Factors Affecting the Transformation of Octacalcium Phosphate to Apatite *in vitro*

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A fresh octacalcium phosphate (OCP) precipitate without drying and three kinds of dried OCP powders were soaked for 3 weeks in 11 kinds of physiological solutions consisting of different combinations of Ca^{2+} , Mg^{2+} , K^+ , Na^+ , HCO_3^- , HPO_4^{2-} , F^- , albumin, collagen and alkaline phosphatase, in three different pH values and at three different temperatures. X-ray diffraction study showed that most of the OCP had been transformed to apatite with low crystallinity after soaking in the solutions without Mg^{2+} . The IR absorption spectra revealed that CO_3 was incorporated in the apatite formed from OCP in the solution without Mg^{2+} , whereas OCP changed little after soaking in the solution containing Mg^{2+} . These results suggest that the presence of Mg^{2+} in the solution is one of the most effective means of inhibiting the transformation of OCP to apatite by interrupting the precipitation process of apatite.

Key words: Octacalcium phosphate, Apatite, Transformation

INTRODUCTION

Octacalcium phosphate ($\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$, hereafter referred to as OCP) has been reported to exist in pathological calcifications and human dental calculi^{1,2)}. In a neutral pH region, OCP transforms to calcium-deficient carbonate apatite which is the most thermodynamically stable calcium phosphate phase^{3,4,5)}. Therefore, OCP has been presumed to be a precursor of biological apatites; *e. g.*, enamel, dentine, cementum, bones, and phosphatic renal stones^{6,7,8)}. On the other hand, a number of authors have concluded on the basis of precipitation and nucleation studies that amorphous tricalcium phosphate (ACP) is formed first and that this then converts to apatite⁹⁾. More recently, it is generally accepted that, in neutral pH regions, ACP is precipitated first and subsequently OCP is initially nucleated on the surface of the ACP particles and then OCP converts to apatite¹⁰⁾. Thus, studying this mechanism under a wide variety of experimental conditions should give some more detail in explanation about the formation of biological apatite.

We developed a new preparation method for a quick preparation of a large amount of OCP by reactions of dicalcium phosphate dihydrate ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, hereafter referred to as DCPD) and calcium carbonate (CaCO_3)¹¹⁾. In our previous study¹²⁾, the transformation of the OCP synthesized by this method was investigated *in vivo* and *in vitro*. It was found that the OCP completely transformed to apatite within 3 weeks in mouse thigh muscle pouches and in 1% NaCl solution, whereas little change was observed in a simulated body fluid. It was concluded that magnesium had a great influence on the rate of transformation to apatite *in*

vitro, as reported by many investigators, and physiological reaction to OCP also had a significant effect *in vivo*. LeGeros *et al.*^{3,13)} demonstrated that the transformation of OCP to apatite is inhibited by Mg^{2+} , citrate or $\text{P}_2\text{O}_7^{4-}$, and facilitated by F^- , CO_3^{2-} , HPO_4^{2-} or Ca^{2+} ions. Many other investigators^{8,14-17)} have also confirmed the effect of Mg^{2+} ions on the transformation of OCP to apatite. However, it is still not clarified which ions inhibit and which stimulate the transformation of OCP. For instance, Eidelman *et al.*⁴⁾ reported that CO_2 slowed the transition of OCP to apatite. There are also some reports about the effect of proteins on the transformation of calcium phosphates. Sauer and Wuthier¹⁰⁾ demonstrated that an early driving force of mineral ion uptake is the binding of Ca^{2+} to proteins and lipids within the vesicle lumen. Wuthier and Eanes¹⁸⁾ suggested that lipids may play a role in the control of normal mineralization *in vivo*. Blumenthal *et al.*¹⁹⁾ reported that type I collagen exerts a considerable inhibitory effect on apatite proliferation. Doi *et al.*²⁰⁾ suggested that the immobilized dentin phosphoprotein could work as a nucleating substrate for the OCP phase in solutions where calcium and phosphate concentrations are sufficiently higher than equilibrium saturation levels for the OCP phase. However, there are still a lot of factors which have to be clarified in this phenomenon; *e. g.*, drying after precipitation, organic substances, and their interaction in the solution, etc.

