Cells and Materials

Volume 5 | Number 1 Article 4

1995

Adsorption of Bovine Serum Albumin onto Octacalcium Phosphate and its Hydrolyzates

O. Suzuki
JGC Corp, Yokohama

H. Yagishita
The Nippon Dental University, Tokyo

M. Yamazaki
The Nippon Dental University, Tokyo

T. Aoba
The Nippon Dental University, Tokyo

Cells and Materials, Vol. 5, No. 1, 1995 (Pages 45-54) 1051-6794/95$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

ADSORPTION OF BOVINE SERUM ALBUMIN ONTO OCTACALCIUM PHOSPHATE AND ITS HYDROLYZATES

O. Suzuki, H. Yagishita[1], M. Yamazaki[1] and T. Aoba[1,*]

JGC Corp., Research and Development Division, 14-1, Bessho 1-chome, Minami-ku, Yokohama, 232, Japan
[1]The Nippon Dental University, Department of Pathology, 1-9-20 Fujimi, Chiyoda-ku, Tokyo 102, Japan

(Received for publication February 15, 1995 and in revised form April 17, 1995)

Abstract

Octacalcium phosphate (OCP) has been advocated to be a precursor of biominerals. In this report, we aimed at investigating OCP hydrolysis and the interaction of OCP and its hydrolyzates (apatitic products) with bovine serum albumin (BSA). A batch of synthetic OCP was allowed to hydrolyze either (a) in deionized water at 70°C or (b) in Tris buffer (pH 7.4) containing 2 ppm fluoride at 37°C. OCP hydrolysis was completed within reasonable experimental periods: after 48 hours at 70°C and after 10 days at 37°C in the presence of fluoride. The adsorption isotherms of BSA onto the original OCP and its various hydrolyzates were determined at pH 7.4 and 37°C. The BSA adsorption onto all the adsorbents was described by a Langmurian model. Remarkable findings were that: (1) when the maximum number of BSA adsorption sites was expressed on basis of unit surface area (N, mols/m^2), the greatest values of N was obtained for the original OCP; (2) the N values became smaller with the advance of OCP-apatite conversion in both hydrolysis systems; (3) the affinity values (K, ml/μmol) were relatively constant for the OCP and the non-fluoridated hydrolyzates, while the K values increased markedly after hydrolysis of OCP in the presence of fluoride. The results of the present work support the hypothesis that the process of precursor precipitation and its subsequent hydrolysis taking place in forming hard tissues may be modulated through the matrix protein-crystals interaction. It seems likely that fluoride ions has a dual role, i.e., acceleration of OCP hydrolysis and, once incorporated into the crystals, enhancement of the protein-mineral interaction.

Key words: Biomineralization, octacalcium phosphate, adsorption, fluoride.

*Person to contact:
T. Aoba, address as above.
Telephone number: 81-3-3261-8311 (ext. 358)
FAX number: 81-3-3264-8399

Introduction

Mineralization events taking place in bone and tooth are in essence organized under controls by the tissue-specific cells and the secreted matrix proteins. However, much still remains to be learned about the mechanism by which the proliferation and growth of biomineral progress in a concerted manner in extracellular spaces. Recent *in vivo* and *in vitro* work provided evidence that the morphologic and structural features of biomineral, such as enamel crystals, are most satisfactorily explained by the epitaxial overgrowth of apatite on the <100> direction of octacalcium phosphate (OCP, $(Ca_8H_2(PO_4)_6 \cdot 5H_2O)$ (Nelson and Barry, 1989; Iijima *et al.*, 1992a; Miake *et al.*, 1993). The OCP is not the most stable phase, but can hydrolyze to basic calcium phosphate under physiological conditions. It is also becoming clear that the major incorporation of impurities (e.g., carbonate ions) into biomineral takes place during the hydrolysis process and that the substituted impurities in turn affect the chemical and physical properties of the crystals (Chickerur *et al.*, 1980; Brown *et al.*, 1987; Siew *et al.*, 1992). Despite the wealth of information as to effects of various ionic regulators (e.g., carbonate, pyrophosphate, citrate, Mg^{2+}, F^-) on OCP hydrolysis (LeGeros *et al.*, 1984, 1989; Bigi *et al.*, 1988; Iijima *et al.*, 1992b; Tung *et al.*, 1992), there is a paucity of information as to whether OCP may interact selectively with macromolecules and whether the precursor hydrolysis may weaken or strengthen the matrix protein-mineral interaction, thereby modulating the subsequent mineralization process. On the basis of these considerations, we prepared OCP and various batches of fluoridated and non-fluoridated OCP hydrolyzates and, using those crystals as adsorbents, investigated their adsorption properties with bovine serum albumin (BSA). The rationale for the use of BSA as adsorbate is that (a) the material is well characterized and commercially available and, from a biological point of view, (b) serum-derived macromolecules are involved in most biomineralization processes, of particular interest in reactive bone induction with calcium phosphate implants.

Indeed, previous studies indicated that selective adsorption of serum constituents, including albumin, occurred onto OCP shortly after its implantation into the subperiosteal space on mouse calvaria (Suzuki *et al.*, 1993).

Materials and Methods

Preparation of OCP and its hydrolyzates

Reagent grade chemicals were used in the current work. A batch of OCP was prepared by direct precipitation at pH 5 to 6 and 60 (± 2)°C according to the method of LeGeros (1985). The precipitates were washed several times with deionized water and then lyophilized. The recovered solid was passed through a USA standard testing sieve (270-mesh, 53 μm pore size). In a series of preliminary experiments, we examined effects of various factors on the kinetics of OCP hydrolysis. The factors tested were solid/solution ratios in equilibration media, temperature (37, 60, 70, and 80°C) and pH (6.0, 7.4, and 8.0), fluoride concentrations in the range of 0 and 10 ppm F as NaF, and equilibration periods covering from 1 hour through 10 days. The nature of solid products was characterized by X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR) as described below. As a consequence of the results obtained in those preliminary trials and with the purpose to secure both fluoridated and non-fluoridated OCP hydrolyzates, OCP hydrolysis was investigated in two solutions: (**A**) deionized water at 70°C in the absence of fluoride and (**B**) in 150 mM Tris buffer at pH 7.4 and 37°C in the presence of 2 ppm fluoride. The details of the procedure used for the hydrolysis were as follows:

