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EFFECTS OF SOME IONS ON THE CONVERSION OF AMORPHOUS CALCIUM PHOSPHATE TO CALCIUM HYDROXYAPATITE IN AQUEOUS MEDIUM

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ABSTRACT. The effects of the ions Mg^{2+}, Ba^{2+}, Pb^{2+}, Sr^{2+}, F^- and Cl^- on the stabilization and conversion of amorphous calcium phosphate (ACP) to calcium hydroxyapatite (HAP) at 298K and a pH of 9.00 ± 0.05 (and also at a pH of 8.00 in the case of Mg^{2+}) have been studied. The order of stabilization of ACP by these ions is $Mg^{2+} > Ba^{2+} > Sr^{2+} > Pb^{2+} \sim F^- \sim Cl^-$. Heat treatment of selected products obtained at various conversion times and plots of crystallinity index against time tend to show the locus of the interactions of the various ions with ACP and the apatitic products. Mg^{2+} appears to block more effectively the growth of apatitic crystals in the 001 directions, while the other ions do not show such directional effect.

INTRODUCTION

Many studies have been carried out on the precipitation of ACP and its subsequent conversion, *in situ*, to HAP, in an attempt to understand the process of mineralization in both normal and pathological tissues [1-3]. The effects of various substances and ions on the precipitation and stablilization of ACP have also been elucidated [4-8]. Thus, it has been suggested [7] that Mg^{2+}, Sr^{2+} and F^- ions act as inhibitors of apatite formation when added to a calcification buffer separately or in combinations. From studies on precipitates obtained at 310K, pH's 7 to 9, in the presence of various ions [2], it was proposed that the order of the ions in decreasing the crystallinity of apatite and promoting the formation of ACP is similar to the order of efficiency in increasing the stability of the precipitate, and is given by;

$$(Mg^{2+} + P_2O_7^{4-}) >> (P_2O_7^{4-}) >> (Mg^{2+} + CO_3^{2-}) >> (Mg^{2+}) >> (CO_3)^{2-}$$

Substances such as phosphocitrate, 1-hydroxyethane, 1,1-diphosphonate, imidodiphosphate and pyrophosphate (all type 1 inhibitors), are known to inhibit both transformation of ACP to HAP and the growth of apatitic crystals. ATP, ADP, phosphonoformate, 2- and 3-phosphoglycerate, Mg^{2+} and citrate (all type 2 inhibitors) on the other hand, are believed to inhibit only the transofrmation of ACP to HAP [8]. However, the problems associated with inhibition in this system include [9]: the qualitative nature of the existing information, difficulty of devising experiments for detecting and describing the effects of inhibitors, even *in vitro*; difficulty in isolating and identifying inhibitors *in vivo*; and uncertainty about the locus of the inhibition effect.

The above problems have led to conflicting mechanisms of inhibition in the literature. For example, from a kinetic study of the transformation of ACP in the presence of Mg^{2+} ions at a pH of 8, Boskey and Posner [6] showed that although the induction time of the transformation increased with increasing concentration of Mg^{2+}, the first order transformation rate constant was independent of Mg^{2+} concentration. The authors concluded that Mg^{2+} did not

appear to poison the HAP crystal and suggested that it is principally the dissolution of ACP that is affected by Mg^{2+}. The stabilization of ACP by Mg^{2+} has been attributed to the ability of this ion to form "stronger" complexes than Ca^{2+} with phosphate ions, thus preventing apatite formation [7]. Similar conclusions have also been reached by Williams and Sallis [8], who proposed that the locus of action of Mg^{2+} and all type 2 inhibitors is at the ACP surface. However, more recently, Nancollas [10] has proposed that the retarding effect of Mg^{2+} could be interpreted in terms of a Langmuir adsorption isotherm representing the effective blocking of growth sites on the HAP seed crystal surface by the presence of Mg^{2+} ions. In contrast, it has been proposed that the crystallization of HAP at low supersaturation in the presence of Sr^{2+} ion is accompained by the incorporation of this ion in an ideal manner. The work of Nancollas and coworkers has been carried out largely at physiological pH.