The present work, therefore, was undertaken to study the effect of the drying condition of OCP, composition of the soaking solution, pH and temperature of the solution on the transformation of OCP to apatite *in vitro*, and the factors with the greatest effect on transformation between *in vivo* and *in vitro* are compared.

MATERIALS AND METHODS

OCP was chemically synthesized from a suspension of DCPD and CaCO_3 . A suspension was prepared containing 0.02 mol of DCPD and 0.0067 mol of CaCO_3 in a vial containing 200 ml of distilled water. The suspension was incubated at 60°C , and stirred by a Teflon-coated magnetic stirrer. The precipitate was filtered after 24 h. Details of the preparation method are the same as those described previously¹¹⁾. To investigate the effect of the dry condition, four kinds of specimens were used for the soaking test: (1) Fresh precipitate without drying, (2) powder dried at room temperature for 10 days in air, (3) powder dried at 60°C for 6 h in air, and (4) lyophilized powder prefrozen at -80°C and dried in a $5\ \mu\text{mHg}$ vacuum.

These four kinds of OCP specimens, 100 mg each, were stored in vials containing 50 ml of test solution. To investigate the effect of the composition of the immersed solution, all OCP powders were soaked in 11 kinds of test solutions for 3 weeks. Table 1 shows the ion concentrations of the test solutions used in this study. These solutions were prepared by dissolving given amounts of reagent-grade chemicals of NaCl, NaHCO_3 , KCl, K_2HPO_4 , $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ into distilled water. The solutions were buffered at a pH of 7.2 with 50 mM trishydroxymethylaminomethane $[(\text{CH}_2\text{OH})_3\text{CNH}_2]$ and an adequate amount of hydrochloric acid (HCl). The simulated body fluid (SBF) in which ion concentrations were similar to those of human blood plasma²¹⁾ is referred to as solution A. Solution B consists of only Na^+ and Cl^- ions; *i. e.*, buffered saline solution. Solution C contains no Ca^{2+} ion; solution D, no Mg^{2+} ion; solution E, neither K^+ ion nor HPO_4^{2-} ions; solution F, no HCO_3^-

Table 1 Ion concentration (mM) of test solutions used

No	Code	Na ⁺	K ⁺	Mg ²⁺	Ca ²⁺	Cl ⁻	HCO ₃ ⁻	HPO ₄ ²⁻	Others
1	<i>A</i>	142.0	5.0	1.5	2.5	148.8	4.2	1.0	—
2	<i>B</i>	137.8	—	—	—	137.8	—	—	—
3	<i>C</i>	142.0	5.0	1.5	—	143.8	4.2	1.0	—
4	<i>D</i>	142.0	5.0	—	2.5	145.8	4.2	1.0	—
5	<i>E</i>	142.0	—	1.5	2.5	145.8	4.2	—	—
6	<i>F</i>	137.8	5.0	1.5	2.5	148.8	—	1.0	—
7	<i>G</i>	142.0	3.0	1.5	2.5	148.8	4.2	—	—
8	<i>H</i>	142.0	5.0	1.5	2.5	148.8	4.2	1.0	F 3.2 mg
9	<i>I</i>	142.0	5.0	1.5	2.5	148.8	4.2	1.0	Albumin 1 g
10	<i>J</i>	142.0	5.0	1.5	2.5	148.8	4.2	1.0	Collagen 4.2 mg
11	<i>K</i>	142.0	5.0	1.5	2.5	148.8	4.2	1.0	ALPase 10 mg

ion; and solution *G*, no HPO₄²⁻ ion. Solution *H* is an SBF-containing F⁻. Solutions *I*, *J*, and *K* are the SBF containing proteins; egg albumin*, bovine collagen (type 1)**, and alkaline phosphatase (calf intestine)#. The vials containing the OCP specimens and the test solutions were incubated at 37°C in a water bath. To investigate the effect of pH of the solution, pH 5.8 and 8.2 of solution *A*, namely SBF, were also prepared by adjusting the amounts of HCl. Furthermore, the solution *A* was incubated at 5 and 62°C in a water bath to investigate the effect of temperature.

