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Influence of Pyrophosphate on the Exchange of Calcium and Phosphate Ions on Hydroxyapatite

A. Jung, S. Bisaz, P. Bartholdi, and H. Fleisch

Pathophysiological Institute, University of Berne, Observatory, University of Genua,
and Laboratory for Experimental Surgery, Davos

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The effect of pyrophosphate on the distribution and the rates of exchange of calcium and phosphate in hydroxyapatite crystals was studied. Analysis of disappearance of ^{45}Ca, ^{32}P [P_i] and ^{32}P [PP_i] from the solution onto the crystals was performed using a compartmental model. The disappearance curves were described by the sum of 4 exponentials, which was interpreted as a four-compartment model in series. Pyrophosphate was present in all the four pools of the crystals. Treatment of crystal with pyrophosphate led to a large decrease of exchangeable phosphate, and two of the four compartments disappeared completely. The quantitative data suggest a replacement of 2 moles of orthophosphate by 1 mole of pyrophosphate. Pyrophosphate had no appreciable effect on the distribution and the fluxes of calcium. The implications of these results for the inhibitory effects of pyrophosphate on the formation and dissolution of apatite crystals are discussed.

Key words: Pyrophosphate — Calcium — Phosphate — Kinetics — Hydroxyapatite.

L'effet du pyrophosphate sur la distribution et les vitesses d'échange du calcium et du phosphate dans les cristaux d'hydroxyapatite a été étudié. Les analyses de disparition du ^{45}Ca, du ^{32}P [P_i] et du ^{32}P [PP_i] de la solution vers les cristaux ont été réalisées en utilisant un modèle de compartimenst. Les courbes de disparition sont formées par la somme de 4 exponentielles, correspondant à un modèle de 4 compartiments en séries. Le pyrophosphate est présent dans les 4 compartiments du cristal. Le traitement du cristal avec le pyrophosphate provoque une diminution nette du phosphate échangeable et deux des quatre compartiments disparaissent totalement. Les données quantitatives suggèrent une substitution de 2 moles d'orthophosphate par mole de pyrophosphate. Le pyrophosphate n'a pas d'effet net sur la distribution et les échanges de calcium. Les conséquences de ces résultats sur les effets d'inhibition du pyrophosphate sur la formation et la dissolution des cristaux d'apatite sont discutées.

Die Wirkung von Pyrophosphat auf die Verteilung und die Austauschrate von Calcium und Phosphat in Hydroxyapatitkristallen wurde untersucht. Das Verschwinden von ^{45}Ca, ^{32}P [P_i] und ^{32}P [PP_i] aus der Lösung und die Anlagerung dieser Ionen auf den Kristallen wurde anhand eines Kompartiment-Modelles studiert. Die Verschwinde-Kurven wurden durch die Summe von 4 Exponentiellen beschrieben und mittels einem Modell von 4 Kompartimenten in Serie interpretiert. Wurde der Lösung Pyrophosphat zugesetzt, so war es in allen 4 Kompartimenten vorhanden. Ferner entstand eine starke Verminderung des austauschbaren Phosphates, wobei 2 der 4 Kompartimente vollständig verschwanden und 2 Mole Orthophosphat mit 1 Mol Pyrophosphat ausgetauscht wurden. Pyrophosphat hatte keine nennenswerte Wirkung auf die Verteilung und die Flüsse des Calciums. Diese Befunde werden im Zusammenhang mit der Hemmwirkung des Pyrophosphates bei der Bildung und Auflösung von Apatitkristallen besprochen.

For reprints: Prof. H. Fleisch, Pathophysiological Institute, University of Berne, Hügelweg 2, CH-3012 Berne, Switzerland.

Introduction

Inorganic pyrophosphate (PP_i) is present in calcified tissues such as bone (Perkins and Walker, 1958; Cartier, 1959) and teeth (Bisaz *et al.*, 1968) and also in body fluids such as blood (Russell *et al.*, 1971), urine (Fleisch and Bisaz, 1962) and saliva (Hausmann *et al.*, 1970). It has been suggested that PP_i has a role in preventing soft tissue calcification (Fleisch and Russell, 1970) and in regulating both formation and dissolution of bone and tooth mineral (Fleisch and Russell, 1970; Fleisch *et al.*, 1966b). Indeed, PP_i has been shown to inhibit ectopic calcification induced by various means in animals (Schibler and Fleisch, 1966; Schibler *et al.*, 1968; Gabbiani *et al.*, 1966) and to reduce bone resorption in rats (Rasmussen *et al.*, 1970). In patients with hypophosphatasia, a disease characterised by an inborn lack of alkaline phosphatase and by rachitic and osteomalacic lesions, plasma PP_i is markedly increased (Russell *et al.*, 1971). Since PP_i has been found to bind strongly to hydroxyapatite crystals (Krane and Glimcher, 1962; Burton *et al.*, 1969; Jung *et al.*, 1973) and to inhibit both their formation (Fleisch and Neuman, 1961) and dissolution (Fleisch *et al.*, 1966a) *in vitro*, the mechanism responsible for these effects of PP_i *in vivo* is thought to be due to actions on apatite crystals. In order to gain a better understanding of the site of deposition and the effects of PP_i on apatite crystals, we have now made a kinetic study with ^{45}Ca, ^{32}P [P_i] and ^{32}P [PP_i]. Neuman and Neuman (1958) and later Pak and Bartter (1967) have shown that the surface of apatite crystal exchanges ions with the surrounding solution. These exchange processes have been described by multiexponential curves and a compartment analysis of the crystals can be made (Neuman and Neuman, 1958; Pak and Bartter, 1967; Avnimelech, 1968). We have analysed our results using such models and have examined the effect of PP_i on the size of the various ion compartments within apatite crystals and on the rates of exchange of ions between them.

