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Effects of Pyrophosphate and Diphosphonates on the Dissolution of Hydroxyapatites Using a Flow System

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Summary. Pyrophosphate and diphosphonate ions have been said to diminish the dissolution of hydroxyapatite crystals, because they lower the equilibrium concentrations of calcium and phosphate ions in the bulk solution around hydroxyapatite crystals in a closed system. However, in a closed system these effects are not necessarily due to an effect on dissolution alone. In this paper we have used a continuous flow system to study the effects of pyrophosphate and two diphosphonates, ethane-1-hydroxy-1,1-diphosphonate and dichloromethane diphosphonate, on the dissolution of hydroxyapatite. All three compounds decreased markedly the rate of dissolution of hydroxyapatite as well as the exchangeable pools of calcium and phosphate ions around the cystals.

Key words: Hydroxyapatite — Dissolution — Pyrophosphate, Diphosphonates — Calcium — Phosphate.

Introduction

The dissolution of hydroxyapatite has been studied in closed ("static") systems, and the calcium and phosphate concentrations in the bulk solution at equilibrium have been taken as the measure of the degree of dissolution [1, 2]. The term dissolution, rather than solubility, has been advised since it has been concluded that hydroxyapatite does not exhibit normal solubility or obey a fixed solubility product [1, 2].

The crystals of hydroxyapatite are of colloidal dimensions, and at the crystal-solution interface there is a considerable electrical field projecting away from the crystal surface. This field gives rise to a bound ion and water layer—the hydration shell [2]—and the amounts of calcium and phosphate ions in the hydration shell can be determined by exchange experiments [2-4]. It can be calculated that the amount of calcium ions in the bulk solution at equilibrium is between 5% and 40% of the total exchangeable calcium ions in the system, depending on the source of the hydroxyapatite and the solid-to-solution ratio [3, 4; this paper]. Therefore it is possible that some or all of the calcium ions which enter the bulk solution in a closed system come from previously formed hydration shells.

Pyrophosphate and diphosphonate ions reduce the equilibrium calcium and phosphate concentrations of precoated hydroxyapatite in a closed system [5, 6]. These results have been taken to indicate an effect of these compounds on the dissolution of hydroxyapatite crystals and are the basis of the suggestion that the inhibition of bone resorption induced by diphosphonates in vivo is due to a decreased rate of dissolution of hydroxyapatite [5-7]. However, pyrophosphate and diphosphonates also reduce the amount of exchangeable calcium and phosphate ions of precoated hydroxyapatite [8, 9], and therefore the equilibrium concentrations of calcium and phosphate ions for precoated hydroxyapatite in a closed system could be dependent on previous changes in the amounts of exchangeable calcium and phosphate ions around the crystals. For this work we have used a flow system to measure the direct effect of pyrophosphate and diphosphonate ions on the rate of dissolution of hydroxyapatite and

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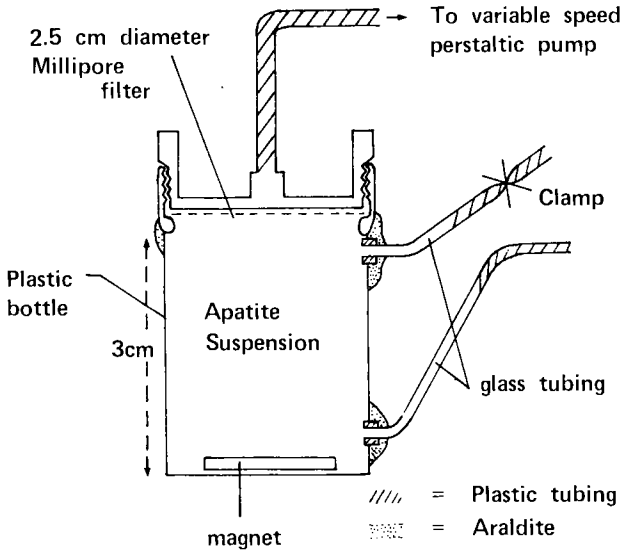

Fig. 1. Diagram of the reaction chamber of the flow system

on the amounts of exchangeable calcium and phosphate ions in the hydration shell during dissolution. These results are compared with those obtained in a closed system using the same hydroxyapatite.

