muscle lactate dehydrogenase A (E.C. 1.1.1.27) appeared to have a low degree of heterogeneity. The preparation exhibited a single major component (isoelectric point, *p*H 8.55) containing more than 80 percent of the protein and 85 percent of the activity. In addition, two minor activities and two inactive components were detected. The ultraviolet absorbing material at *p*H 9.9 is largely the result of an accumulation of electrolysis products from the cathode and was found in all electrofocusing experiments.

All of the enzymes thus far subjected to isoelectric focusing have exhibited some degree of heterogeneity, even though most of them were crystalline proteins and homogeneous by commonly accepted criteria. Some of the heterogeneity detected here is probably the result of degradation or modification of the proteins during isolation. We believe, however, that many of the multiple forms occur intracellularly. The molecular heterogeneity of glyceraldehyde-3-phosphate dehydrogenase and yeast aldolase revealed by electrofocusing can be explained by the random formation of tetrameric and dimeric molecules, respectively, from two similar, but nonidentical subunits, as for aldolase A. The heterogeneity observed in the other enzyme preparations is not so obviously related to the subunit composition. Molecular heterogeneity may arise by selective modification, as for ribonucleases A and B (*9*), or by combinative or conformational variation. Alternatively, these variants could be the products of separate nonallelic genes or simply distinct allelomorphic products derived from a heterozygote. The latter would be more easily reconciled with their apparently strong structural and catalytic similarity and the lack of an obvious physiological function. We consider translational infidelity or ambiguity an unlikely mechanism because of the restricted number of species detected.

Regardless of the origin or function of this molecular heterogeneity, it should be given careful consideration when studying enzyme mechanisms (kinetics) or structure, since interpretations of such studies often depend on homogeneous molecular populations.

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10. The electrofocusing apparatus and ampholytes were obtained from LKB Instruments, Rockville, Maryland. The column was cooled with circulating water at 4°C. Protein solutions were mixed with a small volume of ampholine-sucrose solution and placed at the center of the column. Voltage was increased to 800 volts over a period of 16 hours and allowed to equilibrate an additional 24 to 30 hours. Approximately 200 0.5-ml fractions were collected for assay.
11. Aldolase A was purified from rabbit muscle by the method of B. M. Pogell [*Biochem. Biophys. Res. Commun.* **7**, 225 (1962)], as modified by E. Penhoet, M. Kochman, W. J. Rutter (*Biochemistry*, in press). Glyceraldehyde-3-phosphate dehydrogenase was prepared from rabbit muscle according to M. Kochman and W. J. Rutter [*Biochemistry* **7**, 1671 (1968)]. Yeast aldolase was purified by Dr. R. Kobes from baker's yeast by a modification (*6*) of the method of W. J. Rutter and J. R. Hunsley [*Methods Enzymol.* **9**, 480 (1966)]. Lactate dehydrogenase A was a gift from Drs. V. Hoagland and D. Teller and was prepared by the method of G. T. Cori, W. W. Slein, C. F. Cori [*J. Biol. Chem.* **173**, 605 (1948)]. Twice recrystallized enzyme was passed through DEAE-Sephadex [A. H. Gelderman, H. V. Gelboin, A. C. Peacock, *J. Lab. Clin. Invest.* **65**, 132 (1965)], and the muscle ($M_4$) enzyme was isolated. Yeast enolase, a gift from P. A. Hargrave and Dr. F. Wold, was prepared from baker's yeast by the methods of E. W. Westhead [*Methods Enzymol.* **9**, 670 (1966)] and E. W. Westhead and G. McLain [*J. Biol. Chem.* **239**, 2464 (1964)]. Pyruvate kinase A (M) was prepared from rabbit muscle by the method of T. Tanaka, Y. Harano, F. Sue, H. Morimura [*J. Biochem.* **62**, 71 (1967)]. Enzymatic activities were determined at 25°C on a recording spectrophotometer by the following procedures: for aldolase A, R. Blostein and W. J. Rutter [*J. Biol. Chem.* **238**, 3280 (1963)]; for glyceraldehyde-3-phosphate dehydrogenase, S. F. Velick [*Methods Enzymol.* **1**, 401 (1955)]; for yeast aldolase, O. C. Richards and W. J. Rutter [*J. Biol. Chem.* **236**, 3177 (1961)]; for lactate dehydrogenase, A. Kornberg [*Methods Enzymol.* **1**, 441 (1955)]: for yeast enolase, E. W. Westhead [*ibid.* **9**, 670 (1966)]; and for pyruvate kinase, T. Bucher and G. Pfleiderer [*ibid.* **1**, 435 (1955)].
12. Protein concentrations were determined by absorbance at 280 nm, assuming $E^{1\%}_{cm} = 10$. Since the ampholine carrier compounds absorb at 280 nm, proteins from electrofocusing columns were estimated by subtracting the average baseline absorbance of the ampholine.
13. Supported in part by PHS grant HD-02126.
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## Diphosphonates Inhibit Hydroxyapatite Dissolution in vitro and Bone Resorption in Tissue Culture and in vivo