Material and Methods

Materials. The hydroxyapatite crystals have been shown to have a molar calcium:phosphate ratio of 1.64, an average length in the electron microscope of about 1000 Å and an X-ray diffraction pattern as well as an infrared absorption curve characteristic of well crystallised hydroxyapatite (Fig. 1). No other X-ray diffraction pattern appeared after heating for 15 h at 900°. After heating at 500° for 15 h, a procedure which produces pyrophosphate from calcium deficient apatites (Dallemagne, 1964), the crystals converted only 0.43% of their phosphate into pyrophosphate. Thus, the crystals were predominantly a non-calcium deficient hydroxyapatite and contained no significant amounts of octocalcium phosphate or tri-calcium phosphate hydrate. The surface of the apatite measured by BET nitrogen absorption was 55 m^2/g.

All chemical reagents were obtained from E. Merck AG, Darmstadt, Germany. The carrier-free ^{32}P-orthophosphate (^{32}P [P_i]) and the ^{45}Ca (as $CaCl_2$ with a specific activity between 0.8 and 2.4 mC_i/mmole) were obtained from the Eidg. Institut für Reaktorforschung, Würenlingen, Switzerland. ^{32}P-pyrophosphate (^{32}P [PP_i]) was obtained as the tetrasodium salt from the Radiochemical Centre, Amersham, England and its specific activity was 160 mC_i/mmole.

Disappearance Curves of ^{45}Ca, ^{32}P [P_i] and ^{32}P [PP_i]. In order to obtain a solution equilibrated with apatite, 1.5 g of apatite were stirred 3 days at 4° on a magnetic stirrer in 1 l of a buffered solution of ionic strength of 0.16 containing 0.155 M KCl, 0.01 M diethylbarbituric acid and adjusted to pH 7.4 with KOH. Thereafter the suspension was filtered

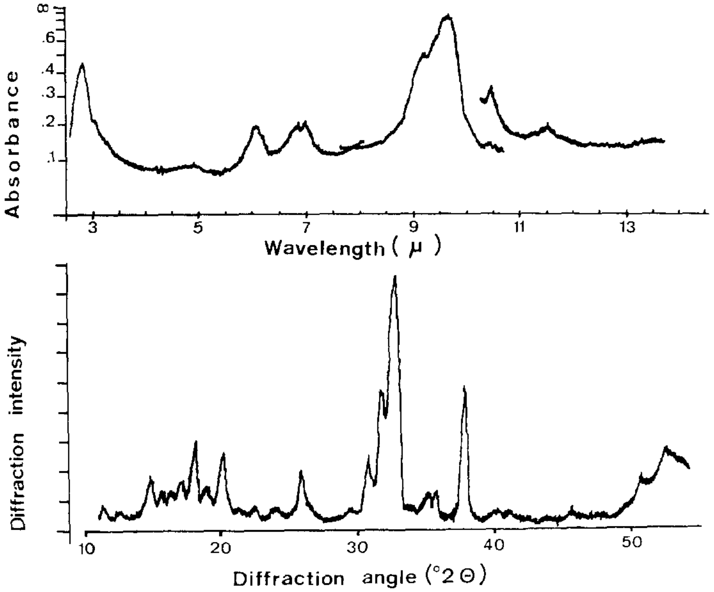

Fig. 1. Characterisation of the apatite by above: infrared spectroscopy (Perkin Elmer, model 221, with an NaCl prism); below: X-ray diffraction (apparatus from Philips)

through a "Membranfilter Sartorius" (pore size 1–1.2 mµ) and filtrate retained; this solution was named solution E (equilibrated).

The kinetic study with ^{45}Ca and ^{32}P [P$_i$] was performed at 4°. Four identical suspensions, each containing 150 mg of fresh apatite in 90 ml of solution E, were made up and equilibrated for 2 h. 1.1 µC$_i$ of ^{45}Ca and 0.8 µC$_i$ of ^{32}P [P$_i$] dissolved in 10 ml of solution E were then quickly added to the suspension. The addition lasted less than 1 sec, and the rate of stirring was so rapid that the mixing was shown to be complete within 5 sec. The final amount of apatite was thus 150 mg/100 ml. After this addition, which corresponds to time zero in the curve, 4 ml of suspension were taken at intervals and immediately filtered by vacuum suction on a "Membranfilter Sartorius". This filtration lasted between 3 and 4 sec, so that the sampling could be repeated every 30 sec for the same suspension. By displacing and pooling the results of the 4 experiments, it was possible to obtain values every 10 sec. This was done up to 1.5 min; afterwards the 4 suspensions were sampled simultaneously at the intervals shown in the figures. Calcium, phosphate, ^{45}Ca and ^{32}P [P$_i$] were measured in the filtrate.