Two studies [11,12] have also shown that at pH values above 9, Mg^{2+} substitutes for Ca^{2+} in HAP to give solid solutions in the complete range, although conflicting results were obtained for the variation of the c unit cell parameter with Mg^{2+} content. This proposal deserves further examination as most of the other studies in the literature indicate that the presence of Mg^{2+} in the mediating solution tends to favour the formation of β-tricalcium phosphate (β-tcp) instead of HAP. Furthermore, it does not appear that there are any systematic studies on the time dependence of the degree of crystallinity of the apatitic precipitates in the presence of ions other than Mg^{2+} ions. In this study, we have followed systematically (for a longer time than ever reported) the precipitation and conversion of ACP in the presence of various ions (at pH values of 8 and 9, and 298 K) by measuring a correlation index of crystallinity [13] from X-ray powder diffraction patterns of products at known reaction times. The correlation index, K expresses numerically the degree of ordering in a given sample relative to some minimum and maximum values observed in a sampling of specimens of the same species. Heat treatment of some products of the conversion obtained at selected reaction times has also been carried out. An attempt is made to contribute towards an understanding of the locus of interaction of a low concentration of each of the ions in the precipitation and conversion of ACP to HAP in aqueous medium.

EXPERIMENTAL

Procedure. ACP was precipitated by mixing pre-buffered (tris-HCl, 0.15 M; pH 9.00 ± 0.05) and equilibrated (298 K) 0.04 M $Ca(NO_3)_2.4H_2O$ solution (0.4 dm^3) with 0.036 M $(NH_4)_2HPO_4$ solution (0.5 dm^3) and stirring vigorously. In separate experiments, 0.001 M (or 2.5% relative to the molar concentration of Ca^{2+}) each of Mg^{2+}, Ba^{2+}, Sr^{2+} and Pb^{2+} respectively were substituted for Ca^{2+} ions in the mediating solution, in all cases, using the nitrates of the metals to avoid changes in the initial number of ions in solution. 0.001 M of the anions F^- and Cl^- were also added in separate experiments at a pH of 9.00 and keeping the initial number of ions in solution constant.

In another experiment, a 0.001 M solution of Mg^{2+} ions was added to the mediating solution without a prior reduction of the initial Ca^{2+} ion concentration. For Mg^{2+}, the experiment was also repeated at a pH of 8.00 substituting a 0.001 M solution of Mg^{2+} for the corresponding amount of Ca^{2+}.

Samples of the precipitated solids in all cases were withdrawn at known reaction times and filtered quickly by suction. The precipitate was washed in each case with chilled ammonia/acetone water (1:1; pH 10.6) and then with acetone to obtain "free-flowing" powder. The time of completion of the acetone wash was recorded, in each case, as the time reaction co-ordinate [14]. The washed solids were dried in a vacuum dessicator over P_2O_5. Each experiment was carried out at least twice.

X-ray analysis. X-ray powder diffraction patterns of the vacuum-dried solids were taken with a Philips PW1050/81 diffractometer controlled by a PW1710 unit, using Nickel-filtered CuKα radiation, λ = 0.15418 nm (40KV, 25 mA). The correlation index of crystallinity, K [13] was determined for each sample from the x-ray powder pattern by linear regression of 8 (002 peak only) to 26 (2θ: 25-35°) sets of points for each value of K. The regression were done on an Olivetti M240 computer. For the determination of K, the sample obtained after 522 minutes was taken as that of maximum crystallinity and the amorphous precipitate first obtained as that of minimum crystallinity. The equation of the line is of the form;

$$Is - Ia = K (Ic - Ia) + B;$$

where Is, Ic and Ia are the numerical values of intensities (arbitrary units) of intermediate, maximum and minimum crystallinity respectively at various 2θ values. B is an intercept on the axis, ideally zero but usually of low values in acceptable experiments. Values of B were generally very low in these experiments.

Theremal analysis. Some selected reaction products were heated at temperatures between 773 K and 1273 K. The x-ray diffraction patterns of the products obtained at the various temperatures were taken and the main crystalline phases present determined.