After soaking in these conditions, the specimens were filtered, rinsed with distilled water, and dried at 60°C for 8 h. The dried specimens were characterized by X-ray diffraction study##, and infrared spectroscopy@. Particle shapes were examined by using a field emission-type scanning electron microscope (SEM)@@.

RESULTS

Fig. 1 shows X-ray diffraction patterns of the lyophilized OCP powders after soaking in the 11 kinds of solutions at 37°C. The OCP completely disappeared and only apatite could be observed after soaking in solutions *B* and *D*, which contained no Mg²⁺, whereas OCP still remained after soaking in all other solutions. Fig. 2 shows X-ray diffraction patterns of the lyophilized OCP powders after soaking in pH 5.8, 7.2, and 8.2 of solution *A* at 37°C (left) and in the solution *A* at 5, 37, and 62°C (right). It was found that X-ray intensities of the OCP crystal decreased at pH 8.2 and at 62°C. Similar X-ray diffraction results were obtained in the soaking tests of fresh OCP without drying, and both the powders dried at room temperature and at 60°C.

* Egg albumin, Tokyo Kasei Kogyo Co., Tokyo, Japan

** 99% formulated by 0.012 HCl, Collaborative Research Inc., Bedford, MA, USA

EIA grade specific activity approximately 3500 u/mg, Zymed Laboratories Inc., San Francisco, CA, USA.

Rotaflex RAD-rC, Rigaku, Tokyo, Japan

@ IR-810, JASCO, Tokyo, Japan

@@ JSM-6400FX, JEOL, Tokyo, Japan

Fig. 1 X-ray diffraction patterns of the lyophilized OCP powder after soaking for 3 weeks in the 11 kinds of solutions at 37°C.

The presence of OCP crystal can easily be detected by using the (010) line at $d=18.7\text{ \AA}$ ($2\theta=4.7^\circ$) (JCPDS 26-1056). The conversion (%) of OCP to apatite was determined by using a calibration curve between the X-ray intensities of (010) line of OCP and the amounts of OCP in the mixtures of the lyophilized OCP powder and an apatite powder. This apatite had a composition of $\text{Ca}_3(\text{PO}_4)_2$ and a similar X-ray diffraction pattern to that of the apatite transformed from OCP. Fig. 3 shows the conversion of the four kinds of OCP (the precipitate without drying and the powders dried in three conditions) after soaking in 11 kinds of solutions. The conversion of OCP in both the solutions *B* and *D* were 50-100% and were higher than those in other solutions (5-30%). Fig. 4 shows the conversion of the four kinds of OCP after soaking in solution *A* at pH 5.8, 7.2, and 8.2 at 37°C (left) and at 5, 37, and 62°C

(right). It was found that the conversion of OCP in pH 8.2 and at 62°C was higher than any other conditions.

Two-way analysis of variance (ANOVA) was performed on the conversion of OCP. ANOVA for each dry condition, kind of solution, pH, and temperature are given in Tables

Fig. 2 X-ray diffraction patterns of the lyophilized OCP powder after soaking for 3 weeks at pH 5.8, 7.2, and 8.2 in solution A at 37°C (left) and in solution A at 5°C, 37°C, and 62°C (right).

Fig. 3 Conversion of the fresh OCP precipitate without drying and the OCP powders dried in three conditions after soaking for 3 weeks in the 11 kinds of solutions at 37°C.