For the pyrophosphate treated apatite (apatite-PP$_i$) another procedure was necessary, since apatite-PP$_i$ has a different solubility from non-treated apatite (Fleisch *et al.*, 1966a). In this case, 150 mg of fresh apatite were suspended in 50 ml of solution E on a magnetic stirrer. Fifty millilitres of solution E containing 22.6 µmoles of PP$_i$ and adjusted to pH 7.4 were then infused into this suspension over 2 h. After 24 h of equilibration, the suspension was filtered and the apatite-PP$_i$ retained. 150 mg of this apatite-PP$_i$ was added to 100 ml of non-equilibrated buffer and stirred for 2 days. The filtrate from this suspension, now equilibrated with apatite-PP$_i$, was termed solution E-PP$_i$.

For the exchanges with ^{45}Ca, ^{32}P [P$_i$] and ^{32}P [PP$_i$], 150 mg of apatite-PP$_i$ was suspended in 90 ml of solution E-PP$_i$ and 4 identical suspensions prepared as previously. Two hours later, ^{45}Ca and ^{32}P [P$_i$] or ^{32}P [PP$_i$], in which case 3 µC$_i$ were used, were added and sampled as discribed previously. Calcium and phosphate, ^{45}Ca, ^{32}P[P$_i$] and ^{32}[PP$_i$], respectively, were measured in the filtrate. Furthermore, in the experiment with ^{32}P [PP$_i$] the amount of non-radioactive PP$_i$ in the solution was analysed before, 12 h after and 36 h after addition of ^{32}P [PP$_i$] to ensure that a steady state was maintained.

Chemical and Radiochemical Measurements. The radioactivity was determined in a liquid scintillation counter Tricarb Packard no. 3375 in a mixture composed of toluene (600 ml), naphthalene (80 g), methyl-cellosolve (400 ml) and butyl-PBD (7 g) (2-(4-t-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazol; Ciba Aktiengesellschaft, Basel, Switzerland) as scintillator. In the exchange experiments, ^{45}Ca and ^{32}P [P_i] were determined in the presence of each other with appropriate corrections. ^{32}P [PP_i] was counted with scintillator, or in 0.5 N HCl without scintillator (using Čerenkow radiation). Not fewer than 2000 counts were collected for each determination, which results in a minimum precision of 2.5%. In order to correct for the hydrolysis of PP_i in P_i during the exchange, P_i was extracted as its phosphomolybdic complex into isobutanol-petroleum ether and radioactivity counted both in the organic and aqueous phases (Hall, 1963). The chemical determination of total phosphate, i.e. P_i plus PP_i, was made after hydrolysis of PP_i by boiling for 30 min in 0.5 N HCl and measuring P_i according to a modification (Bisaz *et al.*, 1968) of the technique of Chen *et al.* (1956). P_i initially present was then given by the difference between total phosphate and PP_i measured radiochemically. Cold PP_i was determined by an isotopic dilution procedure consisting in a separation from P_i on a Dowex anion exchanger and spectrophotometric determination as P_i after hydrolysis in HCl 0.5 N for 30 min at 100° (Russell *et al.*, 1971). Calcium was determined in presence of 1% $LaCl_3$ on an atomic absorption spectrophotometer Perkin-Elmer 290B.

Determination of Exchangeable Ca and P_i at Various pH Values and for Various Amounts of PP_i Bound on the Apatite. In order to determine exchangeable Ca and P_i when pH and the amount of bound PP_i vary, a simplified technique was used. This consisted of measuring the specific activity of ^{45}Ca and ^{32}P [P_i] 30 h after addition of the tracers. Both filtrates and the crystals (dissolved in HCl 0.5 N) were measured. The exchangeable fraction was calculated by dividing the specific activity of the crystal by the specific activity of the solution. This experiment was done at pH 7.0, 7.4 and 7.8 for amounts of added PP_i varying from 0 to 150 μmoles/g of apatite.

Ultracentrifugation. In order to determine whether part of the Ca, P_i and PP_i bound onto the apatite-PP_i crystals was removable by ultracentrifugation, apatite and apatite-PP_i were stirred for 24 h with respectively ^{45}Ca, ^{32}P [P_i] or ^{32}P [PP_i] and centrifuged according to a modification of the technique of Neuman *et al.* (1953).