RESULTS AND DISCUSSION

Fig. 1 shows the effects (at pH 9.00; 298 K) of the cations Pb^{2+}, Sr^{2+}, Ba^{2+} and Mg^{2+} on the stabilization and coversion of ACP, based on the plots of K against time. In this Figure K was derived in each case using the region of the x-ray pattern in the 2θ° range of 25-35°. Similar plots with K derived using the 002 peak of each pattern are presented in Fig. 2. These graphs especially for Mg^{2+} and Ba^{2+} give features reminiscent of secondary crystallization, a known feature in polymer crystallization. The observation of this effect was made possible by adopting a maximum sampling time of about 522 minutes. The effects of added fluoride and chloride ions are shown in Fig. 3. In general the curves show an induction period where no appreciable transformation takes place, followed by a rapid proliferation period marking a quick transformation of the amorphous precipitates to apatitic produets. There is then a gradual tapering off as the apatite becomes more and more crystalline.

Fig. 4 demonstrates the effects of varying the concentration of Mg^{2+} substituted for Ca^{2+} in the mediating solution, together with that due to the addition of 0.001 M Mg^{2+} ions without reducing the initial concentration of Ca^{2+} in the solution. The shape of the graph in the latter case is generally similar to that obtained for the prior substitution of the same amount of Mg^{2+} for Ca^{2+}. This would indicate that with no prior reduction of the Ca^{2+} ion concentration at a pH of 9.00, the Mg^{2+} ions compete less favourably with Ca^{2+} ions at the ACP locus. Plots of the crystallinity index derived from, the 2θ° range of 25-35°, the group of peaks about the 211 peak of HAP, and the 002 peak of the x-ray pattern, against time for prior substitution of 0.001 M Mg^{2+} for Ca^{2+} at a pH of 9.00 are shown in Fig. 5. The plots show a further initial delayed growth of the apatitic product in the 002 direction. Similar plots for the other ions and also in the absence of any added foreign ions did not indicate any significant directional differences. The effect of substitutuing 0.001 M Mg^{2+} for Ca^{2+} in the mediating solution at a pH of 8.00 is demonstrated in Fig. 6, the result being in agreement with literature report [6].

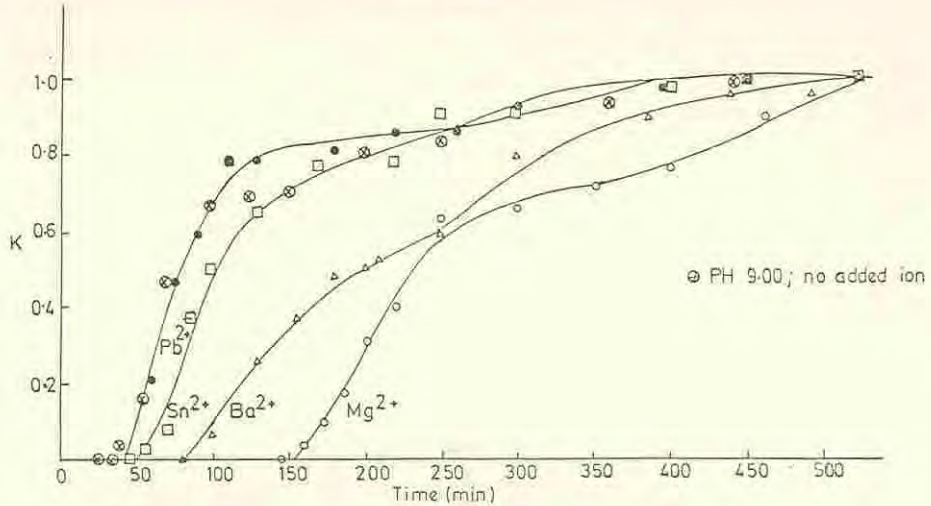

Fig.1 ACP to HAP isotherms at 298K and pH 9.00 showing the effects of various cations (0.001M) substituted for Ca^{2+} in the mediating solutions. The crystallinity index, K refers to the 2θ range of 25° to 35° of the X-ray pattern. The percentage standard deviation in the determination of K was generally less than 5%.