Methods

Preparation of Washed and Precoated Hydroxyapatite

Washed hydroxyapatite was prepared as follows: 10 g of hydroxyapatite, as purchased, was stirred magnetically in 1 liter of buffer at pH 7.0. The buffer was pumped out of the suspension through a 0.45 μm Millipore filter at a flow rate of 100 ml/h and was replaced with fresh buffer at the same rate until 6 liters had been passed through. The resulting crystal suspension was filtered through a 0.45 μm Millipore filter and dried in an oven at 80°C.

Some of the washed hydroxyapatite was precoated with ethane-1-hydroxy-1, 1-diphosphonate (EHDP) or dichloromethane diphosphonate (Cl_2MDP) as described by Jung et al. [9] so that 1.4 mg diphosphonate P was bound to each 150 mg of hydroxyapatite. This precoated hydroxyapatite was filtered off and dried in air.

Measurement of Hydroxyapatite Dissolution in the Flow System

A reaction chamber was made by cementing, with Araldite, a Millipore Swinnex 25 filter holder into a polyethylene bottle (Fig. 1). Before cementing, the lower end of the filter holder was cut away so that the maximum area of filter was exposed to the apatite suspension. The total volume of the reaction chamber was 20 ml.

The total flow system is shown in Figure 2. The volume of fluid between the reservoir and the reaction chamber was 8 ml. To set up the flow system with a hydroxyapatite suspension the sequence of operations was as follows: With the reaction chamber and tubes out of the water bath, tubes B, C, and E were filled with buffer from the reservoir using a 50 ml syringe. Tube B was clamped and fitted to the reaction chamber. The magnet was placed in the reaction chamber and a weighed quantity of hydroxyapatite (usually 20 mg) was added to the chamber. A wetted Millipore filter of 25 mm diameter (0.45 μm pore size) was placed on the flat surface of the filter cap which was then screwed into place with tube D open. Tube E was clamped and tube B was unclamped. The reaction chamber was filled with buffer from the syringe and tube D was clamped. Tube E was unclamped and tube C was clamped. The reaction chamber and tubes were placed in the water bath (at 37°C), and the magnetic stirrer and peristaltic pump were switched on. Aliquots of buffer were collected in a fraction collector for the determination of calcium, phosphate, and pH. The presence of hydroxyapatite in the reaction chamber did not slow the flow rate up to a value of 700 ml/h. The buffer used contained 150 mmol/l NaCl, 10 mg/l neomycin and 5 mmol/l sodium barbital at pH 7.0.

Measurement of Hydroxyapatite Dissolution in the Closed System

A weighed amount of hydroxyapatite (100 or 1000 mg) was added to 100 ml of the buffer (pH 7.0) in a polyethylene bottle and the system was stirred magnetically in a water bath at 37°C. At various times 2.0 to 2.5 ml samples of the suspension were filtered