(**A**) One gram of OCP powder was suspended in 10 ml of the solution, sonicated for 1 minute, and then introduced into a reaction plastic vessel containing 200 ml of deionized water. The reaction vessel was tightly sealed with a rubber stopper and settled in the water bath that was maintained at 70°C. The resulting slurry was continuously agitated with a magnetic stirrer. Samples were withdrawn periodically from the agitated slurry and centrifuged at 12,000 *g*. The supernatant was then carefully removed from the tube for analyses (Ca, P, and pH), while the pellet was freeze-dried and subjected to FTIR analysis. At this high temperature, the OCP hydrolysis was insured to complete within 48 hours. Thus, aliquots, roughly corresponding to one third of the slurry, were taken at short intervals, namely 6, 24 and 48 hours. The suspension recovered was separated by centrifugation. The supernatant was collected and then analyzed with respect to concentrations of the lattice ions (Ca^{2+}, phosphate, H^+, and F^-). The solid obtained as pellet was lyophilized and then passed through the sieve.

(**B**) One gram of the same batch of OCP was incubated in 1 liter of 150 mM Tris buffer (pH 7.4), which contained 2 ppm F, as well as 4 mM azide for bacteriostat. Equilibration and sample collection were carried out in the same manner as described above. At 37°C, OCP hydrolysis proceeded at much slower rates and completed after 10 days incubation. Thus, fractions of the slurry were withdrawn to recover the solids at 1, 3, and 10 days during the equilibration.

In addition to those OCP and hydrolyzates, hydroxyapatite (HAp) synthesized at 80°C was used as adsorbent in the required adsorption experiments. The crystals exhibited long, blade-like appearance. The details of preparation and characterization of that batch of HAp were reported previously (Shimoda *et al.*, 1990).

Adsorption experiment

The adsorbate used was BSA {molecular weight (M.W.): 66 kD; Sigma Chem. Co., St. Louis, MO), which was dissolved in 150 mM Tris buffer at pH 7.4. The general experimental procedures to obtain the adsorption isotherms of BSA onto the various adsorbents were those used previously (Aoba and Moreno, 1985). Each of the adsorbents having different specific surface areas (see Table 1) was accurately weighed to provide the total surface area of 0.12 m^2/g. The weighed crystals were added to 1.5 ml of the experimental solution in polystyrene tubes. Controls without either adsorbent or adsorbate were included in all experiments. Equilibration was conducted by end-over-end rotation at 37°C. In the first series of experiments, the time sequence of BSA adsorption onto either OCP or HAp crystals was examined by preparing a series of the adsorbate-adsorbent mixtures. After equilibration, at specified periods ranging from 15 minutes to 24 hours, each suspension was centrifuged and, thereafter, the supernatant was carefully recovered. BSA concentrations in both the original experimental solution and the supernatant were determined by protein assay (Bio-Rad, Hercules, CA). The amount of BSA adsorbed was obtained by the difference between the analytical values of BSA concentrations before and after equilibration. Part of the recovered supernatant was also used for determination of calcium and total phosphate concentrations, and pH values. In the second series of adsorption experiments, we examined effects of calcium and phosphate concentrations in solution on the BSA adsorption onto OCP and HAp. In those cases, all equilibrations were carried out at a fixed equilibration time (2 hours, see **Results**) otherwise in the same way as described above. In the last series of adsorption experiments, BSA adsorption onto all the adsorbents was examined in a wide range of the equilibrium concentrations so that the adsorption isotherms

Table 1. Chemical composition and specific surface area (SSA) of OCP, its hydrolyzates, and non-stoichiometric hydroxyapatite.

Treatment	Ca wt%	P wt%	F wt%	HPO_4 %P	Ca/P molar ratio	S.S.A. m^2/g
OCP original	32.6	20.1	N.D.[1]	38.3	1.26	16
OCP hydrolyzates						
6 hours at 70°C	31.8	18.9	N.D.	29.0	1.30	26
24 hours at 70°C	34.3	19.0	N.D.	20.0	1.40	41
48 hours at 70°C	35.6	18.8	N.D.	16.0	1.47	45
1 day with 2 ppm F	31.7	18.4	0.22	26.3	1.34	37
3 days with 2 ppm F	32.8	18.3	0.24	20.2	1.39	49
10 days with 2 ppm F	33.4	17.4	0.25	12.3	1.49	58
Hydroxyapatite	37.5	18.7	0.00	10.6	1.55	26

[1]N.D., not detected.

were constructed at 37°C. To this end, the total surface areas of the adsorbents used were varied from 0.06 to 0.18 m^2. Initial concentrations of BSA were in the range of 0.15 through 1.5 mg/ml. In order to minimize dissolution or alteration of the adsorbent crystals during equilibration, the initial adsorbate solution contained 0.5 mM calcium and 0.5 mM phosphate.

X-ray diffraction, Fourier transform infrared spectroscopy, and scanning electron microscopy (SEM)

All adsorbent samples before and after equilibration with BSA were examined by FTIR and, if samples were enough for analysis, by XRD, too. FTIR spectra were obtained by means of a diffuse-reflectance attachment in a Perkin-Elmer 1600 FTIR (Perkin-Elmer, Norwalk, CT). Usually, 64 spectral scans were conducted over the range of 4500 to 450 cm^{-1} with 2 cm^{-1} resolution. X-ray diffraction patterns of those samples were recorded with a D/Max X-ray powder diffractometer (Rigaku USA, Peabody, MA) equipped with a rotating anode and a graphite monochrometer. In order to detect the characteristic (100) reflection of OCP at $2\Theta = 4.75$ degrees, a step-scanning was done at 0.02 degrees interval in the range of 3.5 and 60 degrees. The original batch of OCP and its hydrolyzates were examined in a Hitachi S-4000 SEM (Tokyo, Japan) operating at an accelerating voltage of 2.5 kV. Before observation, powder samples were placed with the aid of a paste on aluminum stubs and then coated with a thin layer (approx. 4 nm) of platinum.