The addition of isotopes was made either directly to the suspension in which the binding of the PP_i to the apatite had been performed, or after resuspension of apatite-PP_i crystals in solution E-PP_i. Ten millilitres of suspension were then filtered on a "Membranfilter Sartorius". This filter was placed on dry absorbant paper at the bottom of a centrifuge tube. Centrifugation was performed for 30 min at 4° in a Spinco-Beckman L_2 (swing-out head SH 25) at 10000 g or 80000 g. The specific activity of ^{45}Ca, ^{32}P [P_i] and ^{32}P [PP_i] was determined on the crystals before and after the centrifugations. Since it is assumed that the isotopes are located in the external layers of the crystals, a removal by ultracentrifugation should lead to a decrease of the specific activity.

Mathematical Analysis of the Disappearance Curves. In each experiment, the percentage of the initial radioactivity remaining in the solution was plotted on semilogarithmic paper as a function of time. Since the results obtained on the 4 parallel studies were very similar, all the points obtained have been grouped in one curve. In order to determine whether a compartment system can be used as a model for the disappearance of Ca, P_i and PP_i, it was necessary to find out whether these curves could be described by a sum of exponentials. The number of exponentials would equal the number of compartments to be analysed over this time period. For this purpose, we have used the digital computer program SAAM 23 of Berman (Berman *et al.*, 1962a, b; Berman, 1965) adapted for the computer CDC 3800. Taking into account all values between 30 sec and 30 h, we found that 4 exponentials would completely describe the data, while three would give a significant systematic deviation. A fifth exponential, while improving slightly the residuals, would not give any significant improvement using a Fisher test. The data between 0 and 30 sec where further fitted by hand on semilog paper (Fig. 3).

To determine the rate constants for ion transfer between the compartments, a model of open compartments in series was chosen (Fig. 2) on the basis that this may give the best representation of the successive crystal layers. The various rate constants were determined

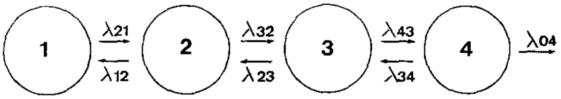

Fig. 2. An open 4-compartment model representing the various pools in the solution (Compartment 1, includes also a fast crystal component) and the crystals (Compartments 2–4). The rate constants λ (time^{-1}) are shown for the various fluxes

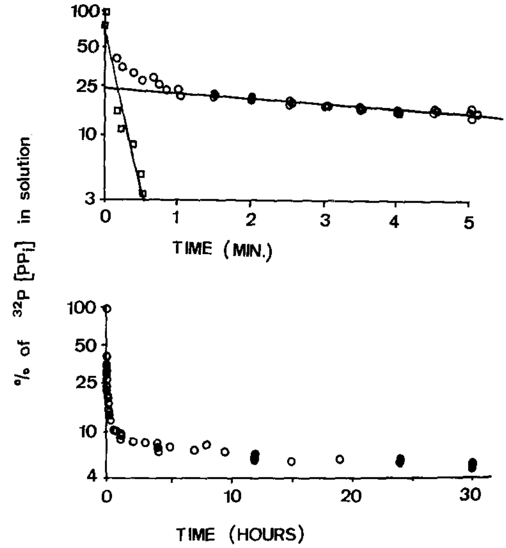

Fig. 3. Above: Disappearance within the first 5 min at 4° and pH 7.4 from solution of ^{32}P [PP$_i$] added to a suspension of apatite-PP$_i$ (150 mg apatite/100 ml solution; 22.6 μmoles PP$_i$ added). The values are expressed as % of the initial ^{32}P [PP$_i$] in the solution (time 0). □ represent the values obtained by peeling, that is by subtracting from the experimental values the values which would lie on a line extrapolated from the points between 1 and 5 min. It is shown that the points in the first minute are practically on a straight line. Below: Disappearance from the solution of ^{32}P [PP$_i$] between 0 and 30 h expressed as % of the initial ^{32}P [PP$_i$] in the solution

by the SAAM program which calculates curves of best fit by an iterative process. The use of first order kinetics for tracer experiments is justifiable under steady state conditions even if the processes themselves do not follow first order kinetics (Robertson, 1957). In fact, the formation and dissolution processes in crystals are not first order processes (Nielsen, 1964), but radioactive ion exchanges in a steady state probably are.

The sizes of the compartment pools were calculated relative to the size of the solution pool, which was determined by measuring the concentration of Ca, P$_i$ and PP$_i$ in the solutions E and E-PP$_i$. Since the points between 0 and 30 sec were practically on a straight line after peeling (Riggs, 1963; Fig. 3), we have assumed that only one compartment is exchanging with the solution in this time period and that the radioactivity which disappeared within the first 30 sec is distributed between this compartment and the solution. The number of experimental points in this time period was not sufficient to allow a precise calculation of the rate constants of exchange between the solution and this fast exchanging crystal compartment. Estimates of these rate constants indicated that they were about 10 times greater than the rate constants of exchange between this compartment and the next succes-