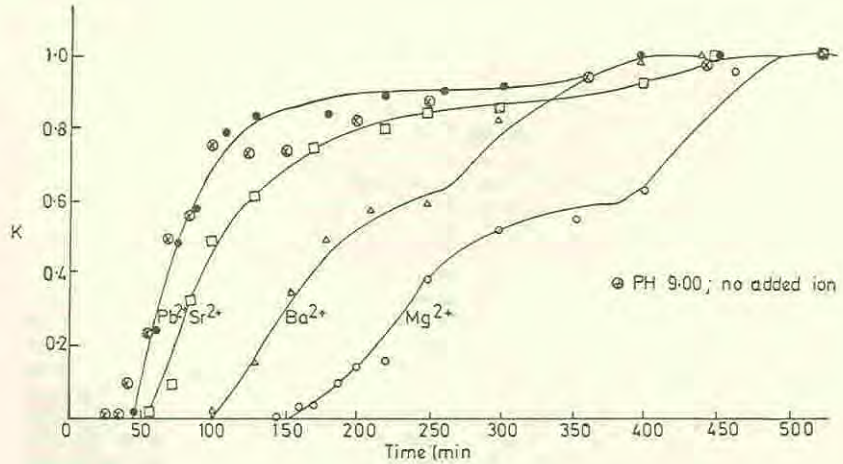

Fig. 2 ACP to HAP isotherms at 298K and pH 9.00 showing the effects of various cations (0.001M), but with the crystallinity index K derived using the 002 peak of the X-ray pattern.

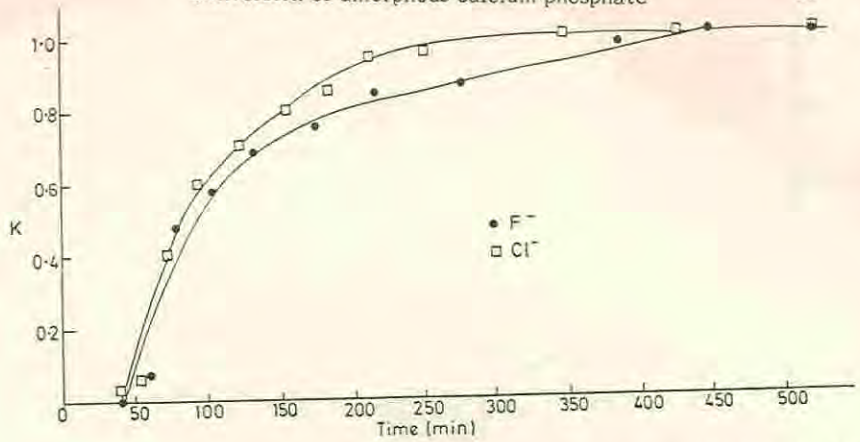

Fig. 3 ACP to HAP isotherms at 298K and pH 9.00 showing the effects of 0.001M F^- and Cl^- ions. K refers to the 2θ range of 25 to 35°.

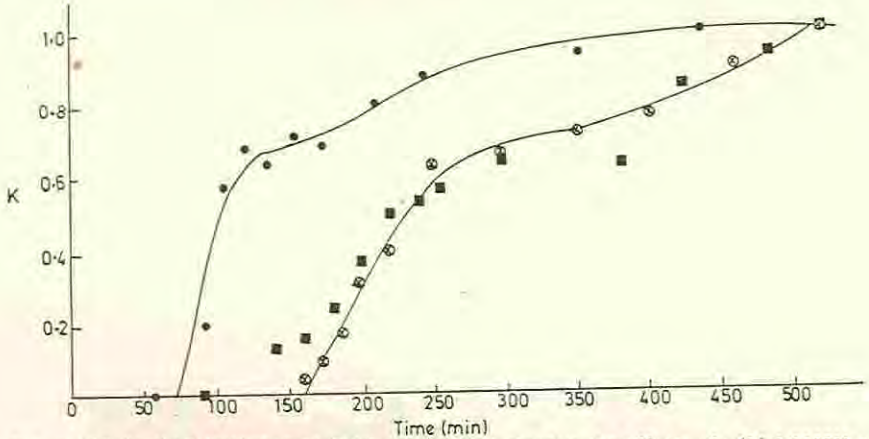

Fig. 4 ACP to HAP isotherms at 298K and pH 9.00 showing the effects of: (●) 0.0005M and (⊗) 0.001M Mg^{2+} ions substituted for Ca^{2+} in the mediating solutions; and (■) 0.001M Mg^{2+} added without reducing the initial Ca^{2+} ion concentration in the mediating solution. K refers to the 2θ range of 25 to 35°.