Chemical analysis and determination of specific surface area (SSA)

Calcium and phosphorus were determined by atomic absorption spectrophotometry and colorimetry (Vogel, 1961), respectively. Fluoride concentration of the recovered supernatant was directly determined with an ion-selective electrode (Orion #10003, Cambridge, MA). The fluoride content of the solid samples was determined according to the microdiffusion procedure with hexamethyldisiloxane (Whitford and Reynolds, 1979; Aoba *et al.*, 1989). Special attention was given to the acid phosphate of the solid samples because even a subtle decrease in the acid phosphate most likely reflects the conversion from OCP to apatite. In practice, the acid phosphate was determined by the procedure reported by Gee and Deitz (1953) after pyrolysis of the solid at 600°C for 24 hours; a full consideration about the pyrolysis conditions and analytical procedures was given elsewhere (Shimoda *et al.*, 1991). Specific surface areas of the original OCP and its hydrolyzates were determined by nitrogen adsorption.

Results

Figure 1 shows X-ray diffraction patterns of the original OCP and its hydrolysis products obtained at 70°C. All reflections observed for the synthesized OCP including the characteristic (100) reflection (Fig. 1A) corresponded well to those expected from the OCP structure (Mathew *et al.*, 1988). OCP-apatite conversion progressed steadily as a function of the incubation time. In Figure 1, the patterns B and C showed that samples removed near the middle of hydrolysis process (6 and 24 hours) were comprised of both OCP and apatite. The (100) reflection of OCP disappeared completely and only apatitic reflections were obtained from the

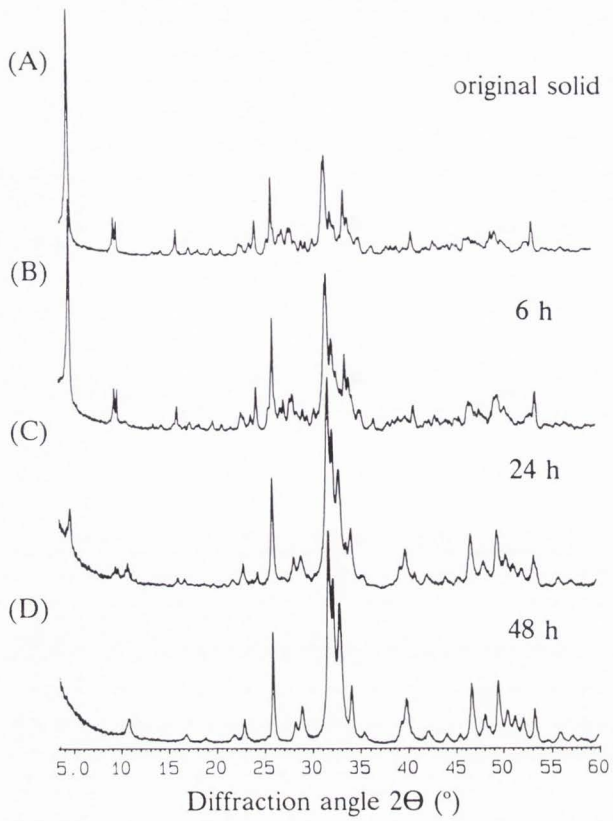

Figure 1. X-ray diffraction patterns of synthetic OCP and its hydrolyzates. Hydrolysis of OCP was conducted at 70°C for 6, 24, and 48 hours. Note a gradual decrease in intensity of OCP (100) reflection concomitant with the appearance of apatite reflections.

---

resulting product after 48 hours hydrolysis (Fig. 1D); the conversion into moderately well crystallized apatite crystals was indicated by their well resolved reflections. As can be seen in Figure 2, the OCP hydrolysis occurred at much slower rates at 37°C even in the presence of 2 ppm F. The (100) OCP reflection disappeared completely after extending the incubation up to 10 days. At 37°C, the resulting apatites yielded broad and overlapped reflections, except for the (002) reflection, indicating their low crystallinity.

Figure 3 shows scanning electron micrographs of the OCP and its hydrolyzates. OCP crystals exhibited platy morphology, several μm in length; usually those crystals showed smooth surfaces and smooth terminals (Fig. 3A). The hydrolyzates obtained at 70°C still retained the original platy morphology (Figs. 3B through 3D). The hydrolyzates obtained at 37°C in the presence of fluoride displayed wide variations in morphology and particle size (Figs. 3E through 3G): some crystals retained the original platy morphology, while others were small in particle size, thin-blade in appearance, having

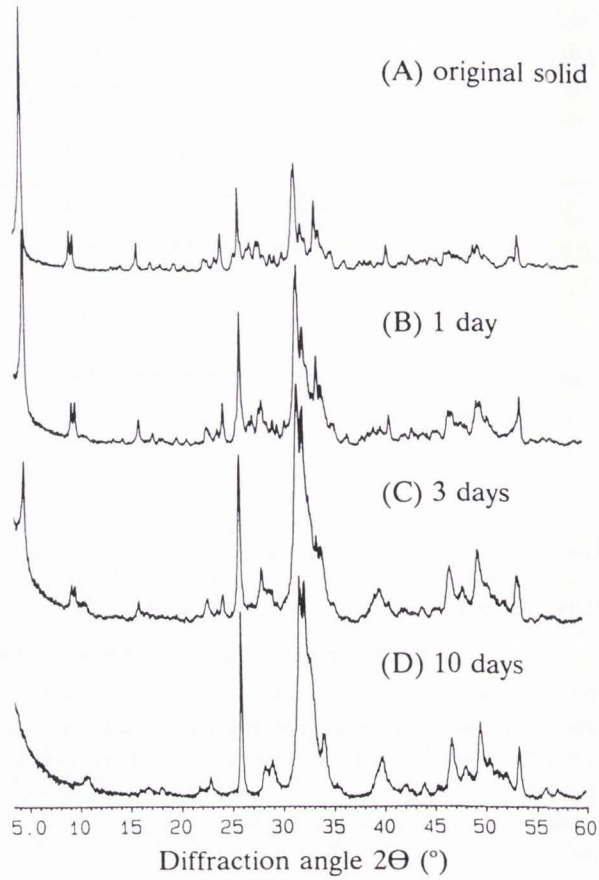

Figure 2. X-ray diffraction patterns of synthetic OCP and its hydrolyzates. Hydrolysis of OCP was conducted at 37°C in 150 mM Tris buffer (pH 7.4) containing 2 ppm F; times indicate the equilibration periods.