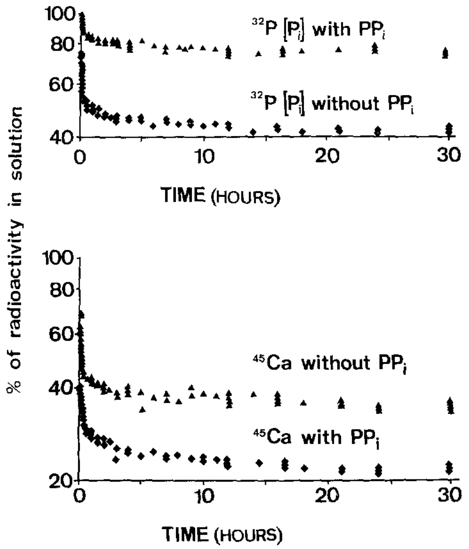

Fig. 4. Above: Disappearance from the solution of ^{32}P [P$_i$] added to a suspension of apatite (Experiment 3) or apatite-PP$_i$ (Experiment 5). Results expressed as % of the initial ^{32}P [P$_i$] in the solution. Below: Disappearance from the solution of ^{45}Ca expressed as % of the initial ^{45}Ca in the solution in the same experiments. Same experimental conditions as in Fig. 3

sive one. Therefore, without introducing a large error, the size of this rapidly exchanging compartment could be calculated knowing the percentage of the initial radioactivity in the solution after 30 sec and the size of the solution pool. In all the further calculations, these two compartments (solution and rapidly exchangeable pool) were treated as a single homogenous compartment. Since we know the rate constants, the sizes of the various other compartments in the series were calculated with the assumption that steady state conditions apply, i.e. the fluxes are equal in both direction.

Results

1. Disappearance Curves

Fig. 4 shows the disappearance of ^{32}P [P$_i$] from the solution into the crystal. The curve decreased further in non-treated apatite than in apatite-PP$_i$. Thus, whereas only 40% of the initial radioactivity remained in the solution in equilibrium with the apatite alone after 30 h, 75% was still present at this time in the case of apatite-PP$_i$. Fig. 4 shows also that for ^{45}Ca the difference was small, and, in contrast to ^{32}P [P$_i$], the curve decreased slightly more for the apatite-PP$_i$ than for the apatite alone. Fig. 3 shows that the major part of the ^{32}P [PP$_i$] disappears from the solution and that only 5% of the initial ^{32}P [PP$_i$] was present after 30 h. In all cases, the curves were similar when the experiments were repeated under the same conditions.

The analysis of the disappearance curves fitting the points between 30 seconds and 30 h by means of the SAAM program could be described by a sum of exponentials of the following type:

$$q/q_0 = A_i e^{-g_1 t} + A_2 e^{-g_2 t} + A_3 e^{-g_3 t} + A_4 e^{-g_4 t},$$

Table 1. Characteristics of the equation $q/q_0 = A_1e^{-g_1t} + A_2e^{-g_2t} + A_3e^{-g_3t} + A_4e^{-g_4t}$ calculated from the disappearance of ^{45}Ca, ^{32}P[P_i] and ^{32}P[PP_i] from the solution. All results expressed as fraction (q/q_0) of the initial dose (q_0) remaining in solution at time t (in min), between 30 sec and 30 h ±SD

	A_1	A_2	A_3	A_4	g_1	g_2	g_3	g_4
Ca (apatite)								
Experiment 1	17.1	10.8	5.3	36.6	0.93	0.100	0.0054	0.000072
(pH~7.6)	±1.6	±1.8	±0.9	±0.6	±0.14	±0.028	±0.0027	±0.000013
Experiment 2	17.2	11.9	7.2	32.7	1.41	0.143	0.0093	0.000058
(pH~7.6)	±0.1	±1.0	±0.6	±0.4	±0.21	±0.023	±0.0017	±0.000010
Experiment 3	12.4	9.7	7.4	38.0	1.35	0.171	0.0163	0.000074
(pH~7.4)	±2.8	±2.1	±1.4	±0.4	±0.60	±0.072	±0.0050	±0.000011
Ca (apatite-PP_i)								
Experiment 4	16.5	9.8	6.0	20.9	1.46	0.140	0.0092	0.000035
(pH~7.4)	±1.0	±0.8	±0.2	±0.3	±0.17	±0.018	±0.0016	±0.000012
Experiment 5	15.8	12.0	6.8	24.6	1.53	0.187	0.0102	0.000086
(pH~7.4)	±2.9	±1.3	±0.5	±0.3	±0.41	±0.032	±0.0015	±0.000009
P_i (apatite)								
Experiment 1	17.1	4.5	6.6	42.7	0.40	0.027	0.0063	0.000028
(pH~7.6)	±0.2	±3.5	±2.8	±1.1	±0.07	±0.020	±0.0042	±0.000019
Experiment 2	17.6	10.2	6.6	37.6	1.20	0.072	0.0022	0.000010
(pH~7.6)	±1.1	±0.5	±1.8	±2.0	±0.13	±0.010	±0.0009	±0.000009
Experiment 3	13.8	8.7	6.2	43.3	0.68	0.062	0.0029	0.000030
(pH~7.4)	±1.6	±1.4	±1.8	±2.2	±0.21	±0.023	±0.0016	±0.000030
P_i (apatite-PP_i)								
Experiment 4	5.1	73.5			0.28	0.000037		
(pH~7.4)	±0.7	±0.4			±0.08	±0.000006		
Experiment 5	9.3	80.6			0.04	0.000053		
(pH~7.4)	±0.4	±0.3			±0.01	±0.000004		
PP_i	14.9	5.8	2.5	6.9	0.51	0.038	0.0038	0.000170
Experiment 6	±0.7	±0.7	±0.6	±0.8	±0.05	±0.010	±0.0025	±0.000070
(pH~7.4)								