Abstract. *Two diphosphonates containing the P–C–P bond, $Cl_2C(PO_3HNa)_2$ and $H_2C(PO_3HNa)_2$ retard the rate of dissolution of apatite crystals in vitro. They inhibit bone resorption induced by parathyroid extract in mouse calvaria in tissue culture and in thyroparathyroidectomized rats in vivo.*

Inorganic pyrophosphate inhibits the precipitation (*1, 2*) and the dissolution (*3*) of hydroxyapatite crystals in vitro. Because pyrophosphate is present in plasma (*4*), teeth (*5*), and bone (*6*), we have suggested (*2*) that it might regulate both the formation and destruction of mineralized tissues in vivo. Although pyrophosphate and longer-chain condensed phosphates can inhibit the deposition of calcium phosphate in chick embryo femurs in tissue culture (*7*) and in the aorta (*8*) and skin (*9*) of the rat, it has not yet been possible to demonstrate an effect on the resorption of living bone. This failure to influence bone resorption may be due to hydrolysis of the P–O–P bond locally in the bone by pyrophosphatase before it can reach its site of action.

Substances were therefore sought that would be related in structure to pyrophosphate but resistant to chemical and enzymatic hydrolysis. Various compounds containing the P–C–P bond have been synthesized and found to have an effect similar to that of condensed phosphates on the precipitation of hydroxyapatite in vitro and on calcification in vivo (*10*). In addition, they were also active when administered orally (*10*). We now describe the effect of two such compounds containing a P–C–P bond, namely, sodium dichloromethylenediphosphonate [$Cl_2C(PO_3HNa)_2$] and sodium methylenediphosphonate [$H_2C(PO_3HNa)_2$], on the dissolution of apatite crystals in vitro and on bone resorption induced by parathyroid extract in tissue culture and

Table 1. Effect of $Cl_2C(PO_3HNa)_2$, $H_2C(PO_3HNa)_2$, and $CH_3(CH_2)_4PO_3HNa$ on resorption of mouse calvaria in tissue culture. Resorption was assessed by a point-counting method with the use of a grid. The resorption in all groups was compared with those subjected to PTE alone, and the significances of the differences were determined by Student's *t*-test. Percentages are given as means ± S.E. The asterisk denotes differences significant at less than 2 percent level.

| Dosage ($\mu$g of P per ml) | Calvaria (No.) | Total bone resorbed (%) |
|---|---|---|
| | *No PTE* | |
| | 16 | 7.8 ± 0.6* |
| | *PTE* | |
| | 85 | 21.3 ± 0.7 |
| | *PTE + $Cl_2C(PO_3HNa)_2$* | |
| 0.01 | 6 | 18.3 ± 2.5 |
| 0.1 | 13 | 10.1 ± 1.0* |
| 1 | 11 | 10.2 ± 0.8* |
| | *PTE + $H_2C(PO_3HNa)_2$* | |
| 0.1 | 10 | 19.4 ± 1.4 |
| 1 | 12 | 16.2 ± 2.4* |
| | *PTE + $CH_3(CH_2)_4PO_3HNa$* | |
| 10 | 9 | 17.6 ± 1.9 |
| 1 | 13 | 24.0 ± 2.0 |

in living rats. A monophosphonate, sodium pentane-1-phosphonate [$CH_3(CH_2)_4PO_3HNa$], which contains only a single C–P bond, as well as pyrophosphate and polyphosphate, was used as a control.

