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Influence of Pyrophosphate on the Transformation of Amorphous to Crystalline Calcium Phosphate

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The transformation of amorphous calcium phosphate into its crystalline form has been studied *in vitro* under various conditions. The transformation was followed by changes in the pH and in the calcium and phosphate content of the solution and by changes in the Ca/P ratio and x-ray diffraction patterns of the solid phase. It was found that inorganic pyrophosphate markedly increased the time required for the transformation under the various conditions used. The addition of intestinal alkaline phosphatase abolished this retarding effect of pyrophosphate on the transformation.

It is proposed that pyrophosphate may be one of the factors that allows part of the bone mineral to persist in a non-crystalline state. The alkaline phosphatase of bone, by virtue of its pyrophosphatase activity, might be able to accelerate the transformation process *in vivo*.

Key words: Pyrophosphate — Calcium Phosphate — Phosphatase — Crystallization — Pyrophosphatase.

La transformation du phosphate calcique de la forme amorphe en forme cristalline a été étudiée *in vitro* dans différentes conditions. On a suivi cette transformation en étudiant la variation des paramètres suivants: le pH de la solution et sa teneur en calcium et en phosphate, ainsi que le rapport Ca/P et la courbe de diffraction aux rayons X de la phase solide. Le pyrophosphate inorganique augmente sensiblement le temps nécessaire à cette transformation dans les différentes conditions choisies. Cet effet s'annule lorsqu'on ajoute en plus du pyrophosphate de la phosphatase alcaline intestinale.

Les auteurs suggèrent que le pyrophosphate pourrait être l'un des facteurs qui permettent à une partie du minéral de l'os de demeurer dans un état non-cristallin. La phosphatase alcaline de l'os, grâce à son activité pyrophosphatasique, serait capable d'accélérer cette transormation *in vivo*.

Die Umwandlung von amorphem in kristallines Calciumphosphat wurde *in vitro* unter verschiedenen Bedingungen studiert. Diese Umwandlung wurde folgendermaßen verfolgt: einerseits in der Lösung durch Änderung des pH's und des Calcium- und Phosphatgehaltes, andererseits in der soliden Phase durch Änderung des Ca/P-Verhältnisses, sowie des Röntgenstrahlendiffraktionsbildes.

Es konnte festgestellt werden, daß unter den verschiedenen Versuchsbedingungen die Anwesenheit von anorganischem Pyrophosphat die zur Umwandlung benötigte Zeit wesentlich verlängert. Wird noch intestinale alkalische Phosphatase zugesetzt, so wird die durch das Pyrophosphat verlängerte Umwandlungszeit aufgehoben.

Es wird vorgeschlagen, daß Pyrophosphat einer der Faktoren sein kann, der Teile des Knochenminerals in einem nicht kristallinen Zustand verbleiben läßt. Die alkalische Knochen-

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phosphatase könnte, dank ihrer Pyrophosphataseaktivität, eine Beschleunigung des Umwandlungsprozesses *in vivo* ermöglichen.

Introduction

Amorphous calcium phosphate can be produced in vitro by rapidly mixing solutions of calcium and phosphate; however, in the presence of an aqueous phase, the salt is unstable and transforms spontaneously into crystalline hydroxyapatite (Eanes *et al.*, 1965; Eanes and Posner, 1965). Recent studies using techniques such as X-ray diffraction, infrared spectroscopy and electron spin resonance have suggested that mineralized tissues such as bone and dentin may contain some of their mineral in an amorphous, i.e. non-crystalline form (Harper and Posner, 1966; Termine, 1966; Termine and Posner, 1966/1967). It has been suggested that amorphous calcium phosphate is the first mineral to deposit *in vivo* and that it later transforms to crystalline apatite (Termine *et al.*, 1967; Richelle, 1967).

In vitro, the speed of transformation of synthetic amorphous calcium phosphate into crystalline calcium phosphate depends on various factors which include pH, temperature and the Ca/P ratio of the solution (Eanes *et al.*, 1967). Under physiological conditions of pH and temperature, this transformation *in vitro* is very rapid and is completed in a few minutes. The question therefore arises of how it is that bone can maintain such a high proportion of its mineral in this apparently unstable form. Since the transformation does not occur in the dry state, relative dehydration may contribute to the relative stability of the salt in vivo. Ions such as Mg^{2+} (Bachra *et al.*, 1965) and carbonate (Bachra *et al.*, 1963) may also be involved in the stabilization since they inhibit the transformation *in vitro*.