2, 3 and 4. In each ANOVA, the kind of solution, pH, and temperature significantly influenced the conversion of OCP ($p < 0.01$). The kind of solution, in particular, had the largest contribution at 83.9%. However, no significant difference between the dry conditions was observed in each ANOVA ($p > 0.05$).

Fig. 5 shows infrared spectra of the lyophilized OCP powder after soaking in the 11 kinds of solutions at 37°C. Several PO_4 bands²²⁾, characterized by OCP around 1000–1200 cm^{-1} ,

Fig. 4 Conversion of the fresh OCP precipitate without drying and the OCP powders dried in three conditions after soaking for 3 weeks at pH 5.8, 7.2, and 8.2 in solution A at 37°C (left) and in solution A at 5°C, 37°C, and 62°C (right).

Table 2 Two-way analysis of variance for conversion (%) of the fresh OCP precipitate without drying and the OCP powders dried in three conditions after soaking for 3 weeks in 11 kinds of solutions

Source	d. f.	Sum of squares	Mean square	Fvalue	Contribution
Solution	10	22649.8	2265.0	17.26 **	83.9
Dry Condition	3	411.8	137.3	1.05	1.5
Error	30	3937.7	131.3		14.6
Total	43	26999.3			100.0

Table 3 Two-way analysis of variance for conversion (%) of the fresh OCP precipitate without drying and the OCP powders dried in three conditions after soaking for 3 weeks at pH 5.8, 7.2, and 8.2 in solution A

Source	d. f.	Sum of squares	Mean square	Fvalue	Contribution
pH	2	4246.3	2123.2	13.47 **	75.5
Dry Condition	3	432.2	144.1	0.91	7.7
Error	6	945.7	157.6		16.8
Total	11	5624.1			100.0

changed to a single band around 1030 cm^{-1} with a small shoulder around 1150 cm^{-1} , and HPO_4^{2-} bands at 869 and 910 cm^{-1} changed to 865 cm^{-1} after soaking in both solutions *B* and *D*, whereas the spectra little changed in other solutions. Furthermore, the CO_3 band^{23,24)} around 1430 cm^{-1} was also observed in the spectra after soaking in solutions *B* and *D*.

Table 4 Two-way analysis of variance for conversion (%) of the fresh OCP precipitate without drying and the OCP powders dried in three conditions after soaking for 3 weeks in solution *A* at 5°C , 37°C , and 62°C

Source	d. f.	Sum of squares	Mean square	Fvalue	Contribution
Temperature	2	1796.6	898.3	36.13 **	77.6
Dry Condition	3	368.1	122.7	4.97	15.9
Error	6	149.2	24.9		6.5
Total	11	2313.9			100.0

Fig. 5 Infrared spectra of the lyophilized OCP powder after soaking for 3 weeks in the 11 kinds of solutions at 37°C .

Although solution *B* contained no carbonate, it seems that atmospheric CO_2 gas dissolved in the solution and HCO_3^- ions might be incorporated in the apatite transformed from OCP. Fig. 6 shows the IR spectra of the lyophilized OCP after soaking in solution *A* at pH 5.8, 7.2, and 8.2 at 37°C (left) and at 5, 37, and 62°C (right). The PO_4 bands slightly changed at pH 8.2 and 62°C. Similar results for IR study were obtained in the soaking tests of fresh OCP without drying and of the powders dried at room temperature and at 60°C. In consideration of the X-ray diffraction study, it is suggested that in the solutions without Mg^{2+} , most of the OCP transformed to carbonate calcium-deficient apatite²⁵⁾ and incorporated HPO_4^{2-} with low crystallinity such as biological apatite.

Fig. 7 shows SEM micrographs of the lyophilized OCP powder after soaking in solutions *A*, *B*, *C*, and *D*. It was found that OCP crystals changed little after soaking in both solutions *A* and *C*, whereas needle-like crystals could be seen in both the solutions *B* and *D*. The morphological changes of OCP in other solutions were slight as in solutions *A* and *C*. From the results of X-ray diffraction and IR study, it seems that the needle-like crystals are the carbonate apatite with low crystallinity.