Hydroxyapatite crystals similar to those used previously (*2, 3*) were equilibrated for 15 hours at 37°C in a solution of 0.155*M* KCl buffered at *p*H 7.0 with 0.01*M* barbital. A concentrated solution of $H_2C(PO_3HNa)_2$, $Cl_2C(PO_3HNa)_2$, $CH_3(CH_2)_4PO_3HNa$ or pyrophosphate dissolved in the same buffer was added slowly with stirring over 2 hours until the final amount added represented 5 percent of the total phosphate of the apatite present (*3*). The crystals were filtered and then resuspended in a similar buffer containing no calcium, phosphate, phosphonates, or pyrophosphate. Control crystals, treated identically except that no phosphonates or pyrophosphate had been added, were resuspended in a similar manner. Addition of the diphosphonates and pyrophosphate to apatite crystals resulted in reduction of the rate at which calcium and phosphate entered the solution (Fig. 1); the monophosphonate had no effect.

Calvaria from 3-day-old mice were cultured individually for 5 days in 2 ml of modified BGJ medium (*11*) containing heat-inactivated horse serum (5 percent). The medium was changed each day. Parathyroid extract (PTE Parathormone, Lilly) added at a concentration of 0.2 unit/ml induced detectable resorption in such bones. This resorption, as assessed quantitatively by a point counting method, could be inhibited by $Cl_2C(PO_3HNa)_2$ or $H_2C(PO_3HNa)_2$ at concentrations as low as 0.1 and 1.0 $\mu$g of P per milliliter, respectively (Table 1); $CH_3(CH_2)_4PO_3HNa$, in contrast, was not inhibitory even at concentrations as high as 10 $\mu$g of P per milliliter (Table 1). Pyrophosphate and polyphosphates also have no effect at similar concentrations. Histologic and electron microscopic surveys failed to reveal any toxic action of the phosphonates on the cultured bones; however, this does not exclude subtle metabolic effects.

Male Wistar rats (100 to 130 g) were thyroparathyroidectomized surgically and maintained for about 4 days on a diet poor in calcium and phosphate. The drinking water was supplemented with 2 percent (weight to volume) calcium gluconate. For 36 hours before the experiment the rats were allowed to drink distilled water only. On the day of the experiment a blood sample was taken, and the animals were then injected subcutaneously with 50 units of PTE per 100 g of body weight. Six hours later a second blood sample was taken, and its calcium concentration was compared with the first. Parathyroid extract induced a significant rise in plasma calcium (Table 2). This rise could be completely prevented by $Cl_2C(PO_3HNa)_2$ and $H_2C(PO_3HNa)_2$ at doses of 10 mg of P per kilogram of body weight, given subcutaneously daily for 3 days up to but not including the day on which the parathyroid extract was given

Table 2. Influence of $H_2C(PO_3HNa)_2$, $Cl_2C(PO_3HNa)_2$, and $CH_3(CH_2)_4PO_3HNa$, given at 10 mg of P per kilogram body weight per day, on the rise in plasma calcium induced by 50 units of parathyroid extract (PTE) per 100 g of body weight in thyroparathyroidectomized rats. The significances of the differences in plasma calcium between the animals treated with PTE alone and the other groups were determined by Student's *t*-test. SC, subcutaneous.