The present study was designed to test whether inorganic pyrophosphate could also stabilize amorphous calcium phosphate. The rationale was as follows. Pyrophosphate is known to inhibit calcium phosphate precipitation *in vitro* probably by inhibiting crystal growth (Fleisch and Neuman, 1961; Fleisch *et al.*, 1966). The mechanism of conversion of non-crystalline to crystalline calcium phosphate is thought to involve dissolution of the amorphous salt followed by a crystallization of calcium phosphate from the overlying supersaturated solution (Eanes and Posner, 1965). Once some crystals of calcium phosphate form in this solution the conversion of the remainder of the amorphous salt follows rapidly, apparently by an autocatalytic mechanism. If this mechanism is correct, pyrophosphate should retard the conversion of the amorphous salt by inhibiting the crystallization of calcium phosphate in the overlying solution.

This study shows that inorganic pyrophosphate can inhibit the conversion of amorphous to crystalline calcium phosphate *in vitro*. This observation may be of physiological relevance since pyrophosphate is present in plasma (Fleisch and Bisaz, 1962), and in bone (Cartier, 1957; Perkins and Walker, 1957) and tooth (Bisaz, Russell and Fleisch, in press) mineral.

Materials

The reagents were all of analytical grade, generally from Merck, Darmstadt, Germany. $Na_4P_2O_7$ was from J. A. Benckiser, Ludwigshafen am Rhein, Germany, and the calcichrome was from British Drug Houses Ltd., Poole, England. The phosphatase was a highly purified

calf intestinal alkaline phosphatase from Boehringer, Mannheim, Germany. This enzyme is known to be a pyrophosphatase (FERNLEY and WALKER, 1966, 1967; RUSSELL *et al.*, 1967). ^{32}P-labelled tetrasodium pyrophosphate was obtained from the Radiochemical Centre, Amersham, England.

Methods

Amorphous calcium phosphate was prepared according to the method of EANES *et al.* (1965) by rapidly mixing 40 ml of 6.25 mM-$CaCl_2$. $2H_2O$ with 60 ml of 2.5 mM-$(NH_4)_2HPO_4$. Both solutions were adjusted before mixing to pH 10.5, 9.0 or 8.5 depending on the experiment. The preparation and subsequent incubation were carried out at room temperature (about 22°) or in a cold room at 4°. The mixtures were continuously shaken for up to 15 days and aliquots were withdrawn when required. The aliquots (5 ml) were filtered through Sartorius-Membranfilters M. F. 50 pore size 0.45—0.60 μm (Sartorius Membranfilter GmbH., Göttingen, Germany) in order to retain the precipitate. Calcium, phosphate and pH were measured in the filtrate. The precipitate on the filter was first washed with 2 N-NH_4OH and was then dissolved in 0.5 ml of 0.5 N-HCl and diluted to 2.5 ml with distilled water. Calcium and total phosphate (included pyrophosphate when added) were measured to determine the molar Ca/P ratio of the precipitated material. In some experiments, X-ray diffraction analysis was carried out on the precipitate, in which case the volumes of the original solutions were increased twentyfold.

In the experiments performed in the presence of pyrophosphate an aqueous solution of 0.805 mM-$Na_4P_2O_7$ was added drop by drop immediately after the calcium chloride and ammonium phosphate solutions had been mixed. The amount of pyrophosphate-P added in the various experiments ranged from 1.1 to 16.5% of the initial orthophosphate-P in the solid phase. The latter was determined by measuring the orthophosphate in the filtrate and subtracting it from the orthophosphate added initially as ammonium phosphate. In order not to change the original ratio of the liquid/solid phase, a volume of the suspension equal to the volume of the solution of pyrophosphate added was withdrawn; this was filtered, the filtrate discarded and the solid phase put back into the solution.

In the experiments with phosphatase the enzyme was added (1 mg/100 ml solution) immediately after the pyrophosphate. Since the pH of the solution decreased slightly after this addition it was readjusted with 2 N-NH_4OH to the initial value. The hydrolysis of pyrophosphate by the enzyme was checked in one experiment by measuring the hydrolysis of radioactive ^{32}P-pyrophosphate added instead of non-labelled pyrophosphate.