---

pointed terminals. The latter suggested *de novo* precipitation of apatite crystals on the platy templets, mostly due to acceleration of the kinetic rates by fluoride ions.

Table 1 shows the chemical composition and SSA of the OCP and its hydrolyzates. The composition of the OCP batch was Ca-deficient, having the Ca/P molar ratio of 1.26. The acid phosphate (38% of the total P) was slightly higher than that expected for the OCP stoichiometry, although analyses by XRD and FTIR ruled out contamination of dicalcium phosphate. Regarding the high acid phosphate content, Mathew *et al.* (1988) recently reported a new formula of OCP, $Ca_{16}H_{4+x}(PO_4)_{12}(OH)_x \cdot (10\text{-}x)H_2O$. According to this formula, the authors stated that the maximum acid phosphate assessed by pyrolysis can correspond to about 40% of the total phosphorus. At this time, we do not have further information about the stoichiometry and structure of the OCP batch used. The apatite products were non-stoichiometric, exhibiting low Ca/P values (around 1.48). It was also found that: (a) the acid phosphate

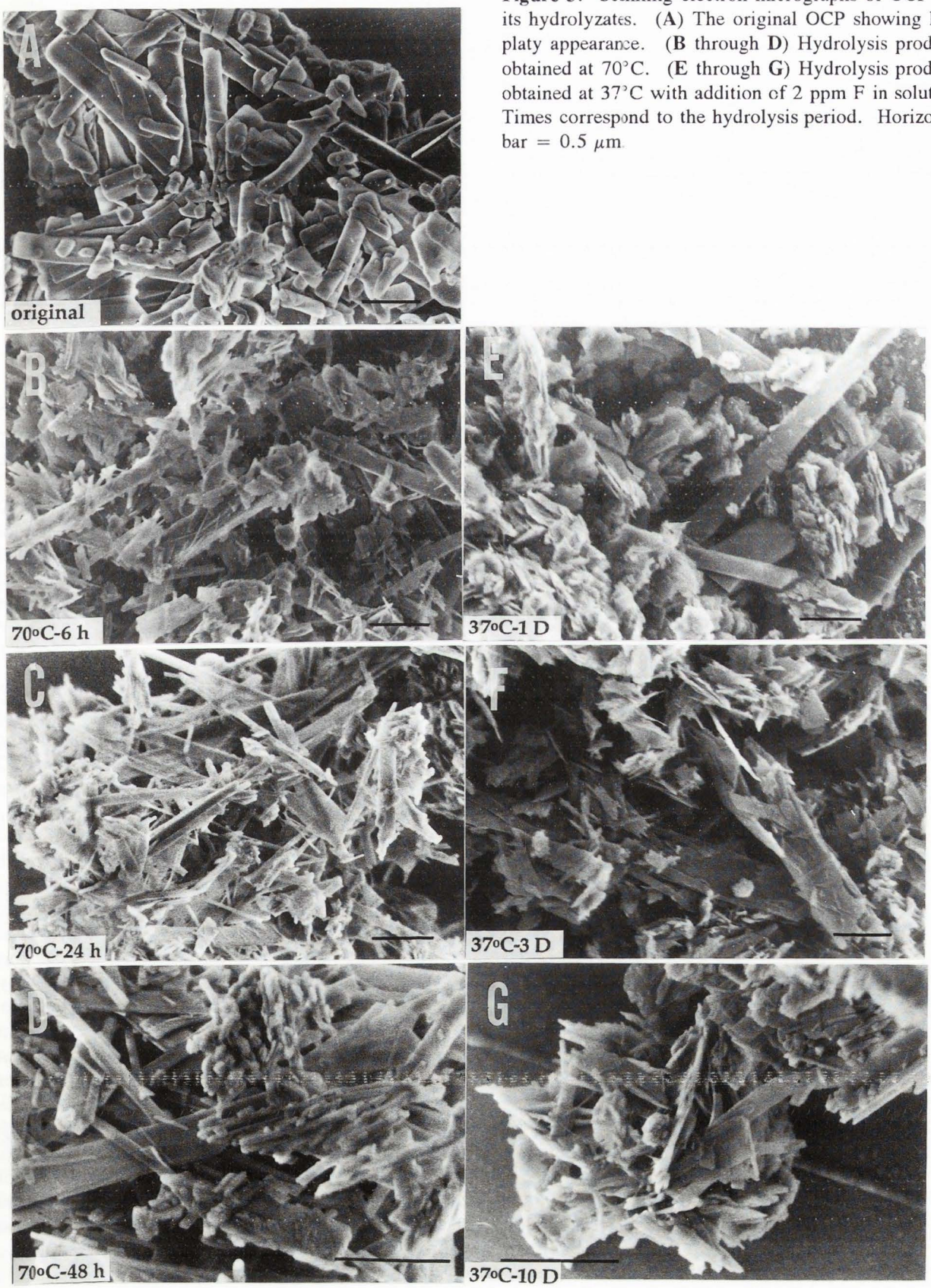

Figure 3. Scanning electron micrographs of OCP and its hydrolyzates. (**A**) The original OCP showing long platy appearance. (**B** through **D**) Hydrolysis products obtained at 70°C. (**E** through **G**) Hydrolysis products obtained at 37°C with addition of 2 ppm F in solution. Times correspond to the hydrolysis period. Horizontal bar = 0.5 μm.