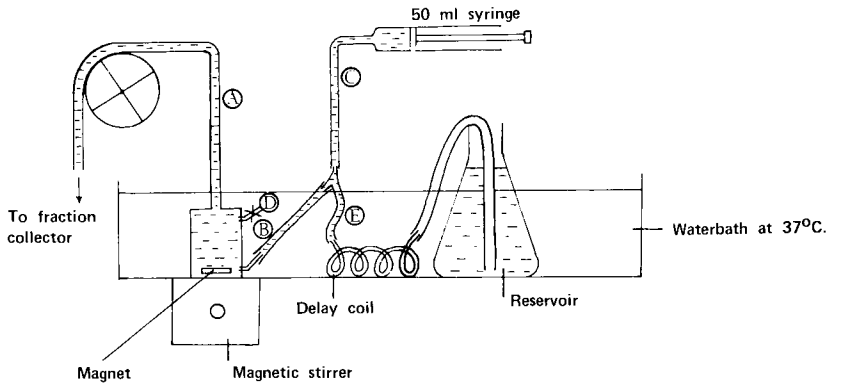

Fig. 2. Diagram of the total flow system for hydroxyapatite suspensions

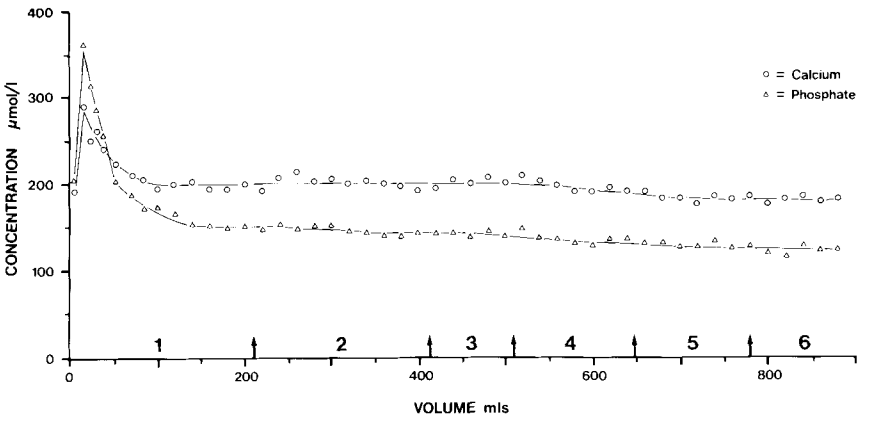

Fig. 3. Calcium and phosphate concentrations in the effluent buffer from nonwashed hydroxyapatite in the flow system at a solid-to-solution ratio of 10 g/l. The flow rates (ml/h) were 244, 119, 55, 90, 208, and 471 in sections 1–6, respectively

through a Millipore filter (pore size 0.45 µm) in a Swinnex hypodermic adaptor, and the filtrate was kept for the determination of calcium, phosphate, and pH. The calcium and phosphate concentrations did not increase after 6 days and the hydroxyapatite suspension was taken to be in equilibrium.

Measurement of the Exchangeable Calcium and Phosphate of Hydroxyapatite

In the closed system the amounts of exchangeable calcium and phosphate of hydroxyapatite were calculated from the amount of ^{45}Ca or ^{32}P-phosphate in the bulk solution 90 min after adding a known amount of isotope to a hydroxyapatite suspension at equilibrium [3, 4]. It was assumed that after 90 min the specific activities in the bulk solution and in the exchangeable pool were equal and the amount of exchangeable calcium of the hydroxyapatite was calculated using the following equation [1–3]:

$$\text{Ca exchangeable} = \text{Ca in bulk solution} \times \frac{^{45}\text{Ca on apatite}}{^{45}\text{Ca in bulk solution}}$$

The amount of exchangeable phosphate was calculated in the same way.

For the flow system ^{45}Ca or ^{32}P-phosphate was added to the buffer entering the suspension together with a stable calcium or phosphate concentration of 5 µmol/l (as carrier). After about 90 min the inflow and outflow of radioactivity were equal and it was assumed that a steady state had been reached such that the specific activities in the exchangeable layer of hydroxyapatite and the bulk solution were equal. The exchangeable pool was calculated using the equation above.

Chemical Techniques and Radioactivity Measurements

Stable calcium was measured by atomic absorption spectrophotometry with a concentration of 1% lanthanum chloride and 5% hydrochloric acid in the diluted sample. Stable phosphorus was measured by a modification [10] of the method of Chen et al. [11]. Radioactivity was measured in a Packard Tri-Carb liquid scintillation counter. Infrared absorption spectra of hydroxyapatite samples were recorded using a Perkin-Elmer Infrared spectrophotometer, model 621. X-ray diffraction peaks of hydroxyapatite samples were recorded with a Siemans Kristalloflex diffractometer. The surface area of hydroxyapatite samples was measured by the Ströhlein indirect method based on BET absorption.

Materials

The hydroxyapatite was purchased from E. Merck AG, Darmstadt, W. Germany. The diphosphonates were a gift from Proctor and Gamble Co., Cincinnati, Ohio, U.S.A. All other reagents were of the highest grade available from E. Merck AG, Darmstadt, W. Germany.

