4 parallels the finding of Francois [14] that the amount of acidic calcium phosphate (revealed as pyrophosphate) decreased in rat bone with time, and that young epiphyseal bone contained a higher proportion of acidic calcium phosphate than did older, more mature diaphyseal bone. Thus, a relatively high proportion of acidic calcium phosphate seems to be present where there is active mineral synthesis. On the basis of petrographic examination of dental calculus, Brown et al. [1] suggested that the sequence brushite → OCP → OHAp was involved in calculus formation. Schroeder and Bambauer [25] found that the acidic calcium phosphates were most abundant in new calculus deposits and that the more basic calcium phosphates were most abundant in old calculus. This is an example in which acidic calcium phosphates formed first in an oral fluid and then hydrolyzed to a more insoluble basic calcium phosphate.

It is known that fluoride *in vitro* accelerates the hydrolysis of OCP to apatite [21, 22]. Thus, as an increasing

amount of fluoride is administered to rats, the amount of *in vivo* acidic calcium phosphate in nascent tooth mineral should decrease faster than in controls without fluoride supplementation. Indeed, Figure 4 indicates just such a phenomenon. The fluoride effect was also dose dependent (Fig. 5). As the serum fluoride concentration increased, the amount of tooth pyrophosphate decreased. Furthermore, it was shown (Fig. 3) that the amount of fluoride in the nascent incisor increased with time in fluoride-supplemented animals. This implicates fluoride as the causative agent in accelerating the disappearance of acidic calcium phosphate in developing incisors.

Magnesium has been found to inhibit both formation of OCP from amorphous calcium phosphate [26] and its hydrolysis to OHAp *in vitro* [1, 22]. Thus, the lower acidic calcium phosphate content in magnesium-deficient enamel and the higher content in the magnesium-supplemented enamel are consistent with *in vitro* observations. The results establish, also, that the magnesium effect is dose dependent. The magnesium effect is not as linear as is that with F^-, probably because of difficulty in tightly controlling the magnesium level in serum.

Although these and other data strongly support acidic calcium phosphate involvement as a precursor to OHAp, they do not explicitly distinguish between brushite and OCP as the precursor. Thermodynamically, OCP is favored over brushite as the precursor to bioapatite [27]. Furthermore, it is very unlikely that the ribbon or fiber-like crystallites that form during the rapidly mineralizing stage of enamel formation [9] could have an epitaxial relationship to brushite as they do with OCP [28].

The technique described here should prove to be a powerful new tool for studying the effects of other biological components and surface-active agents on crystal growth and dissolution phenomena related to alveolar bone loss, osteoporosis, osteomalacia, and cardiovascular and other pathological mineral deposits.

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