In order to measure the amount of pyrophosphate bound to the precipitate at different concentrations of pyrophosphate in solution, ^{32}P-labelled pyrophosphate diluted with carrier to a specific activity of 0.605 mC/mM was used. Equal amounts of such ^{32}P-labelled pyrophosphate were added successively to the initial amorphous suspension at 22°, at pH 10.5 and 9.0. After each addition the mixture was allowed to equilibrate for 10—15 minutes, then a sample was taken, filtered and the radioactivity measured in the filtrate. From this activity, both the amount of pyrophosphate free in the solution and the amount bound to the amorphous material could be calculated and the binding curve constructed.

Calcium was measured by titration with 5 mM-EDTA in an EEL titrator (Evans Electroselenium Limited, Halstead, Essex) with calcichrome (cyclo-tris-7-(1-azo-8-hydroxy-naphthalene-3.6-disulphonic acid, British Drug Houses Ltd.) as indicator. Phosphate was determined spectrophotometrically as phosphomolybdate by a modification of the technique of CHEN *et al.* (1956), in which HCl was substituted for H_2SO_4 and in which color development was carried out for 10 minutes at 100°; solutions containing pyrophosphate were first hydrolyzed for 30 minutes at 100° in 0.5 N-HCl.

The amount of hydrolysis of the ^{32}P-labelled pyrophosphate bound on the precipitate to ^{32}P-orthophosphate was measured by a method adapted from that of HALL (1963), in which ^{32}P present as orthophosphate was separated from that present as pyrophosphate by extraction as phosphomolybdic acid into a mixture of isobutanol and petroleum ether (4:1 V/V).

pH was measured on a Methrom (Herisau, Switzerland) Compensator E 388 pH-Meter. Radioactivity due to ^{32}P was measured in a windowless Methane gas-flow counter (FH 407, Frieseke and Hoepfner, Erlangen-Bruck, Germany). X-ray diffraction analysis was carried

out on a Siemens scintillation counter recording diffractometer, using conditions and techniques described elsewhere (EANES *et al.*, 1965; HARPER and POSNER, 1966).

Results

The first experiments were performed at pH 10.5 and room temperature as described by EANES *et al.* (1965). The transformation was followed by a fall in calcium concentration in the solution (see e.g. Fig. 1). The concentration of phosphate increased in the solution during the transformation but the pH changed

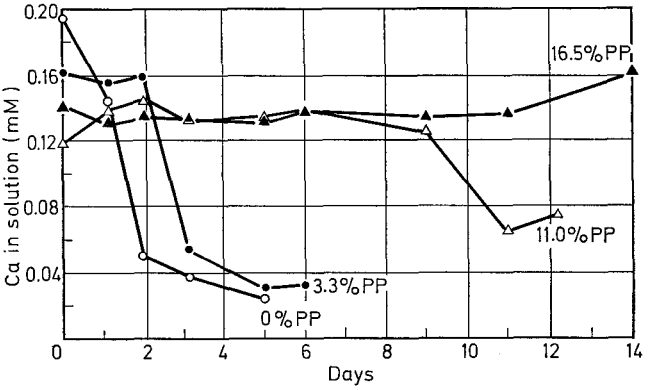

Fig. 1. Effect of inorganic pyrophosphate on the transformation of amorphous into crystalline calcium phosphate at pH 10.5 and 22° C as represented by the change in calcium concentration in the solution. The various amounts of pyrophosphate-P added (as % of the initial orthophosphate-P of the solid phase) are given at the end of each curve