Table 1 lists approximate suspension life times of ACP (Prepared under the stipulated conditions) and as derived from Figs. 1, 3 and 6. The order of stabilization of ACP by the various ions is:

$$Mg^{2+} > Ba^{2+} > Sr^{2+} > Pb^{2+} \sim F^- \sim Cl^-$$

The crystalline phases obtained by heat treatment of selected ACP and apatitic products previously prepared at a pH of 9.00 (and also at a pH of 8.00 for Mg^{2+}) are shown in Tables 2 and 3. Both ACP and the apatitic products obtained under all experimental conditions used are converted completely to β-tricalcium phosphate (β-tcp) at 1073 K. The presence of Ba^{2+} ions leads to a further destablization of the apatitic phase promoting its conversion to α-tcp at the relatively low temperature of about 1273 K. The products of heat treatment at 773 K are of particular interest as they seem to reflect more on the milieu from which the products were derived. First, Table 2 shows that at a pH of 8, all samples obtained in the presence of Mg^{2+} were completely converted

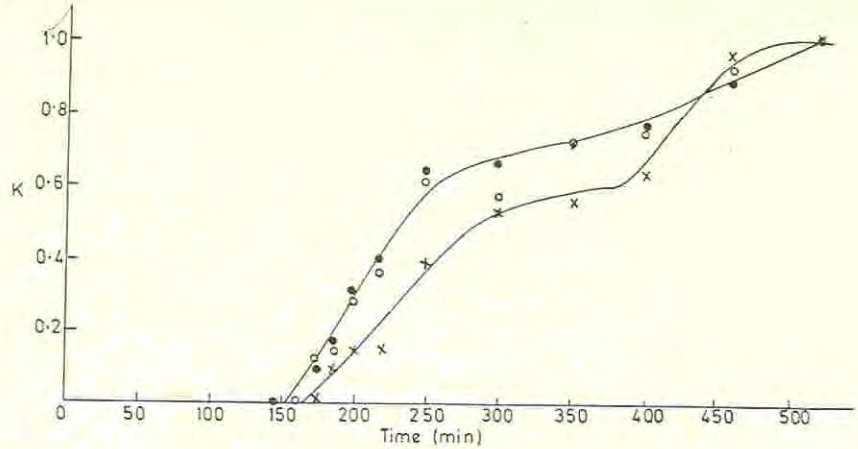

Fig. 5 ACP to HAP isotherms at 298K and pH 9.00 with 0.001M Mg^{2+} initially substituted for Ca^{2+}, but with K derived from: (●) 2θ range of 25 to 35°; (X) the 002 peak only; and (O) the unresolved group of peaks about the 211 peak of the apatite X-ray pattern.

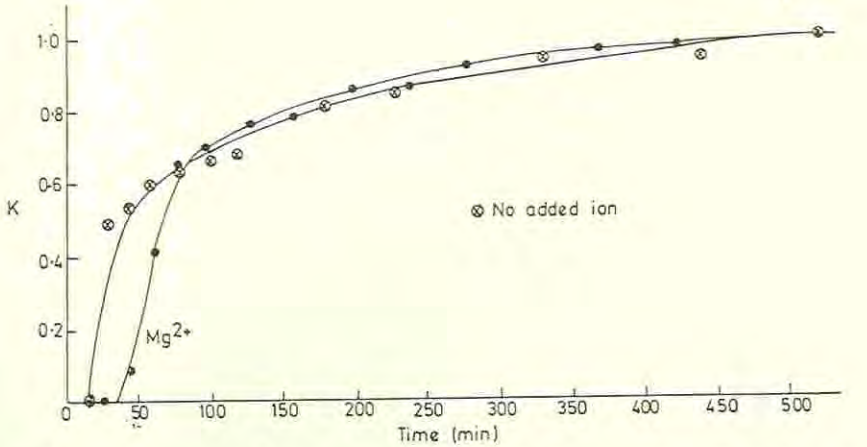

Fig. 6 Effect of 0.001M Mg^{2+} initially substituted for Ca^{2+} at 298K and pH 8.00. K refers to the 2θ range of 25 to 35°.

to β-tcp at 773 K, in contrast to samples without added Mg^{2+} which showed the concurrent presence of both the apatitic and β-tcp phases at this temperature. Second, at a pH of 9.00, the ACP and apatitic samples up to 352 minutes of reaction time obtained in the presence of Mg^{2+} were also completely converted

Table 1. Approximate suspension life times of ACP in the presence of various ions at 298 K and (a) pH 9.00 (b) pH 8.00.