where q/q_0 is the fraction of the initial dose of radioactivity in the solution. The values of the constants A_1, A_2, A_3, A_4 and g_1, g_2, g_3, g_4 are given in Table 1. In all cases the disappearance curves were described by a sum of 4 exponentials, except for P_i on apatite-PP_i, where the best fit is with 2 exponentials.

2. Determination of the Rate Constants and the Pools

Table 2 gives the various rate constants for Ca, P_i and PP_i on apatite and apatite-PP_i for the different experiments. The goodness of the fit is given by the variances obtained for the parameter values. Fig. 5 gives for the experiments

Table 2. Rate constants (in min^{-1} ± SD) of the exchanges between the different compartments of the crystal for ^{45}Ca, ^{32}P[P_i] and ^{32}P[PP_i]

	λ_{21}	λ_{12}	λ_{32}	λ_{23}	λ_{43}	λ_{34}	λ_{04}
Ca (apatite)							
Experiment 1	0.361	1.094	0.181	0.166	0.0055	0.0064	0.00053
(pH∼7.6)	±0.030	±0.176	±0.052	±0.032	±0.0016	±0.0018	±0.00012
Experiment 2	0.362	0.909	0.112	0.123	0.0089	0.0088	0.00037
(pH∼7.6)	±0.028	±0.125	±0.035	±0.031	±0.0026	±0.0019	±0.00007
Experiment 3	0.274	0.962	0.133	0.149	0.0145	0.0142	0.00050
(pH∼7.4)	±0.042	±0.292	±0.092	±0.082	±0.0080	±0.0050	±0.00012
Ca (apatite-PP_i)							
Experiment 4	0.308	0.638	0.079	0.087	0.0080	0.0075	0.00016
(pH∼7.4)	±0.018	±0.073	±0.024	±0.024	±0.0031	±0.0020	±0.00007
Experiment 5	0.445	0.952	0.144	0.142	0.0086	0.0081	0.00040
(pH∼7.4)	±0.031	±0.121	±0.035	±0.026	±0.0020	±0.0010	±0.00006
P_i (apatite)							
Experiment 1	0.250	0.993	0.126	0.110	0.0029	0.0030	0.00005
(pH∼7.6)	±0.032	±0.219	±0.048	±0.030	±0.0012	±0.0013	±0.00019
Experiment 2	0.308	0.881	0.052	0.063	0.0019	0.0020	0.00002
(pH∼7.6)	±0.016	±0.068	±0.009	±0.009	±0.0001	±0.0007	±0.00019
Experiment 3	0.138	0.511	0.043	0.057	0.0028	0.0025	0.00025
(pH∼7.4)	±0.013	±0.089	±0.019	±0.020	±0.0012	±0.0014	±0.00030
P_i (apatite-PP_i)							
Experiment 4	0.0051	0.114	0.0008[a]				
(pH∼7.4)	±0.0014	±0.039	±0.0003				
Experiment 5	0.0037	0.031	0.0005[a]				
(pH∼7.4)	±0.0004	±0.004	±0.00006				
PP_i							
Experiment 6	0.257	0.262	0.050	0.049	0.0065	0.0046	0.00070
(pH∼7.4)	±0.013	±0.034	±0.020	±0.020	±0.0024	±0.0014	±0.00015

[a] In this case the model was a 2-compartment one and this rate constant is λ_{02}.

3, 5 and 6 the size for the various pools in µmoles per g of apatite (150 mg of apatite were suspended in 100 ml solution). It can be seen a) that the fluxes of Ca are only little influenced by the PP_i bound; b) that the quantity of Ca in the various pools is also little influenced by the presence of PP_i; c) that the 4 compartments of P_i in the apatite are reduced to 2 compartments in the apatite-PP_i and that they contain much less P_i than before, the total amount being 20 µmoles instead of 143; d) that the PP_i is distributed in the 4 compartments and that the quantity of PP_i in the 4 compartments is equal to the amount of P_i (both evaluated as P) which has disappeared during the uptake of PP_i by the crystals. Thus, 2 moles of P_i have been replaced by 1 mole of PP_i.