only little. At room temperature the transformation was found in 4 experiments to start between 7 and 25 hours after mixing the reagents (Table). This is in agreement with the findings of EANES *et al.* (1965). As shown in Fig. 1 and Table, addition of sufficient pyrophosphate to bring the amount of pyrophosphate-P on the precipitate to 3.3, 11 and 16.5% of the initial orthophosphate-P of the precipitate delayed the onset of transformation until 2—3 days, 8—9 days and more than 16 days respectively. Further evidence for the inhibition of the transformation by pyrophosphate was seen in the changes in the molar Ca/P ratio of the precipitate (Fig. 2). Soon after mixing this ratio was 1.46, a value which is in agreement with those found by EANES *et al.* (1965). After 3 days in the presence of 11.3% pyrophosphate it remained low at 1.39, whereas in the absence of pyrophosphate it had increased to 1.70 indicating that in this case the transformation had taken place (Fig. 2). That the changes in the calcium of the solution and the Ca/P ratio of the solid really represented the crystallization of the amorphous material was confirmed by examining the X-ray diffraction patterns of the solid. As shown in Fig. 2, one hour after preparation the precipitate was non-crystalline both in the presence and absence of 11.3% pyrophosphate. However, after 3 days the X-ray diffraction pattern indicated that crystallization had occurred only in the absence of pyrophosphate. Thus, several independent methods for measuring the conversion of amorphous calcium phosphate to its crystalline form all indicate that pyrophosphate inhibits the process.

Table. *Times needed for transformation*[a] *of amorphous to crystalline calcium phosphate under various conditions*

pH of initial solutions	pH after mixing	Temperature	%PP added	P'ase added (μg/ml)	Transformation time[a]	%PP Hydrolysis
10.5	—	room[c]	0	—	11—25 hr	—
10.5	—	room	0	—	7—20 hr	—
10.5	—	room	0	—	10—24 hr	—
10.5	10.28	room	0	—	16—24 hr	—
10.5	10.32	room	3.3	—	48—72 hr	10% at 6 days
10.5	10.19	room	11	—	8—9 days	8% at 12 days
10.5	10.38	room	16.5	—	>16 days	5% at 16 days
—	—	—	—	—	—	—
9.0	8.73	room	0	—	0—15 hr	—
9.0	8.52	room	0	—	7—8 hr	—
9.0	8.67	room	0	—	7—8 hr	—
9.0	8.56	room	0	—	4—5 hr	—
9.0	8.70	room	0	—	6—7 hr	—
9.0	8.64	room	0	—	10—24 hr	—
9.0	8.72	room	1.1	—	0—15 hr	—
9.0	8.64	room	5	—	2—4 days	—
9.0	8.77	room	5.3	—	24—39 hr	—
9.0	8.74	room	10	—	> 50 hr	—
9.0	8.61	room	10	—	2—5 days	19% at 5 days
9.0	8.72	room	10	—	>22 hr	—
9.0	8.64	room	10	—	11—12 days	—
9.0	8.71	room	10.5	—	5—6 days	—
9.0	8.36	room	0	5	2—3 hr	—
9.0	8.70[b]	room	0	5	6—7 hr	—
9.0	8.70[b]	room	10	5	8—22 hr	57% at 22 hr
9.0	8.27	room	0	10	2—3 hr	—
9.0	8.67[b]	room	0	10	6—7 hr	—
9.0	8.74[b]	room	10	10	20—26 hr	—
9.0	8.61[b]	room	10	10	16—25 hr	70% at 25 hr
8.5	7.83	room	0	—	1—1½ hr	—
8.5	7.55	4°	0	—	14—24 hr	—
8.5	7.76	4°	0	—	5—8 hr	—
8.5	7.47	4°	0	—	> 4 hr	—
8.5	7.65	4°	1	—	14—24 hr	—
8.5	7.70	4°	5	—	3—4 days	—
8.5	7.70	4°	10	—	13—14 days	—

[a] Transformation time is defined as the time when there was a detectable fall of calcium in the solution.

[b] The pH of these solutions was adjusted after the addition of phosphatase to bring the pH to the same value as that of the control solution.

[c] About 22°C.

Similar experiments were performed at lower pH and at a lower temperature (Table). In agreement with previous results (EANES *et al.*, 1967), the transformation was faster the lower the pH and the higher the temperature. The Table shows that the inhibitory effect of pyrophosphate on the transformation was present under all conditions tested.