Fig. 6 Infrared spectra of the lyophilized OCP powder after soaking for 3 weeks at pH 5.8, 7.2, and 8.2 of solution *A* at 37°C (left) and in solution *A* at 5°C, 37°C, and 62°C (right).

Fig. 7 SEM micrographs of the lyophilized OCP powder after soaking for 3 weeks in solutions A, B, C, and D at 37°C.

DISCUSSION

In the present study, it was concluded that Mg^{2+} ions were the most effective inhibitor of the transformation of OCP to apatite *in vitro*, and the presence of other ions and proteins were not statistically significant. It is known that the presence of Mg^{2+} ions cause some reduction in the crystallization of OCP³⁾. In the aqueous system, Mg^{2+} ions are substituted into the Ca^{2+} sites, although Mg^{2+} ions do not enter solid phases to any great extent. Mg^{2+} ions induce a clear contraction along the c-axis dimension of apatite, because Mg^{2+} ions have a small radius of 0.65 Å, as opposed to the 0.99 Å of Ca^{2+} ions²⁶⁾. In contrast, most apatite in which some anions have been substituted show contraction along the a-axis; *e. g.*, CO_3^{2-} ions substitute PO_4^{3-} sites of apatite and F^- ions substitute OH^- sites. These metal ions have also smaller ionic radii than the PO_4^{3-} and OH^- . Biological apatite generally grows along c-axis in biological systems. Therefore, it seems that Mg^{2+} ions can most strongly affect the crystallization of calcium phosphates, since Mg^{2+} ions have a greater influence on the lattice arrangement along the c-axis than do other ions. One of the effects of the low crystallinity by Mg^{2+} ions may be surface poisoning²⁷⁾. Mg (or some of its complexes) can poison the surface of apatite nuclei by being absorbed into the active growth sites, so that growth either stops or proceeds at a rate so slow that other phases can nucleate. This process most likely depends on the Mg concentration in solution and on the surface structure of crystal faces.

Apatite obtained by the transformation from OCP in this study was not stoichiometric apatite ($\text{Ca/P}=1.67$), but Ca-deficient apatites such as biological apatite. Brown *et al.*⁶⁾ proposed that the transformation of OCP to apatite in biological systems can proceed either by *in situ* hydrolysis or by dissolution of OCP and precipitation of apatite incorporating ions present in the micro-environment, or both. Recently, LeGeros *et al.*¹³⁾ concluded that its transformation occurred by the process of dissolution of OCP and subsequent precipitation of Ca-deficient apatites, incorporating CO_3^{2-} , HPO_4^{2-} , or F^- present in solution. We also concluded that the transformation of OCP to apatite is predominantly determined by the dissolution of OCP and the precipitation of apatite on the surface of the OCP, and that the incorporation of Mg^{2+} interferes with the precipitation process because of surface poisoning.

The results in this study are consistent with the hypothesis that both higher pH and higher temperature of the solution significantly promoted the transformation of OCP to apatite, and this promotion may be due to the chemical and thermodynamic equilibrium^{28,29)}, because of the involvement of dissolution and precipitation.

Furthermore, OCP obtained in different drying conditions showed nearly the same behavior in the transformation. When drying living tissue, lyophilized specimens generally show a relatively large specific surface area. However, the lyophilized OCP powder in the present study showed nearly the same morphology with those of powders dried at room temperature and at 60°C. Then, it seems that the similarity in morphology may cause similar chemical behavior, such as dissolution and precipitation in the solution, although each OCP absorbed a different amount of water. This may explain why the drying conditions did not significantly affect the transformation of OCP to apatite.