Results

Characteristics of the Nonwashed and Washed Hydroxyapatites

The calcium to phosphorus molar ratio of the nonwashed hydroxyapatite was 1.65. No differences between nonwashed and washed hydroxyapatite could be detected by X-ray diffraction or by infrared absorption techniques. Both were highly crystalline apatites with about 2% calcium carbonate, and the mean crystal size was the same. The washed hydroxyapatite had a smaller surface area (52.6 m^2/g) than the nonwashed hydroxyapatite (58.7 m^2/g).

Dissolution of Hydroxyapatite in the Flow System

Studies on Nonwashed and Washed Hydroxyapatite. Figure 3 shows an experiment with nonwashed hydroxyapatite at a solid-to-solution ratio of 10 g/l and an initial flow rate of 244 ml/h. There were peaks of calcium and phosphate concentration in the early samples before a steady state was reached. The pH of the effluent buffer was 6.7 in

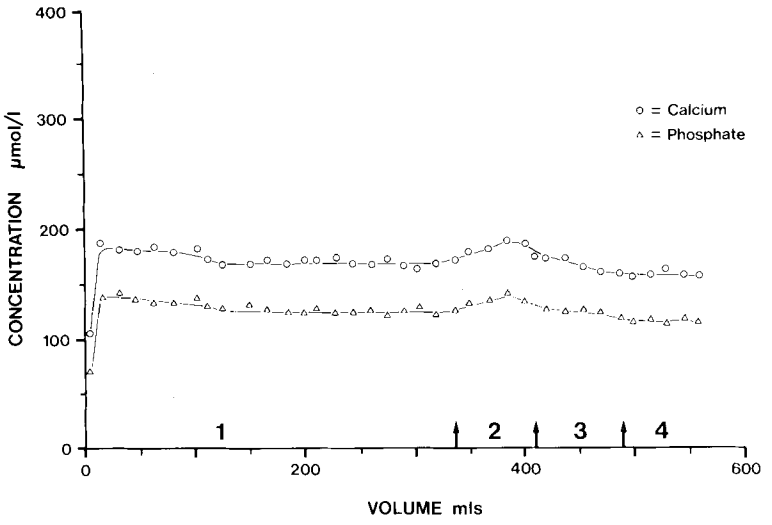

Fig. 4. Calcium and phosphate concentrations in the effluent buffer from washed hydroxyapatite in the flow system at a solid-to-solution ratio of 10 g/l. The flow rates (ml/h) were 253, 48, 246, and 542 in sections 1–4, respectively

these early samples, rising to 7.10 at steady state. In the steady state there was a constant difference of 0.1 pH units between the inflowing and outflowing buffer.

When the buffer was pumped through washed hydroxyapatite at a solid-to-solution ratio of 10 g/l, there were no early peaks of concentration of calcium and phosphate ions (Fig. 4), but there was the same difference of 0.1 pH units between the inflowing and outflowing buffer. The concentrations of calcium and phosphate ions in the effluent buffer at steady state were approximately the same for washed and nonwashed hydroxyapatite.

When the solid-to-solution ratio was reduced to 1 g/l, the steady-state concentrations were less than those obtained at 10 g/l (Table 1) and there was no detectable pH difference between inflowing and outflowing buffer. Figures 3 and 4 show also that changing the flow rate within the range 50 to 500 ml/h had little effect on the concentrations of calcium and phosphate ions in the effluent buffer.

Effects of Pyrophosphate and Diphosphonates. The effects of pyrophosphate and diphosphonates were studied at a solid-to-solution ratio of 1 g/l (where there was no detectable change of pH). The pH 7.0 buffer was pumped through the suspension at 420 ml/h for about 15 min when the buffer was changed to one which contained the sodium salt of either pyrophosphate, EHDP, or Cl_2MDP, each at a concentration of 10 µmol/l. All three compounds decreased the calcium and phosphate concentrations in the effluent buffer, and this effect was maximal after about 100 ml of buffer (5 times the volume of the reaction chamber) had been pumped through. The effect wore off slowly when buffer without pyrophosphate or diphosphonate was reintroduced, but eventually the concentrations of calcium and phosphate ions in the effluent buffer rose to those observed in the control experiment where buffer without additives was pumped throughout. Figure 5 shows the results of an experiment with Cl_2MDP and a control experiment. The results of all the experiments are summarized in Table 2.