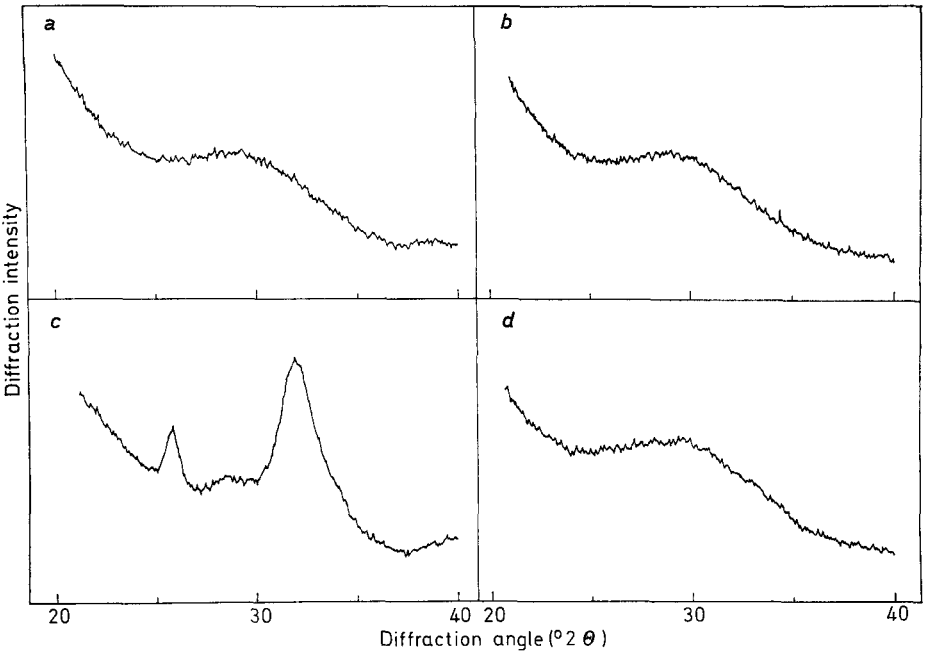

Fig. 2a—d. X-ray diffraction pattern (Copper Kα radiation) of precipitate obtained at pH 10.5 and 4° C. a 1 hour after mixing without added PP_i (Ca in filtrate, 0.15 mM; molar Ca/P ratio of precipitate, 1.46); b 1 hour after mixing in presence of added PP_i (11.3% of the initial orthophosphate-P of the solid phase) (Ca in filtrate, 0.12 mM; Ca/P ratio of precipitate, 1.44); c after 3 days without PP_i (Ca in filtrate, 0.01 mM; Ca/P ratio of precipitate, 1.70); d after 3 days with PP_i (11.3% of the initial orthophosphate-P of the solid phase) (Ca in filtrate, 0.11 mM; Ca/P ratio of precipitate, 1.39)

The amounts of pyrophosphate used to produce inhibition in these experiments were rather large and accounted for several % of the initial orthophosphate in the solid phase (Table). In view of our original hypothesis that pyrophosphate might be effective because it could prevent formation of calcium phosphate crystals in the overlying solution it was of interest to know how much of the added pyrophosphate remained in the solution. Fig. 3 shows the binding curves for the adsorption of pyrophosphate onto amorphous calcium phosphate. Most of the pyrophosphate added to the system was adsorbed by the precipitate and only very small amounts remained free in the solution. The ability of the amorphous material to bind pyrophosphate was very great and was only a little less at pH 9.0 than at pH 10.5.

Since apatite crystals (KRANE and GLIMCHER, 1962; FLEISCH *et al.*, 1966) and amorphous calcium phosphate (BURLEY, 1965) are known to be able to hydrolyze P-O-P bonds, the amount of the added pyrophosphate hydrolyzed to orthophosphate during the incubation was measured as described in methods. The results obtained (see Table) show that at room temperature the hydrolysis did not exceed 20% under the conditions used.

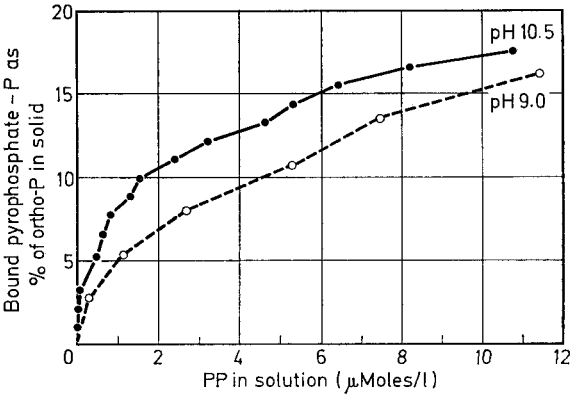

Fig. 3. Binding of added pyrophosphate onto amorphous calcium phosphate at 22° C at pH 10.5 and 9.0. Each point was determined after 10—15 minutes equilibration. The solid phase contains 144 μMoles orthophosphate at pH 10.5 and 119 μMoles orthophosphate at pH 9.0 in a volume of 100 ml