In comparison with the results *in vivo* in our previous report¹²⁾, it seems that physiological reactions have a great effect on the transformation of OCP to apatite *in vivo*. Although the body fluid around the implanted OCP contains Mg^{2+} ions, the physiological environment seems to negate their influence and promote transformation. The physiological reaction is not simple inorganic or organic chemical reaction, since major proteins contained in the body fluid showed no significant effect *in vitro* in this study. Furthermore, implantation of biomaterials always involves the formation of a surgical wound. Consequently, the initial interfacial reaction can involve the interaction of the implanted material surfaces with serum components present at the wound site. For instance, the normal pH of the body fluid is about neutral, in the range of 7.2 to 7.4. At injured sites, the pH shifts to acidic values of about 5.2. In hematoma, it can drop to 4.0. In case of infection, the pH becomes alkaline.³⁰⁾ Furthermore, the temperature around the implant rises due to some physiological reactions related to fever. The physiological reaction around the implant changes as the surgical wound heals and as the test animal grows. Therefore, it appears that the difference between the transformation behavior of OCP to apatite *in vivo* and *in vitro* is due to unknown factors in the highly complex *in vivo* environment.

CONCLUSION

Solutions without Mg^{2+} , high pH, and high temperature significantly promoted the transformation of OCP to apatite, although its transformation was not significantly influenced by the

drying conditions. Especially, the presence of Mg^{2+} in the solution had the greatest influence on transformation. The effect of the presence of other ions and some proteins were not statistically significant. These results suggest that the transformation of OCP to apatite *in vitro* is mainly determined by the dissolution of OCP and the precipitation of apatite, and the incorporation of Mg^{2+} interrupts the precipitation process because of surface poisoning.

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本号掲載論文の和文抄録

新しいデンチンボンディング材の引張接着強さとギャップ形成について

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新しいデンチンボンディング材を用いて, 象牙質の前処理, プライミング処理の影響について, 引張試験, ギャップテストを行って検討した。引張試験の結果, 前処理およびプライマー処理の有無によって接着強さに有意な差が認められ, リン酸による前処理と HEMA, グルタルアルデヒドを含むプライマーの組み合わせで 151.3 kgf/cm^2 と最も高い接着強さが得られた。一方, ギャップテストの結果, すべての群においてギャップの形成が認

められたが, その様相は様々であり, ばらつきが大きかった。また, 引張接着強さとギャップ形成との間には, 統計学的な相関は認められなかった。以上より, 新しいボンディング材はどの群においてもほぼ良好な接着強さを示すと思われるが, 酸処理およびプライマー処理の併用によって最も高い接着強さが得られ, ギャップを最小にできることがわかった。

実験室的にリン酸八カルシウムのアパタイトへの転化に影響を与える因子

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調製したままの乾燥前のリン酸八カルシウム (OCP) 沈殿物と乾燥条件の異なる 3 種の OCP 粉末を 3 週間, 11 種の生理的溶液に浸漬した。この溶液は Ca^{2+} , Mg^{2+} , K^+ , Na^+ , HCO_3^- , HPO_4^{2-} , F^- , アルブミン, コラーゲン, アルカリフォスファターゼより構成されている。また, ある溶液で pH を 3 つに変えた場合, 温度を 3 段階に変えた場合についても浸漬実験をした。X 線回折の測定結果より, OCP は Mg^{2+} を含まない溶液への浸漬後は結晶性の低いアパタイトにほとんど転化していた。その X 線回折の強度変化より求めた OCP の転化量について

行った分散分析結果より, OCP のアパタイトへの転化は溶液の種類, pH, 温度に対して有意差があり (危険率 1 % 以下), 一方乾燥条件についての有意差は認められなかった (危険率 5 % 以上)。また, 赤外分光分析スペクトルより, Mg^{2+} を含まない溶液中で OCP から転化するアパタイトは CO_3 が含まれているが, Mg^{2+} を含む溶液中ではほとんど変化がなかった。これらの結果は溶液中での Mg^{2+} の存在が OCP からアパタイトへの転化に対し, 最も効果的な因子であると考えられた。