When hydroxyapatite precoated with EHDP was studied in the flow system at a solid-to-solution ratio of 1 g/l, the calcium and phosphorus concentrations in the effluent buffer (63 and 39 µmol/l, respectively), were similar to those obtained when a buffer containing EHDP was pumped through a suspension of untreated hydroxyapatite (see Table 2), and changing the flow rate between 65 and 380 ml/h had little effect on these concentrations.

Effect of Pyrophosphate on the Exchangeable Calcium and Phosphate of Hydroxyapatite. The exchangeable calcium and phosphate of washed hydroxyapatite (solid-to-solution ratio of 1 g/l; pH 7.0) was measured under conditions of steady-state dissolution at a flow rate of 150 ml/h. The exchangeable calcium was 342 µmol/g and the exchangeable phosphate was 290 µmol/g of hydroxyapatite. When pyrophosphate was added to the buffer at a concentration of 10 µmol/l, the exchangeable calcium was reduced to 210 µmol/g and the exchangeable phosphate was reduced to 32 µmol/g of hydroxyapatite.

Table 1. Steady-state effluent concentrations (duplicate experiments) at two solid-to-solution ratios of washed hydroxyapatite suspension in the flow system

Solid-to-solution ratio	Calcium µmol/l	Phosphate µmol/l
1 g/l	130; 127	92; 86
10 g/l	194; 180	149; 136

Table 2. Steady-state concentrations of calcium and phosphate in the effluent buffer without, and with, the addition of pyrophosphate (PPi), EHDP, or Cl_2MDP at concentrations of 10 μmol/l to the buffer (pH 7.0)[a]

Additive	Calcium (μmol/l)			Phosphate (μmol/l)		
	Without	With	% Reduction	Without	With	% Reduction
EHDP	135	53	61	96	35	63
Cl_2MDP	127	67	47	87	46	47
PPi	130	71	45	93	55	41

[a] In each case the solid-to-solution ratio was 1 g/l (washed hydroxyapatite)

Dissolution of Hydroxyapatite in a Closed (Static) System

Dissolution of Washed and Nonwashed Hydroxyapatite and the Effect of Precoating Washed Hydroxyapatite with Diphosphonates. The results of the closed system dissolution studies on the two hydroxyapatite samples at two solid-to-solution ratios are shown in Table 3. At the solid-to-solution ratio of 10 g/l, the nonwashed hydroxyapatite gave increased calcium and phosphate concentrations, with lower pH, than the nonwashed hydroxyapatite. For the same solid-to-solution ratio all the steady-state concentrations of calcium and phosphate ions were higher in the closed system than in the flow system.

When the washed hydroxyapatite was precoated with EHDP or Cl_2MDP, the steady-state concentrations of both calcium and phosphate ions were reduced to about 75% (range 70–83%; $N = 3$) of the mean values obtained with noncoated washed hydroxyapatite. The two diphosphonates gave similar results.

Exchangeable Calcium and Phosphate in the Closed (Static) System and the Effect of Precoating Washed Hydroxyapatite with Diphosphonates. The exchangeable calcium of washed hydroxyapatite was measured as 366 μmol/g (range 290–440; $N = 5$), while the exchangeable phosphate was 203 μmol/g (range 161–223; $N = 4$). The ratio of exchangeable ions for washed and nonwashed hydroxyapatite was 0.89 (range 0.85–0.93; $N = 4$) for both calcium and phosphate, i.e., washing reduced these exchangeable ions by about 10%.

The exchangeable calcium of the washed hydroxyapatite coated with diphosphonates was 86% (range 70–109%; $N = 4$) of the value for washed hydroxyapatite. The exchangeable phosphate of the washed hydroxyapatite coated with diphosphonates was 20% (range 10–37%; $N = 4$) of the value obtained for washed hydroxyapatite. The two diphosphonates gave similar results.