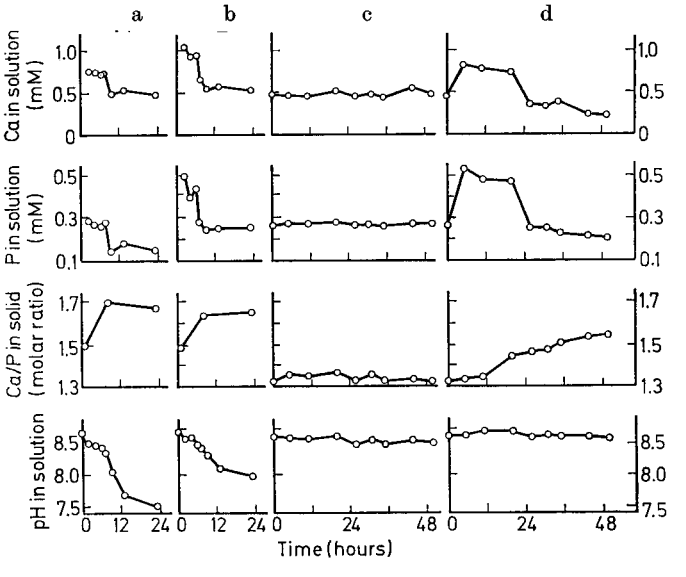

Fig. 4a—d. Influence of alkaline phosphatase on the transformation of amorphous into crystalline calcium phosphate at pH 9.0 and 22° C. a Without pyrophosphate or phosphatase; b without pyrophosphate but with 10 μg phosphatase/ml; c with pyrophosphate (10% of the initial orthophosphate-P of the solid phase) but no phosphatase; d with pyrophosphate (10%) and 10 μg phosphatase/ml

In order to see whether the inhibition of transformation could be relieved by removing the pyrophosphate some experiments were performed in which a highly purified alkaline phosphatase was added. This enzyme had previously been shown to also be a pyrophosphatase (Fernley and Walker, 1966, 1967; Russell *et al.*, 1967). The pH chosen for the original solutions of calcium chloride

and ammonium phosphate was 9.0 since after mixing this gave a pH of about 8.6 at which the pyrophosphatase activity of the enzyme was close to its maximum. Fig. 4 and the Table show that the phosphatase was able to relieve the inhibition. For instance incubation of a precipitate containing 10.5% of its initial phosphorus as pyrophosphate with phosphatase shortened the transformation time from 5 to 6 days to about 16—26 hours. The hydrolysis of the pyrophosphate in the presence of 5 μg/ml of enzyme was 57% after 22 hours, with 10 μg/ml of enzyme 70% after

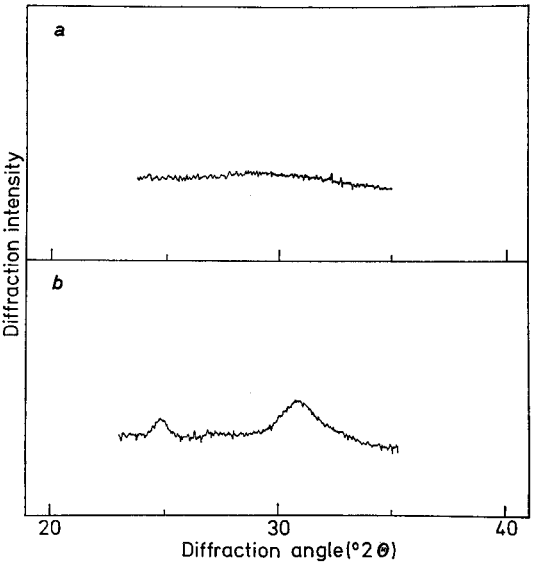

Fig. 5a and b. Influence of alkaline phosphatase (10 μg/ml) on x-ray diffraction patterns (Copper Kα radiation) of precipitate obtained at pH 9.0 and 22° C after 50 hours in presence of pyrophosphate (10% of the initial orthophosphate-P of the solid phase).
a Without phosphatase; b with phosphatase