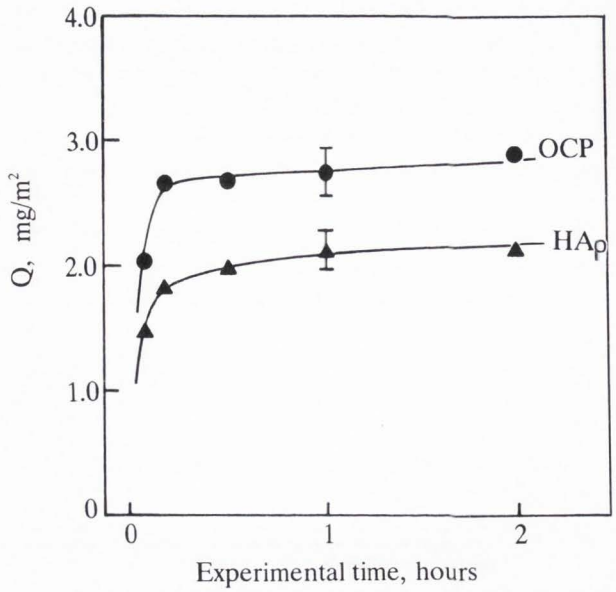

Figure 4. Time course of bovine serum albumin (BSA) adsorption onto octacalcium phosphate (OCP) and hydroxyapatite (HAp) at 37°C. The ordinate, Q, represents the amount of BSA adsorbed onto the solid. Note that a plateau of the adsorption was achieved after 30 minutes equilibration in both systems.

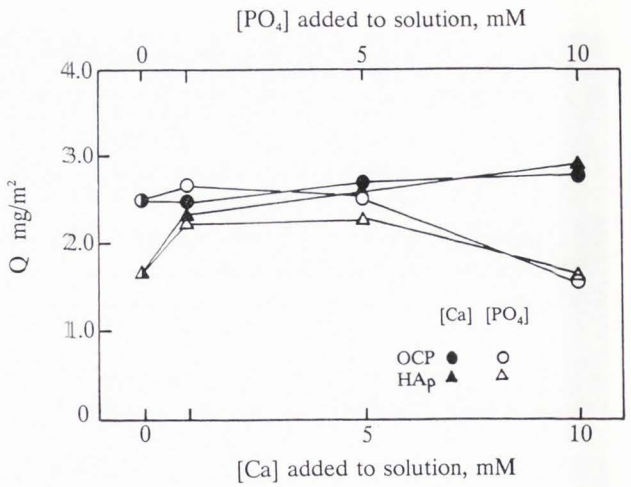

Figure 5. Effects of concentrations of calcium and phosphate ions in experimental solution on BSA adsorption onto OCP and HAp at 37°C. Other experimental conditions were: the initial BSA concentration = 1.5 mg/ml, the total surface area of the adsorbents = 0.06 m^2/ml, and the equilibration time = 2 hours. Note that, except for a marked decrease of adsorption in the presence of the highest concentration of phosphate, BSA adsorption levels were relatively constant, regardless of wide variations of solute concentrations in the experimental solutions.

---

decreased consistenly with the advance of OCP hydrolysis, (b) nevertheless, the resulting apatite crystals still contained high acid phosphate as observed for enamel crystals at early developmental stages (Shimoda *et al.*, 1991), and (c) the fluoride contents of all hydrolyzed products obtained at 37°C were constant, regardless of the incubation time. The fluoride contents obtained (0.22-0.25% weight) correspond roughly to substitution of fluoride into one tenth of the OH sites on the assumption of its homogeneous distribution in the apatite lattice. Consistent with those results obtained from solid samples, analysis of the experimental solution proved that most of the fluoride in solution was removed, decreasing sharply from the initial 2 ppm to barely detectable levels (below 0.01 ppm), within 1 hour upon introduction of the crystals in the solution. The OCP hydrolysis was also accompanied by an increase of the SSA. The finding that the hydrolyzates obtained at 70°C had lower values of SSA than the hydrolysis products at 37°C is also consistent with their differences in morphology and crystallinity obtained by SEM and XRD. As shown in Table 1, the HAp exhibited non-stoichiometric composition, containing 10.6% total P as acid phosphate.

Figure 4 illustrates the time course of BSA adsorption onto OCP and HAp. The amount of protein molecules adsorbed onto the solid surface increased rapidly in the first 15 minutes, thereafter remaining constant

---

over the range of time studied. In those experiments, no calcium and phosphate were added to the original adsorbate solution. At the end of equilibration using OCP as adsorbent, some of the lattice ions were released from the adsorbent crystals; the Ca and PO_4 concentrations increased up to 0.5 mM and 0.7 mM, respectively, which were explained by dissolution, at maximum 5%, of the total OCP crystals used. In the equilibration systems using HAp, both Ca and PO_4 concentrations were much lower, close to 0.1 mM or below. Since the buffer solution at the high concentration was used for equilibration, changes in pH values were only marginal, if any.

Figure 5 illustrates the effects of calcium and phosphate concentrations in solution on BSA adsorption onto OCP and HAp. Fairly constant adsorption of BSA onto OCP was obtained over the range of Ca and PO_4 concentrations tested, whereas the addition of the highest PO_4 concentration gave rise to a marked decrease of the protein adsorption onto both OCP and HAp. At present, we do not have an unequivocal explanation for the observed effect of phosphate ions, although one may argue that high concentrations of negatively charged (mostly divalent) ions may cause electrostatic modification of apatite surfaces or changes of protein conformation in solution. Nevertheless, it is noteworthy that the addition

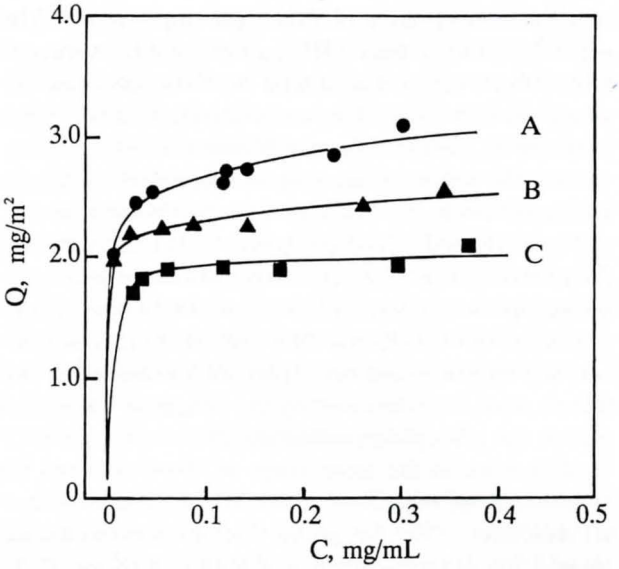

Figure 6. Adsorption isotherms of BSA onto synthetic OCP and its hydrolyzed products at 37°C. Equilibration was carried out in 150 mM tris buffer containing 0.5 mM Ca and 0.5 mM PO_4. Adsorbents used were: (**A**) OCP, (**B**) intermediate products comprising OCP and apatite, which were obtained after 1-day equilibration in the presence of 2 ppm F, and (**C**) apatite crystals converted from OCP after 10-day equilibration in the presence of 2 ppm.