Discussion

The use of the flow system with nonwashed hydroxyapatite at a solid-to-solution ratio of 10 g/l showed early peaks of the concentration of calcium and phosphate ions in the effluent buffer which were not seen with washed hydroxyapatite (Figs. 3 and 4). During these peaks the pH of the effluent buffer was substantially less (at pH 6.7) than that of the inflowing buffer (pH 7.0). In the closed system the calcium and phosphate concentrations at equilibrium for both 1 and 10 g/l solid-to-solution ratios for washed hydroxyapatite were similar, whereas those for nonwashed hydroxyapatite at the 10 g/l ratio were higher (Table 3); in the latter case the pH of

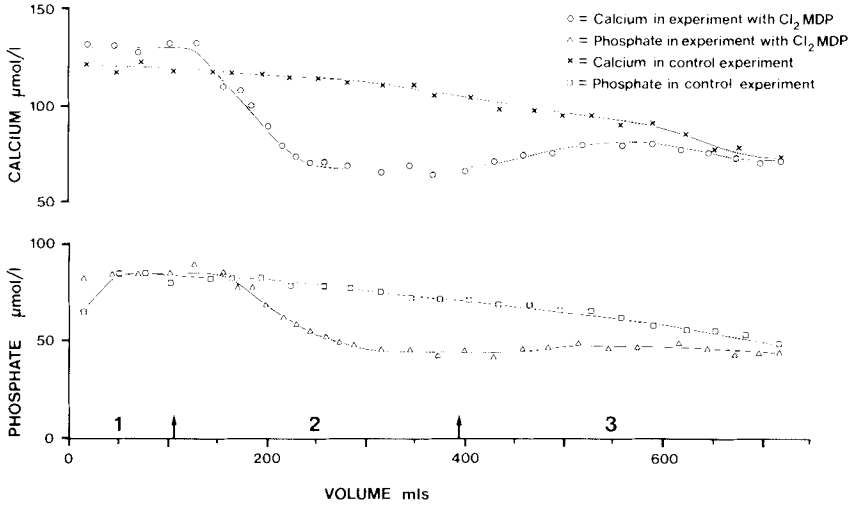

Fig. 5. Effect of Cl_2MDP at a concentration of 10 μmol/l on the calcium and phosphate concentrations in the effluent buffer from washed hydroxyapatite in the flow system at a solid-to-solution ratio of 1 g/l. The 3 sections are *1*, buffer without Cl_2MDP; *2*, buffer plus Cl_2MDP; *3*, buffer without Cl_2MDP. In the control experiment buffer without Cl_2MDP was used throughout. In both experiments the flow rate was 420 ml/h

Table 3. Dissolution of washed and nonwashed hydroxyapatite in a closed (static) system[a]

Solid to solution ratio	Calcium μmol/l	Phosphate μmol/l	pH
10 g/l			
Washed	267 [242-290] (4)	195 [177-209] (4)	7.14 [7.09-7.19] (4)
Nonwashed	500 [461-535] (4)	340 [306-368] (4)	6.76 [6.72-6.80] (4)
1 g/l			
Washed	256 [230-272] (9)	184 [168-197] (9)	7.15 [7.10-7.21] (9)
Nonwashed	259 [245-266] (6)	199 [184-213] (6)	7.15 [7.06-7.24] (6)

[a] The mean values are shown with the ranges in brackets; the numbers of estimations are in parentheses

the bulk solution at equilibrium was substantially reduced (to pH 6.76). The simplest explanation for these effects is that the nonwashed hydroxyapatite (that is, as purchased) contained some residual acid, possibly in the hydration shell. However, the amount of acid released into the bulk solution was not enough to change the pH of the buffer for nonwashed hydroxyapatite at the 1 g/l ratio in the closed system (Table 3). The surface area and the exchangeable calcium and phosphate of the hydroxyapatite were reduced by about 10% during the washing process, which may have been due to the complete dissolution of some small crystals, but X-ray diffraction and infrared absorption studies did not detect any chemical or physical differences between nonwashed and washed hydroxyapatite. These results led us to choose the washed hydroxyapatite to study the effects of adding pyrophosphate or diphosphonates to the buffer in the flow system.