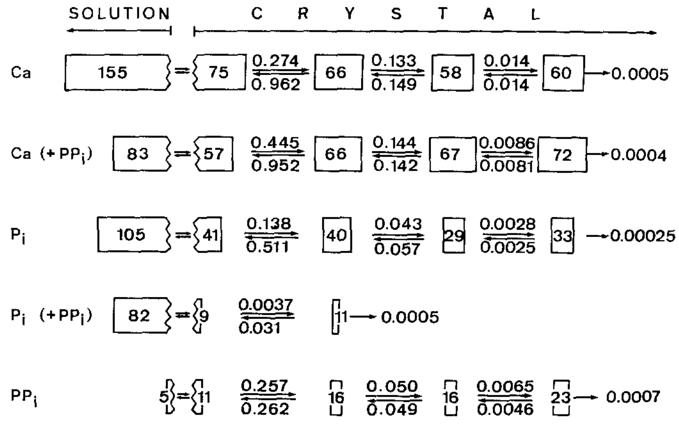

Fig. 5. Compartmental model of the distribution of Ca, P$_i$ and PP$_i$ in apatite and apatite-PP$_i$ (150 mg apatite/100 ml solution; 22.6 µmoles PP$_i$ added). The pool sizes are given in µmoles Ca or P per g of apatite, the rate constants in min^{-1}. The first pool represents the solution, the second is a crystal pool exchanging with the solution in the first 30 sec, the next ones are pools within the crystal

Table 3. Influence of pH and various additions of PP$_i$ on the exchangeable calcium and phosphate of apatite

Crystals	µmoles PP$_i$ added per g apatite	pH = 7.0		pH = 7.4		pH = 7.8	
		Ca	P$_i$	Ca	P$_i$	Ca	P$_i$
Apatite	0	4.1%	4.3%	4.2%	4.2%	3.9%	3.4%
Apatite-PP$_i$	54	—	—	4.0%	2.4%	—	—
	108	—	—	4.1%	1.4%	—	—
	151	3.8%	0.85%	3.9%	0.65%	3.9%	0.40%

3. Exchangeable Ca and P$_i$ for Various Amounts of PP$_i$ Bound and at Various pH's

Table 3 shows that increasing amounts of PP$_i$ on the apatite do not influence greatly the amount of exchangeable Ca, whereas they diminish strongly the amount of exchangeable P$_i$. These results are in agreement with those obtained by the compartment analysis. In the latter case, also, the presence of PP$_i$ influenced little the amount of Ca in the different crystal compartments, but strongly diminished the amount of P$_i$ in these compartments. Furthermore, the exchangeable P$_i$ diminishes in parallel with the amount of PP$_i$ bound.

Variations between pH 7.0 and 7.8 do practically not change the amount of exchangeable Ca. Exchangeable P$_i$, however, is reduced at alkaline pH for both apatites.

4. Ultracentrifugation

Table 4 gives the results of the specific activity of Ca, P$_i$ and PP$_i$ in apatite and apatite-PP$_i$ before and after ultracentrifugation. When crystals had been

Table 4. Specific activity (cpm/μmole) of ^{45}Ca, ^{32}P[P$_i$] and ^{32}P[PP$_i$] in apatite and apatite-PP$_i$ before and after ultracentrifugation

Crystals		Initial specific activity	Specific activity after ultracentrifugation ± SD of 3 determinations	
			10000 g	80000 g
Ca	Apatite	404	384 ± 8	404 ± 16
	Equilibrated apatite-PP$_i$	268	272 ± 12	264 ± 4
	Non-equilibrated apatite-PP$_i$	1344	1372 ± 24	1008 ± 40
P$_i$	Apatite	90	94 ± 5	97 ± 7
	Equilibrated apatite-PP$_i$	57	55 ± 3	57 ± 2
	Non-equilibrated apatite-PP$_i$	236	238 ± 3	235 ± 2
PP$_i$	Equilibrated apatite-PP$_i$	344	371 ± 5	383 ± 4
	Non-equilibrated apatite-PP$_i$	184	180 ± 1	180 ± 5

resuspended in solution E-PP$_i$ after addition of PP$_i$, even a centrifugal force of 80000 g did not change the specific activities of either apatite or apatite-PP$_i$, indicating that neither Ca, nor P$_i$, nor PP$_i$ are removed by these forces. However, if the crystals were not re-equilibrated after PP$_i$ addition, the specific activity of Ca decreased after centrifugation at 80000 g. The specific activities of P$_i$ and PP$_i$ were, however, not changed. Thus, some Ca bound on apatite-PP$_i$ was lost by ultracentrifugation. The amount lost by ultracentrifugation was calculated in the following way: If one assumes that the external layer taken away by ultracentrifugation has the same specific activity as the solution, then, with the difference between the specific activity before and after ultracentrifugation being about 360 cpm per μmole of crystal Ca and the specific activity of the solution 14400 cpm/mole Ca, the 360 cpm represent 0.025 μmoles Ca/μmole of crystal Ca, that is 2.5% of total crystal Ca.