Table 2. Adsorption of bovine serum albumin onto octacalcium phosphate, its hydrolyzates, and non-stoichiometric hydroxyapatite.

Crystals/ Treatment	Adsorption Parameters N μmol/m^2	K ml/μmol	(r^2)
OCP original	0.054	1,520	(0.975)
OCP hydrolyzates			
6 hours at 70°C	0.053	1,660	(0.986)
24 hours at 70°C	0.049	1,340	(0.977)
48 hours at 70°C	0.046	1,220	(0.950)
1 day with 2 ppm F	0.047	1,040	(0.975)
3 days with 2 ppm F	0.038	2,350	(0.987)
10 days with 2 ppm F	0.033	5,940	(0.978)
Hydroxyapatite	0.043	1,980	(0.975)

of 0.5 mM of both calcium and phosphate ions to the adsorbate solution is desirable to provide saturation with respect to OCP, thereby minimizing dissolution or alteration of adsorbent crystals during equilibration, without unforeseen influence of solute ions on BSA adsorption.

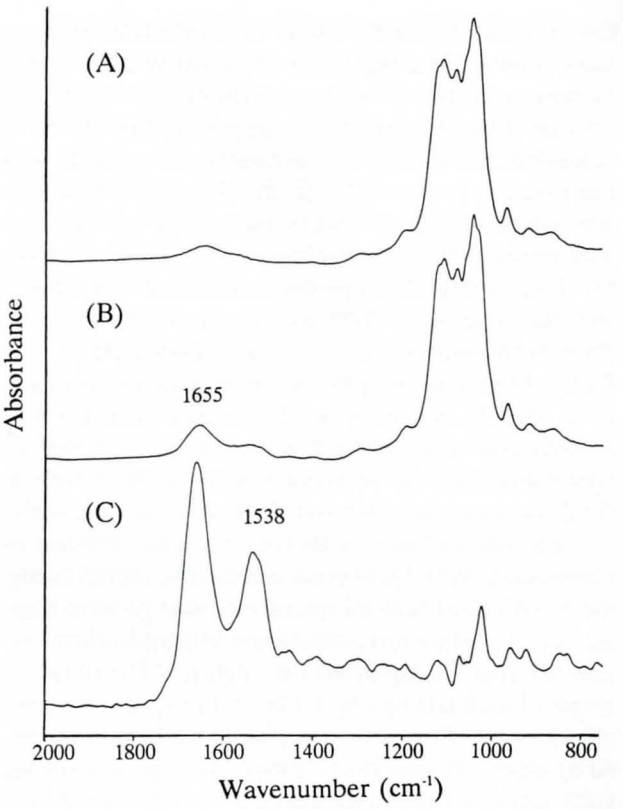

Figure 7. FTIR absorption spectra of synthetic OCP taken (**A**) before and (**B**) after equilibration with 1.5 mg BSA/ml for 2 hours. The spectrum shown in (**C**) was obtained by subtraction procedure, (**B**) - (**A**). Note that there occurred no appreciable changes in OCP spectral feature after equilibration with BSA. Two prominent absorption bands stemming from the adsorbed BSA were discerned at 1655 and 1538 cm^{-1}.

Figure 6 gives the adsorption isotherms of BSA onto the OCP and its fluoridated hydrolyzates (obtained after 1 and 10 days hydrolysis), which were determined in the presence of 0.5 mM Ca and 0.5 mM PO_4 at pH 7.4 and 37°C. In all cases, the experimental data yielded typical Langmurian isotherms (Moreno *et al.*, 1978) which were characterized by an initial increase in adsorption with the equilibrium concentration of BSA, followed by a plateau indicating a proximity to adsorption saturation. On the basis of this adsorption model, the adsorption parameters were determined according to the equation:

$$Q = \{CNK/(1 + K)\} \quad (1)$$

where Q is the amount of BSA adsorbed onto the solid, C is the BSA concentration in equilibration, N is the maximum number of adsorption sites per unit of surface area, and the parameter K reflects the affinity that BSA molecules have for the adsorption sites. Table 2 shows

the values of N and K, which were calculated by non-linear least squares regression of the experimental data. In most cases, the correlation coefficients of regressions (r) were 0.98. Remarkable findings were that (**1**) the N values became smaller for the hydrolyzed products than that obtained for the OCP, (**2**) the fluoridated hydrolyzates exhibited slightly lower values of N as compared with the non-fluoridated products, and (**3**) the K values for the non-fluoridated products decreased consistently with the progress of OCP-apatite conversion, while the fluoridated apatites had significantly higher affinity for BSA. At present, no plausible explanation is available as to why the N values should decrease with the progressive conversion of OCP to apatite, although the obtained difference in the adsorption sites surely reflects the distinct structures between OCP and apatite crystals.

Since the crystal structure of metastable OCP and its intermediate hydrolyzed products could be altered during the protein equilibration, special care was given to characterize the nature and composition of the adsorbents recovered after the equilibration. Figure 7 shows an example of such attempts by FTIR. FTIR spectrum of the original OCP (Fig. 7A) corresponded well to that reported by others (Fowler *et al.*, 1966). The spectrum of the OCP recovered after equilibration with the initial concentration of 1.5 mg BSA/ml (Fig. 7B), was identical to that of the original crystals, except for the appearance of amide bands in the wave-number range of 1,400 and 1,700 cm^{-1}, which stemmed from the adsorbed BSA molecules. Similar analyses by FTIR, as well as XRD, also confirmed that no apparent structural changes were discerned for all hydrolyzates before and after the equilibration with BSA (data not included).