The amounts of calcium and phosphate ions removed from the crystal suspension at a solid-to-solution ratio of 1 g/l could be greater than the total amount of exchangeable ions (Table 4), so that dissolution had occurred. The exchangeable calcium and phosphate pools of hydroxyapatite, previously demonstrated in closed systems [2, 4, 8, 9], remained under conditions of continuous dissolution in the flow system. Therefore, calcium and phosphate ions were moving from the crystal, through the hydration shell and the bulk solution, and out of the reaction chamber. At constant flow rate, under steady-state conditions, the concentration of ions in the effluent buffer is directly proportional to the rate of dissolution. Pyrophosphate, EHDP, and Cl_2MDP each diminished the calcium and phosphate concentrations in the effluent buffer so that a new steady state was reached (Table 2, Fig. 5), showing that the rate of dissolution of hydroxyapatite had been diminished by each of these compounds. These compounds also reduced the exchangeable calcium and phosphate ions in the hydration layer during dissolution in the flow system. These effects in the flow system have a similar pattern to previous results obtained with precoated re-equilibrated hydroxyapatite in the closed system [8, 9; this paper]. The most probable explanation of these effects is that pyrophosphate and diphosphonates are adsorbed on the surface of the crystal and block the preferred sites of dissolution [9].

These results support the earlier suggestion [5, 6], previously based on closed system results, that the inhibition of bone resorption by diphosphonates in vivo [6, 12, 13] is due to this inhibitory effect on dissolution, although it is possible that cellular mechanisms also play a role.

Table 4. Amounts of calcium and phosphate removed from the reaction chamber in the control experiment of Fig. 5 as a percentage of (a) the amount of total exchangeable calcium and phosphate and (b) the amount of total calcium and phosphate in the reaction chamber at the beginning of the experiment

Volume, ml	Percentage (a)		Percentage (b)	
	Calcium	Phosphate	Calcium	Phosphate
100	129	111	6.2	7.1
200	257	220	12.3	14.2
300	380	324	18.2	20.8

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References

1. Neuman, W.F., Neuman, M.W.: Chem. Rev. **53**:1-45, 1953
2. Neuman, W.F., Neuman, M.W.: The Chemical Dynamics of Bone Mineral. University of Chicago Press, Chicago, 1958
3. Pak, C.Y.C., Bartter, F.C.: Biochim. Biophys. Acta **141**:401-409, 1967
4. Robertson, W.G., Morgan, D.B.: Biochim. Biophys. Acta **230**:495-503, 1971

5. Fleisch, H., Russell, R.G.G., Straumann, F.: Nature **312**:901-903, 1966
6. Russell, R.G.G., Mühlbauer, R.C., Bisaz, S., Williams, D.A., Fleisch, H.: Calcif. Tissue Res. **6**:183-196, 1970
7. Fleisch, H., Russell, R.G.G., Bisaz, S., Bonjour, J.P.: Hard tissue growth, repair and remineralisation, Ciba Found. Symp. **11**:331-348, 1973
8. Robertson, W.G., Morgan, D.B., Fleisch, H., Francis, M.D.: Biochim. Biophys. Acta **261**:517-525, 1972
9. Jung, A., Bisaz, S., Bartholdi, P., Fleisch, H.: Calcif. Tissue Res. **13**:27-40, 1973
10. Bisaz, S., Russell, R.G.G., Fleisch, H.: Arch. Oral Biol. **13**:683-696, 1968
11. Chen, P.S., Toribara, T.Y., Warner, H.: Anal. Chem. **28**:1756-1758, 1956
12. Reynolds, J.J., Minkin, C., Morgan, D.B., Spycher, D., Fleisch, H.: Calcif. Tissue Res. **10**:302-313, 1972
13. Schenk, R., Merz, W.A., Mühlbauer, R., Russell, R.G.G., Fleisch, H.: Calcif. Tissue Res. **11**:196-214, 1973

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