Discussion

The disappearance curve of ^{45}Ca and ^{32}P [P$_i$] from the solution onto the crystals in the time period examined (30 sec to 30 h) was best described by an equation containing the sum of 4 exponentials. Our results agree with those obtained by Pak and Bartter (1967) for Ca. However, Neuman found 3 exponentials for Ca and P$_i$ between 0 and 45 h (Neuman and Neuman, 1958). These differences are probably explained by the fact that, in Neuman's experiments, no determinations were available during the first hour, and a straight line was drawn back to zero time during this time lapse. Our results show, however, that at least 2 exponentials are present within this period. The points obtained in the first 30 sec were few. However, all lie on a straight line on semi-log paper after peeling (Riggs, 1963) back to time zero. This means that the disappearance curve can be represented by the sum of no more than five exponentials from zero to 30 h. Four of these exponentials have been accurately calculated, while this could not be done for the fifth.

It is not possible to give definite spatial location to each compartment. However, it is reasonable to assume that the disappearance curve corresponds to the successive passage of ^{45}Ca and ^{32}P [P_i] through a population of Ca and P_i molecules described by an open 4 compartment model in series. These compartments would correspond to successive layers from the solution to the crystal interior. Such models with successive layers have been suggested repeatedly (Neuman and Neuman, 1958; Pak and Bartter, 1967; Težak, 1970). It is interesting that the number of compartments is the same for Ca, P_i and PP_i, and that the exponents are in the same order of magnitude for the 3 ions.

Quantitative data for pool sizes and rate constants have been obtained by analysing the disappearance curve with the SAAM model fitting. PP_i diminishes greatly the P_i present in all compartments, and 2 of the 4 pools were no longer even detectable. It can be calculated that 2 moles of P_i are displaced as 1 mole of PP_i is bound. Similar heteroionic exchanges have been found between orthophosphate and citrate, and between orthophosphate and carbonate (Pak and Diller, 1969). Comparison between the amount bound and the amount exchangeable within 30 h as calculated by the model, shows that about 50% of the PP_i is not exchangeable. This finding is in agreement with observations that only about 80% of the bound PP_i is hydrolysed by yeast pyrophosphatase (Krane and Glimcher, 1962; unpublished results). Thus, part of the PP_i added is unavailable for exchange or for enzyme hydrolysis.

PP_i has no large influence on the amounts and distribution of Ca when the apatite is re-equilibrated after the addition of PP_i. Thus it seems that PP_i takes the place of P_i in the various compartments without attracting Ca as a counter ion. However, if the apatite is not re-equilibrated, the amount of exchangeable Ca has been found to be increased (Robertson and Morgan, 1971). It seems therefore that treatment with PP_i attracts a pool of Ca which is lost by such a bland procedure as re-equilibration. This is supported by the fact that if non-re-equilibrated crystals are ultracentrifuged, Ca is lost when centrifuged at 80000 g. On the other hand, no P_i and no PP_i are lost when centrifuged at this speed.

Increasing pH has been found to have little influence on the exchangeable Ca but to decrease the amount of exchangeable P_i. It is likely that an increase in pH would lead to an increase of the negativity of the surfaces by a gain of OH^- ions or a loss of H^+ ions. This would tend to repell the phosphate ions. However, it is not known why the exchangeable calcium is not changed by an increase in pH.

The effect of PP_i on the P_i fluxes is difficult to interpret since one does not know to what compartment the pool remaining after treatment with PP_i has to be compared. However, the fluxes out of the last compartment, which might represent the incorporation into the crystal lattice, have not been changed by PP_i. The Ca fluxes are not changed by PP_i either. These results are difficult to reconcile with the known fact that PP_i diminishes growth (Fleisch *et al.*, 1966b; Fleisch and Neuman, 1961) and dissolution (Fleisch *et al.*, 1966a) of apatite crystals. It is likely that the rates obtained under steady state conditions are not related in any simple way to rate of crystal growth and dissolution under non-steady state conditions. It is known indeed that the dissolution of apatite crystals does not follow first order kinetics (Zimmermann, 1966; Gray, 1966), and this is probably partly accounted for when geometrical parameters defining the surface to volume rates are introduced in the calculation of the free energy of dissolution and growth (Best, 1959). The growth of crystals does not generally follow first order kinetics either (Nielsen, 1964).

The observation that the fluxes are not altered by PP_i argues against the existence of a diffusion barrier by a layer of Ca-pyrophosphate, as has been suggested for fluoride (Francis *et al.*, 1968). It is more likely that the decrease of growth and dissolution of apatite crystals, brought about by pyrophosphate, are explained by the pyrophosphate ions having some effect on the phosphate ions in the crystal surface. By replacing the orthophosphate at the surface, pyrophosphate might partially block the sites of nucleation of calcium phosphate and, hence, growth. The same mechanism could explain the diminished dissolution, as has been demonstrated for other crystals, where crystal poisons adsorbing on the surface block the preferred sites of dissolution (Mutaftschiev *et al.*, 1965; van Damme, 1965).

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