Discussion

The present studies provide further evidence that the synthesized OCP crystals can be converted into apatite crystals within reasonably short periods. The temperature markedly affected the hydrolysis kinetics and the crystallinity of the apatite crystals formed. Another factor of probable biological importance is the fluoride ion concentration in solution: (**a**) The well-grown OCP crystals remained stable at 37°C and pH 7.4 over 10 days even in agitated solutions containing low concentrations of F^- such as 0.2 ppm or below (data not included); it is of interest that the precipitating OCP crystals were hydrolyzed concurrently even at such low fluoride concentrations, advancing a solid-solution transformation (Mura-Galelli *et al.*, 1992). (**b**) Higher concentrations of fluoride in the range of 1 and 10 ppm were required to complete the hydrolysis of the synthesized OCP within 10 days at the physiological temperature. The resulting fluoridated apatite crystals were Ca-deficient, exhibiting platy or thin-blade appearance. The general feature of their XRD patterns and the values of SSA (50-60 m^2/g) are similar to those obtained for young enamel mineral at early developmental stages (Aoba *et al.*, 1987; Aoba and Moreno, 1990).

All the results of the present adsorption studies, in agreement with the previous reports (Moreno *et al.*, 1977; Hlady and Füredi-Milhofer, 1978), indicate that BSA behaves as a relatively strong adsorbate onto calcium phosphate crystals. Most interestingly, the adsorption behavior of BSA onto OCP and the hydrolysis products was similar to that onto HAp: all the adsorption isotherms were described well by the Langmuir adsorption model; the adsorption parameters obtained for the OCP crystals were in the same range as those obtained for synthetic HAp, regardless of the difference in their crystal structures. The foregoing findings provide the rationale that serum albumin is a strong inhibitor of the crystal growth of calcium phosphates *in vitro* (Robinson *et al.*, 1992), although its biological significance still remains open. Previously, Eidelman *et al.* (1987) suggested that serum constituents with molecular weight above 50 kD may facilitate hydrolysis of OCP to an apatitic product. Our current work ascertained that the OCP hydrolysis was retarded markedly by preadsorption with BSA or bone matrix proteins even in solution containing 10 ppm fluoride (data not included).

An expected, but still interesting finding was that the enhancement of the adsorption bond onto fluoridated hydrolyzates took place without affecting the parameter N. Similar enhancing effects of fluoride on the adsorption of salivary or enamel proteins have been reported previously using a series of fluoridated apatites having various magnitudes of fluoride substitution (Moreno *et al.*, 1978; Tanabe *et al.*, 1988). The observation that the adsorption affinity was maximized for the completely hydrolyzed crystals, leads to the proposition that fluoride ions modulate the protein adsorption uniquely onto apatite surfaces, most probably by lowering surface energy and thereby facilitating water desorption requisite for protein adsorption (Tanabe *et al.*, 1988). The above proposition was partially supported by the finding that fluoride ions in soluble state even at concentration of 10 ppm did not yield distinctive effects on the BSA adsorption onto OCP or apatite crystals (data not included).

The current work using BSA as adsorbate provided some insight into protein-OCP (or its hydrolyzates) interaction. It is obviously of interest to learn about whether tooth or bone-specific proteins, e.g., non-collagenous proteins in enamel, dentin, and bone, may exhibit strong adsorption onto OCP as well as apatite surfaces. Preliminary evidence was obtained in adsorption studies using enamel matrix proteins, in which the secreted amelogenins adsorb selectively onto OCP as well

as onto hydroxyapatite (Aoba *et al.*, 1987). As reported by others (Doi *et al.*, 1993; Füredi-Milhofer *et al.*, 1994), dentin and bone matrix proteins appear to interact with OCP, affecting the precipitation process. Taken together, it is reasonable to assume that early extracellular events in tooth and bone formation involves the interaction between matrix proteins and OCP precursor, which in turn may affect the precursor hydrolysis, consequently, the mineralization process. In that whole process, fluoride ions seem to have a dual role, i.e., acceleration of OCP hydrolysis and, once incorporated into the crystals, enhancement of the protein-mineral interaction.

Acknowledgements

The authors are grateful to Mr. G. Tanabe of Japan Fine Ceramics Co. Ltd. for his great help in preparation of octacalcium phosphate. We also thank Ms. F. Mitsuhashi for her valuable help in SEM studies. This study was supported in part by Grant DE8670 from the National Institute of Dental Research.

References

Aoba T, Collins J, Moreno EC. (1989). Possible function of matrix proteins in fluoride incorporation into enamel mineral during porcine amelogenesis. J. Dent. Res. **68**: 1162-1168.

Aoba T, Fukae M, Tanabe T, Shimizu M, Moreno EC. (1987). Selective adsorption of porcine amelogenins onto hydroxyapatite and their inhibitory activity on hydroxyapatite growth in supersaturated solutions. Calcif. Tissue Int. **41**: 281-289.

Aoba T, Moreno EC. (1985). Adsorption of phosphoserine onto hydroxyapatite and its inhibitory activity on crystal growth. J. Colloid Interface Sci. **106**: 110-121.

Aoba T, Moreno EC. (1990). Changes in the nature and composition of enamel mineral during porcine amelogenesis. Calcif. Tissue Int. **47**: 356-364.

Bigi A, Gazzano M, Ripamonti A, Roveri N. (1988). Effect of foreign ions on the conversion of brushite and octacalcium phosphate into hydroxyapatite. J. Inorg. Biochem. **32**: 251-257.

Brown WE, Eidelman N, Tomzaic BB. (1987). Octacalcium phosphate as a precursor in biomineral formation. Adv. Dent. Res. 1: 306-313.

Chickerur NS, Tung MS, Brown WE. (1980). A mechanism fof incorporation of carbonate into apatite. Calcif. Tissue Int. **32**: 55-62.

Doi Y, Horiguchi T, Kim SH, Moriwaki Y, Wakamatsu N, Adachi M, Shigeta H, Sasaki S, Shimokawa H. (1993). Immobilized DPP and other proteins modify OCP formation. Calcif. Tissue Int. **52**:139-145.

Eidelman N, Chow LC, Brown WE. (1987). Calcium phosphate phase transformation in serum. Calcif. Tissue Int. **41**: 18-26.

Fowler BO, Moreno EC, Brown WE. (1966). Infrared spectra of hydroxyapatite, octacalcium phosphate. Arch. Oral Biol. **11**: 477-492.