25 hours. The addition of the enzyme to amorphous material that did not contain pyrophosphate had no effect on the time of transformation, but did cause some solubilization of the amorphous salt. X-ray diffraction data (Fig. 5) from the phosphatase experiments again confirmed that the changes seen in calcium, phosphate and pH in the filtrate and in the Ca/P ratio of the solid truly represented a transformation of amorphous to crystalline calcium phosphate. Thus, X-ray diffraction analysis of the precipitate containing 10% pyrophosphate showed that only amorphous material was present 50 hours after the start of the experiment; in the same material treated with phosphatase crystallization had occurred at this time. Alkaline phosphatase is therefore able to destroy the inhibitory action of pyrophosphate on the transformation of amorphous to crystalline calcium phosphate.

Discussion

The results show that pyrophosphate is able to inhibit the transformation of amorphous calcium phosphate into its crystalline form *in vitro*. In these experiments we have only studied the effects of pyrophosphate added after the calcium

and phosphate have been allowed to precipitate. We have not yet studied the effects of pyrophosphate added before mixing calcium and phosphate and therefore coprecipitated with the amorphous material. The reason for adding pyrophosphate after precipitation was based on the hypothesis that *in vivo* the amorphous material might be in contact with extracellular fluid serving as a continual source of pyrophosphate. The possible importance of having a continual supply of pyrophosphate is that the pyrophosphate on the amorphous material spontaneously hydrolyses (Table). Pyrophosphate trapped within the interior of such material might therefore be expected to be destroyed with time.

There are several possible mechanisms for the inhibitory action of pyrophosphate. The binding studies indicate that pyrophosphate added to the system is present both on the precipitated amorphous material and in the solution, albeit at much lower concentration. The inhibition of the transformation could be brought about by pyrophosphate at either or both of these sites.

The pyrophosphate on the amorphous material might retard the transformation by reducing the rate of dissolution of the amorphous material, in a manner similar to that described for apatite crystals *in vitro* (FLEISCH *et al.*, 1966). The entry of calcium into the solution and the tendency to form calcium phosphate crystals in the solution would thus be less. In support of this possibility the calcium concentration in the solution overlying the amorphous calcium phosphate was lower in the presence of pyrophosphate than in its absence (Fig. 1 initial values and Fig. 4 columns c and d compared with a and b).

An alternative explanation of the inhibition of transformation is that it is due to the pyrophosphate in the solution, which would prevent the appearance of the crystals of calcium phosphate thought to be necessary to initiate the conversion process. This possibility is strengthened by the observation that the pyrophosphate began to inhibit the transformation when the solid amorphous phase contained about 3—5% of its orthophosphate-P as pyrophosphate-P. Under the defined conditions used in these experiments, the concentration of pyrophosphate remaining in the solution was then about 0.3 μM. This concentration is in the same range as is required to inhibit *in vitro* precipitation of calcium phosphate crystals from solution (FLEISCH, 1964). From the physiological point of view, it is perhaps significant that these concentrations are also within the same range as those in plasma (FLEISCH, 1964).

The inhibition of transformation by pyrophosphate could be of physiological importance and pyrophosphate might be one of the factors that stabilizes amorphous calcium phosphate in mineralized tissues *in vivo*. This possibility is strengthened by the observation of RICHELLE (personal communication) that when bone is separated into fractions of different specific gravity the lightest fraction, which contains the greatest percentage of amorphous calcium phosphate, is rich in pyrophosphate. In this lightest fraction pyrophosphate may constitute up to 20% of the total P and this amount is of the same order as those used in the present experiments.

Under physiological conditions the regulation of the transformation by pyrophosphate could be under enzymatic control. This might provide a new role for bone alkaline phosphatase, namely that of destroying pyrophosphate and hence allowing the amorphous phase to crystallize. The possibility that bone alkaline

phosphatase can act as a pyrophosphatase has gained strength recently with the demonstration that not only bacterial alkaline phosphatases (Heppel *et al.*, 1962) but also mammalian alkaline phosphatases (Eaton and Moss, 1967; Moss *et al.*, 1967; Fernley and Walker, 1966, 1967; Russell *et al.*, 1967) can function as pyrophosphatases. Current work in our laboratory has shown that bone alkaline phosphatase is also probably a pyrophosphatase (Russell *et al.*, 1967).

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