(a)

ion*	Suspension life time (min)
No added ion	39
Mg^{2+}	158
Mg^{2+}**	125
Mg^{2+} (0.0005 M)	75
Ba^{2+}	85
Sr^{2+}	58
Sr^{2+} (0.002 M)	76
Pb^{2+}	44
F^- (0.001 M)	43
Cl^- (0.001 M)	42

(b)

No added ion	16
Mg^{2+}	40

* Unless otherwise stated, 0.001 M of each cation was substituted for Ca^{2+} in the initial mediating solution. The anions were previously substituted for OH^- in the mediating solution.

** 0.001 M Mg^{2+} was added to the mediating solution without reducing the initial Ca^{2+} concentration.

Table 2. Crystalline phases obtained by heat treatment of selected products of the ACP - HAP reaction at pH 9.00 and 298K: (i) without added Mg^{2+}; (ii) with 0.001 M Mg^{2+} substituted for Ca^{2+}; and at pH 8.00; (iii) without added Mg^{2+}; (iv) with 0.001 M Mg^{2+} substituted for Ca^{2+}.

Sample with time (min) in parentheses	Temperature (K) 773	873	1073	1273
		pH 9.00 (i)		
ACP (0-44)	HAP>β-tcp	β-tcp>HAP	β-tcp	-
HAP (522)	β-tcp>HAP	β-tcp>HAP	β-tcp	-
		(ii)		
ACP (0-158)	β-tcp	β-tcp	β-tcp	-
HAP (300-352)	β-tcp	β-tcp	β-tcp	-
HAP (522)	β-tcp>HAP	β-tcp	β-tcp	-
		pH 8.00 (iii)		
ACP (0-16)	HAP>β-tcp	β-tcp>HAP	β-tcp	β-tcp
HAP (522)	β-tcp>HAP	β-tcp	β-tcp	β-tcp
		(iv)		
ACP (0-30)	β-tcp	β-tcp	β-tcp	β-tcp
HAP (170)	β-tcp	β-tcp	β-tcp	β-tcp
HAP (522)	β-tcp	β-tcp	β-tcp	β-tcp

tcp - tricalcium phosphate

Table 3. Crystalline phases obtained by heat treatment of selected products of the ACP-HAP reaction at pH 9.00 and 298K with: (i) 0.001M Ba^{2+}; (ii) 0.001M Sr^{2+} and (iii) 0.001M pb^{2+} substituted for Ca^{2+}.

sample with time (min) in parentheses	Temperature (K) 773	873	1073	1273
		(i)		
ACP (0-78)	HAP> β-tcp	β-tcp>HAP	β-tcp	α-tcp> β-tcp
HAP (200)	HAP> β-tcp	β-tcp>HAP	β-tcp	α-tcp> β-tcp
HAP (522)	HAP*	β-tcp	β-tcp	α-tcp> β-tcp
		(ii)		
ACP (0-35)	HAP> β-tcp	β-tcp>HAP	β-tcp	β-tcp
HAP (170)	HAP> β-tcp*	β-tcp>HAP	β-tcp	β-tcp
HAP (522)	HAP*> β-tcp	β-tcp	β-tcp	β-tcp
		(iii)		
ACP (0-50)	β-tcp>HAP	β-tcp>HAP	β-tcp	β-tcp
HAP (180)	HAP> β-tcp*	β-tcp>HAP	β-tcp	β-tcp
HAP (522)	HAP*> β-tcp	β-tcp	β-tcp	β-tcp

* This phase was less crystalline than the corresponding previous phases at the stipulated temperature.

to β-tcp at this temperature. The sample at 522 minutes under the same reaction conditions gave both an apatitic and β-tcp phases, as did the sample at the same reaction time prepared without added Mg^{2+}. The 522 - minute sample corresponds to the second plateau in Fig. 2 for Mg^{2+}.