Füredi-Milhofer H, Moradian-Oldak J, Weiner S, Veis A, Mintz KP, Addadi L. (1994) Interactions of matrix proteins from mineralized tissues with octacalcium phosphate. Connect. Tissue Res. **30**: 251-264.

Gee A, Deitz VR. (1953). Determination of phosphate by differential spectrophotometry. Anal. Chem. **25**: 1320-1324.

Hlady V, Füredi-Milhofer H. (1978). Adsorption of human serum albumin on precipitated hydroxyapatite. J. Coll. Int. Sci. **69**: 460-468.

Iijima M, Tohda H, Moriwaki Y. (1992a). Growth and structure of lamellar mixed crystals of octacalcium phosphate and apatite in a model system of enamel formation. J. Crystal Growth **116**: 319-326.

Iijima M, Tohda H, Suzuki H, Yanagisawa T, Moriwaki Y. (1992b). Effects of F- on apatite-octacalcium phosphate intergrowth and crystal morphology in a model system of tooth enamel formation. Calcif. Tissue Int. **50**: 357-361.

LeGeros RZ. (1985). Preparation of octacalcium phosphate (OCP): A direct fast method. Calcif. Tissue Int. **37**: 194-197.

LeGeros RZ, Kijkowska R, LeGeros JP. (1984). Formation and transformation of octacalcium phosphate, OCP: A preliminary report. Scanning Electron Microscopy. **1984**; IV: 1771-1777.

LeGeros RZ, Daculsi G, Orly I, Abergas T, Torres W. (1989). Solution-mediated transformation of octacalcium phosphate (OCP) to apatite. Scanning Microsc. **3**: 129-138.

Mathew M, Brown WE, Schroeder LW. (1988). Crystal structure of octacalcium bis(hydrogenphosphate)tetrabis(phosphate)pentahydrate, $Ca_8(HPO_4)_2(PO_4)_4{\cdot}5H_2O$. J Crystallographic Spectroscopic Res. **18**: 235-250.

Miake Y, Shimoda S, Fukae M, Aoba T. (1993). Epitaxial overgrowth of apatite crystals on the thin-ribbon precursor at early stages of porcine enamel mineralization. Calcif. Tissue Int. **53**: 249-256

Moreno EC, Kresak M, Zahradnik RT. (1977). Physicochemical aspects of fluoride-apatite systems relevant to the study of dental caries. Caries Res. **11** (Suppl. 1): 142-171.

Moreno EC, Kresak M, Hay DI. (1978). Adsorption of two human parotid salivary macromolecules on hydroxy-, fluorhydroxy- and fluorapatites. Arch. Oral Biol. **23**: 523-533.

Mura-Galelli MJ, Narusawa H, Shimada T, Iijima

M, Aoba T. (1992). Effect of fluoride on precipitation and hydrolysis of octacalcium phosphate in an experimental model simulating enamel mineralization during amelogenesis. Cells and Materials **2**: 21-230.

Nelson DGA, Barry JC. (1989). High resolution electron microscopy of non-stoichiometric apatite crystals. Anat. Rec. **224**: 265-276.

Robinson C, Kirkham J, Brookes SJ, Shore RC. (1992). The role of albumin in developing rodent dental enamel: A possible explanation for white spot hypoplasia. J. Dent. Res. **71**: 1270-1274.

Shimoda S, Aoba T, Moreno EC. 1991). Changes in acid phosphate content in enamel mineral during porcine amelogenesis. J. Dent. Res. **70**: 116-1523.

Shimoda S, Aoba T, Moreno EC, Iiake Y. (1990). Effect of solution composition on morphological and structural features of carbonated calcium apatites. J. Dent. Res. **69**: 1731-1740.

Siew C, Gruninger SE, Chow LC, Brown WE. (1992). Procedure for the study of acidic calcium phosphate precursor phases in enamel mineral formation. Calcif. Tissue Int. **50**: 144-148.

Suzuki O, Nakamura M, Miyasaka Y, Kagayama M, Sakurai M. (1993). Maclura ponfera agglutinin-binding glycoconjugates on converted patite from synthetic octacalcium phosphate implanted into subperiosteal region of mouse calvaria. Bone and Tineral **20**: 151-166.

Tanabe T, Aoba T, Moreno EC, Iikae M. (1988). Effect of fluoride in the apatitic lattice on adsorption of enamel proteins onto calcium apatites.. Dent. Res. **67**: 536-542.

Tung MS, Tomazic B, Brown WE (1992). The effects of magnesium and fluoride on the hydrolysis of octacalcium phosphate. Archs. Oral Biol. **37**: 585-591.

Vogel AJ. (1961). Quantitative Inrganic Analysis. 3rd ed. John Wiley & Sons, New York. 810.

Whitford GM, Reynolds KE. (1979). Plasma and developing enamel fluoride concentrations during chronic acid-base disturbances. J. Dent. Res. **58**: 2058-2065.

Discussion with Reviewers

R.Z. LeGeros: What is the pH of the deionized water at 70°? (**A**) and (**B**) solutions differ in different aspects; (**a**) presence of F; (**b**) composition: deionized water vs. Tris buffer; (**c**) temperature, 70°C vs. 37°C; (**d**) pH: ? vs. 7.4. There appears to be no variable in common between solutions A and B. How can one compare these variables?

Authors: We did not measure the pH value at 70°C. The experimental solutions, having no variables in common, were selected to secure two types of OCP hydrolyzates, with and without fluoride substitutions. As the reviewer knows (and we described in the text), the hydrolysis rate of OCP in the absence of fluoride at 37°C is quite slow. With this restriction, we forced to conduct one hydrolysis experiment at the high temperature. At 37°C, we chose other conditions to simulate the physiological media.

Reviewer III: In the introduction, the statement "serum-derived macromolecules are involved in most biomineralization processes" is not accurate. Usually, the active organic matter is synthesized by specialized cells, e.g., osteoblasts, ameloblasts, etc.

Authors: It was established that serum derived proteins, e.g., α2-HS-glycoproteins, are involved with tissue-specific matrix proteins. Thus we do not agree with the reviewer's comment.