| Treatment | | Animals (No.) | Plasma calcium (mg per 100 ml) (mean ± SE) | | Mean change ± SE | *P* |
|---|---|---|---|---|---|---|
| Material | Route | | Before PTE | Six hours after PTE treatment | | |
| No PTE | | 14 | 6.59 ± .26 | 6.27 ± .33 | −0.32 ± .29 | <.01 |
| PTE alone | | 47 | 6.27 ± .15 | 8.22 ± .29 | +1.95 ± .24 | |
| PTE + $H_2C(PO_3HNa)_2$ | SC | 14 | 5.92 ± .41 | 6.10 ± .33 | +0.18 ± .25 | <.01 |
| PTE + $H_2C(PO_3HNa)_2$ | Oral | 14 | 5.13 ± .35 | 6.29 ± .46 | +1.16 ± .28 | <.05 |
| PTE + $Cl_2C(PO_3HNa)_2$ | SC | 14 | 5.41 ± .40 | 5.70 ± .34 | +0.29 ± .16 | <.01 |
| PTE + $Cl_2C(PO_3HNa)_2$ | Oral | 14 | 6.05 ± .34 | 7.11 ± .48 | +1.06 ± .26 | <.05 |
| PTE + $CH_3(CH_2)_4PO_3HNa$ | SC | 14 | 5.30 ± .40 | 7.13 ± .68 | +1.83 ± .38 | |

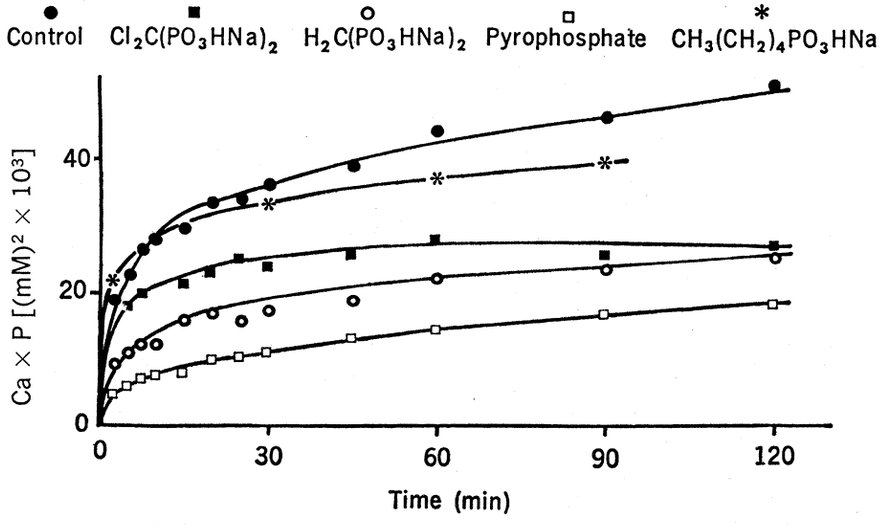


Fig. 1. Effect of pyrophosphate, two diphosphonates [$Cl_2C(PO_3HNa)_2$ and $H_2C(PO_3HNa)_2$] and a monophosphonate [$CH_3(CH_2)_4PO_3HNa$] on the dissolution of hydroxyapatite crystals in vitro.

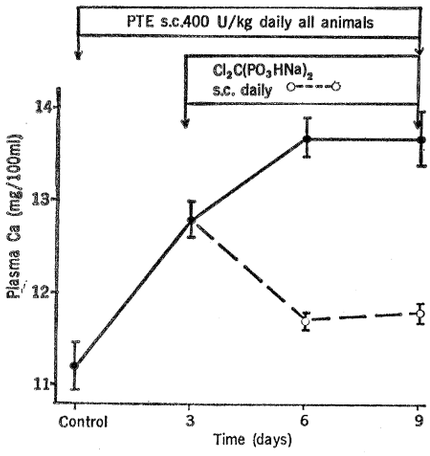


Fig. 2. Influence of $Cl_2C(PO_3HNa)_2$ (5 mg of P per kilogram of body weight, subcutaneously) on the hypercalcemia of rats treated for 9 days with parathyroid extract.