At 773 K products obtained in the presence of 0.001 M Pb^{2+} and Sr^{2+} at a pH of 9.00 gave both the apatitic and β-tcp phases in the range of samples treated (Table 3). In the case of Ba^{2+}, the 522 - minute sample gave only an apatitc phase of decreased crystallinity (Table 3). The apatitic phases obtained on heat treatment of samples at 522 minutes previously prepared in the presence of added Sr^{2+} and Pb^{2+} also showed slightly reduced crystallinity when compared with phases obtained from samples of shorter reaction times. Broadening in X-ray diffraction peaks and reduction in the intensity of the peaks usually indicate a reduction in crystallite size and/or increase in strain in the crystal. Such effects were observed by Legeros et. al. [15] on the incorporation of Ba^{2+}, Pb^{2+} and Sr^{2+} into the apatite lattice.

As can be seen from Figs. 1 to 3 and Table 1, the presence of Pb^{2+}, Sr^{2+}, F^- or Cl^- does not greatly affect the conversion of ACP to apatitic products. This result is in agreement with the finding that Pb^{2+}, F^- and Sr^{2+} do not greatly affect the conversion of octacalcium phosphate (OCP) and brushite (DCPD) into HAP [16]. The result is also consistent with the fact that these ions are readily incorportated into the apatite lattice. However, the slight tendency of Sr^{2+} to stabilize ACP has been attributed to a possible initial competition of Sr^{2+} with Ca^{2+}, preventing the formation of appropriate clusters and nuclei and delaying the initiation of crystal growth due to the larger size of the Sr^{2+} ion [7]. This may not be fully correct, as Pb^{2+} at relatively lower concentrations compared to Ca^{2+} conveniently fits into site 2 of the apatite lattice with minor disruption, according to the equation:

$$Ca_{10}(PO_4)_6(OH)_2 + nPb^{2+} \rightarrow Ca_{10-n}\,Pbn(PO_4)_6(OH)_2 + nCa^{2+}$$

Examination of the results indicates that Ba^{2+} and Mg^{2+} of the four cations studied cause the greatest stabilization of ACP and reduction in initial conversion rates to apatitic products at a pH value of 9. Both cations have radii that are most different from that of Ca^{2+} (99 pm) on the high (Ba^{2+}; 135 pm) and low (Mg^{2+}; 65 pm) sides. The apatitic phase of reduced crystallinity obtained on heating the Ba^{2+} substituted sample at 522 minutes at 773 K overnight (see Table 3) suggests that the second proliferation region in Figs. 1 and 2 is probably due to the growth of a Ba -incorporated apatitic phase initially formed in the period corresponding to the first plateau. A similar process may be invoked to explain the shapes of the plots in these Figures for Mg^{2+}. However, the phases obtained on heating the Mg^{2+} substituted 522 -minute sample (Table 2) are similar to those obtained by heating a sample obtained after the same reaction time but containing no added Mg^{2+}. In the former case, the apatitic phase is relatively much less than the β-tcp. Thus, the plots obtained for the Mg^{2+} substituted sample (pH = 9.00) could be due partly to the incorporation of a little amount of Mg^{2+} into the apatite lattice [11,12], and partly to a rejection of some Mg^{2+} from the surface of the apatite lattice, resulting in the unblocking of growth sites, particularly in the 001 direction (Fig. 2). Isotopic labelling experiments have shown that up to about 90% of added Mg^{2+} could be rejected from the apatite surface [5]. Such rejection and unblocking of growth sites may also be responsible, at least in part, for the shape of the isotherm in the case of samples with Ba^{2+}. This would be consistent with the observation that the amount of Ba^{2+} found in some solid solutions of Ca-Ba hydroxyapatites was always significantly less than the concentration initially added to the mediating solutions [18]. It would therefore appear that at a pH of 9.00 and 298 K both Mg^{2+} and Ba^{2+} show inhibiting action which is not only limited to the ACP locus [6,8], but also block growth sites on the HAP crystal surface [9] with a possible incorporation of some amounts of these ions into the apatitie lattice. For Mg^{2+} some incorporation will also be in accord with the fact that the overall shape of the growth isotherm at a pH of 9.00 (Fig. 4) is independent of the way of introducing the Mg^{2+} ions into the mediating solution [19]. From both Fig. 6 and Table 2, it appears that at a pH of 8.00 the locus of Mg^{2+} is at the ACP as previously suggested [6] and any interaction of the ion with the apatitic product is a surface effect.

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