(Table 2). The compounds were also effective when given orally; $CH_3(CH_2)_4$-$PO_3HNa$ had no significant effect on the increase in plasma calcium under similar conditions (Table 2). Pyrophosphate and polyphosphates given at 10 and 30 mg also have no effect. The $Cl_2C(PO_3HNa)_2$ can also reverse the hypercalcemia in rats given prior treatment with PTE for several days (*12*) (Fig. 2).

Our experiments indicate that diphosphonates and pyrophosphate retard the dissolution of hydroxyapatite crystals in vitro. The diphosphonates, but not pyrophosphate and polyphosphates, also inhibit the resorption of living bone in several experimental systems. For instance, diphosphonates can prevent the development of immobilization osteoporosis in rats (*13*). Furthermore, some diphosphonates reduce plasma calcium values in normal or thyroparathyroidectomized rats fed a low calcium diet (see for example Table 2). This indicates that the diphosphonates can reduce bone resorption both in the presence or the absence of parathyroid hormone.

The diphosphonates, which contain a P–C–P bond, differ from pyrophosphate and longer-chain condensed phosphates, which contain P–O–P bonds, in that the latter do not inhibit bone resorption in tissue culture and in vivo. It is possible that this difference is due to the hydrolysis of the condensed phosphates when they are given parenterally or added to tissue culture. They would therefore be unable to reach their potential site of action in bone. Pyrophosphatases from bone and other tissues are unable to split the phosphonates in vitro and it may be that phosphonates act on bone in tissue culture and in vivo because they are resistant to such hydrolysis. The simplest explanation of the mechanism of action of diphosphonates on bone resorption is that they retard crystal dissolution in a manner similar to that of pyrophosphate (*3*). Other mechanisms are, however, possible.

To our knowledge the diphosphonates are still the only substances, apart from thyrocalcitonin (*14*), that can significantly inhibit bone resorption in vivo, although fluoride (*15*), orthophosphate (*16*) and estrogens (*17*) have been studied for their potential therapeutic effect in this respect. Since diphosphonates appear to be relatively nontoxic they might prove valuable in the treatment of osteoporosis and other human diseases that involve increased resorption of bone.

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## Diphosphonates Inhibit Formation of Calcium Phosphate Crystals in vitro and Pathological Calcification in vivo

Abstract. *Two diphosphonates containing the P–C–P bond, $CH_3C(OH)(PO_3HNa)_2$ and $H_2C(PO_3HNa)_2$, inhibit the crystallization of calcium phosphate in vitro and prevent aortic calcification of rats given large amounts of vitamin $D_3$. The diphosphonates therefore have effects similar to those described for compounds containing the P–O–P bond but are active when administered orally.*

Low concentrations of pyrophosphate inhibit the precipitation of hydroxyapatite crystals in vitro (*1*, *2*). Pyrophosphate is present in plasma at concentrations which are inhibitory in vitro (*3*), and therefore this compound might be one of the physiological agents responsible for preventing the deposi-

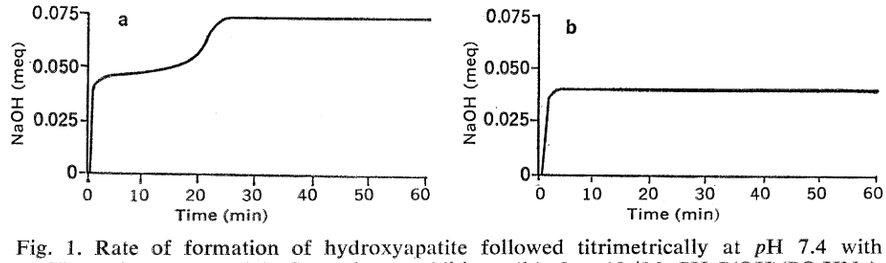


Fig. 1. Rate of formation of hydroxyapatite followed titrimetrically at *p*H 7.4 with a *p*H-stat instrument. (a) Control, no addition; (b) $2 \times 10^{-4} M$ $CH_3C(OH)(PO_3HNa)_2$ present.