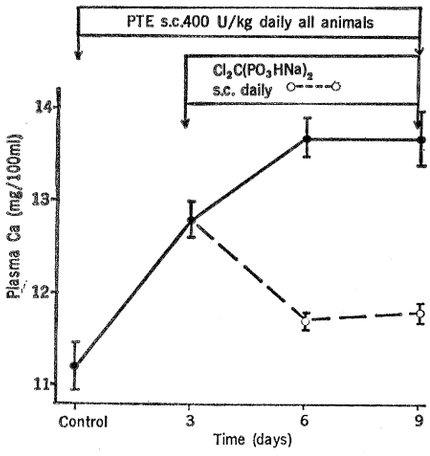


Fig. 2. Influence of $Cl_2C(PO_3HNa)_2$ (5 mg of P per kilogram of body weight, subcutaneously) on the hypercalcemia of rats treated for 9 days with parathyroid extract.

(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-

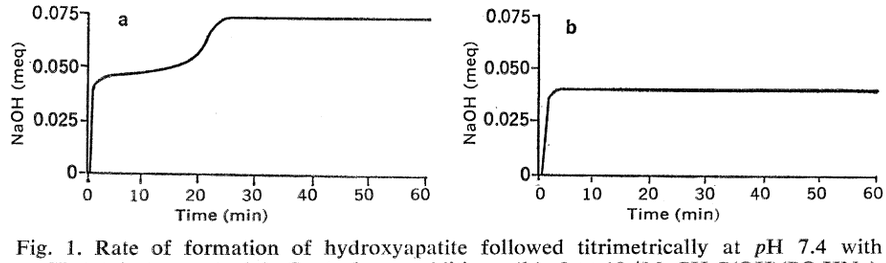


Fig. 1. Rate of formation of hydroxyapatite followed titrimetrically at *p*H 7.4 with a *p*H-stat instrument. (a) Control, no addition; (b) $2 \times 10^{-4} M$ $CH_3C(OH)(PO_3HNa)_2$ present.

tion of calcium phosphate in soft tissues in vivo (*3*). In rats, pyrophosphate and longer-chain condensed phosphates (polyphosphates) indeed inhibit aortic calcification induced by excess vitamin $D_3$ (*4, 5*) and skin calcification induced by various other means (*6*). However pyro- and polyphosphates do not inhibit the nephrocalcinosis produced by the vitamin $D_3$ (*5*) and are not active when given orally. This is attributed to the hydrolysis of the P–O–P bond by pyrophosphatases in the kidney and intestine. Therefore, compounds related to pyrophosphate in structure but stable to chemical and enzymatic hydrolysis were sought and tested for their effect on precipitation of hydroxyapatite and on heterotopic calcification in vivo. Of the many compounds synthesized and tested, those that possess the P–C–P bond instead of the P–O–P bond seem to meet this requirement. Two such diphosphonates, namely sodium ethane-1-hydroxy-1,1-diphosphonate [$CH_3C(OH)(PO_3HNa)_2$] and sodium methylene diphosphonate [$H_2C(PO_3HNa)_2$], have been examined, and their effects were compared with those of a monophosphonate, sodium *n*-pentane-1-phosphonate [$CH_3(CH_2)_4PO_3HNa$], which possesses only a single C–P bond. We studied the effects of these compounds on the precipitation and crystallization of hydroxyapatite in vitro and on the calcification of rat aortas in vivo.

The rate of formation of hydroxyapatite was followed titrimetrically by the consumption of base by means of a radiometer *p*H-stat instrument. Solutions of calcium chloride and sodium phosphate were mixed to obtain a final concentration of 4 mmole/liter in each at zero time. The amount of NaOH required to keep the *p*H constant at 7.4 was then continuously recorded as a function of time. In this system precipitation and crystal growth occur in two distinct stages (Fig. 1a). The first, which corresponds to the initial rise in consumption of NaOH, represents the formation of amorphous calcium phosphate; the second, which corresponds to the second rise in consumption of NaOH, represents the crystal growth of hydroxyapatite. At one-tenth to one-fiftieth the initial concentrations of calcium or phosphate, $CH_3C(OH)(PO_3HNa)_2$ (Fig. 1b) and $H_2C(PO_3HNa)_2$ as well as inorganic pyrophosphate completely inhibit the second stage but do not influence the first. In contrast, the monophosphonate [$CH_3(CH_2)_4PO_3HNa$] and disodium ethylenediamine-

Table 1. Influence of $CH_3C(OH)(PO_3HNa)_2$, $H_2C(PO_3HNa)_2$ and $CH_3(CH_2)_4PO_3HNa$, given orally or subcutaneously, on aortic calcification induced by vitamin $D_3$ in rats. In all cases, the dosage was 10 mg of P per kilogram of body weight. Because the calcium values were not normally distributed, the significances (*P*) of the differences from the vitamin $D_3$–treated controls were determined by Student's *t*-test, with the use of the log calcium values. The numbers of animals are in parentheses. Control 1, no vitamin $D_3$; control 2, vitamin $D_3$ only. All other rats were treated with vitamin $D_3$ and phosphonates.

| Substance | Calcium in aorta ($\mu$g/dry weight) | | |
|---|---|---|---|
| | ($\mu$g/mg) | (log $\mu$g/g) | *P* |
| Control 1 | 5.4 ± 2.6 (26) | 3.25 ± 0.10 | <.001 |
| Control 2 | 68.3 ± 7.6 (51) | 4.64 ± 0.07 | |
| *Subcutaneous administration* | | | |
| $CH_3C(OH)(PO_3HNa)_2$ | 2.2 ± 0.4 (9) | 3.33 ± 0.06 | <.001 |
| $H_2C(PO_3HNa)_2$ | 2.5 ± 1.1 (13) | 3.18 ± 0.11 | <.001 |
| $CH_3(CH_2)_4PO_3HNa$ | 79.1 ± 25.3 (9) | 4.59 ± 0.22 | >.6 |
| *Oral administration* | | | |
| $CH_3C(OH)(PO_3HNa)_2$ | 2.7 ± 0.6 (6) | 3.38 ± 0.09 | <.001 |
| $H_2C(PO_3HNa)_2$ | 3.8 ± 1.5 (17) | 3.28 ± 0.11 | <.001 |
| $CH_3(CH_2)_4PO_3HNa$ | 64.0 ± 16.5 (8) | 4.66 ± 0.15 | >.9 |

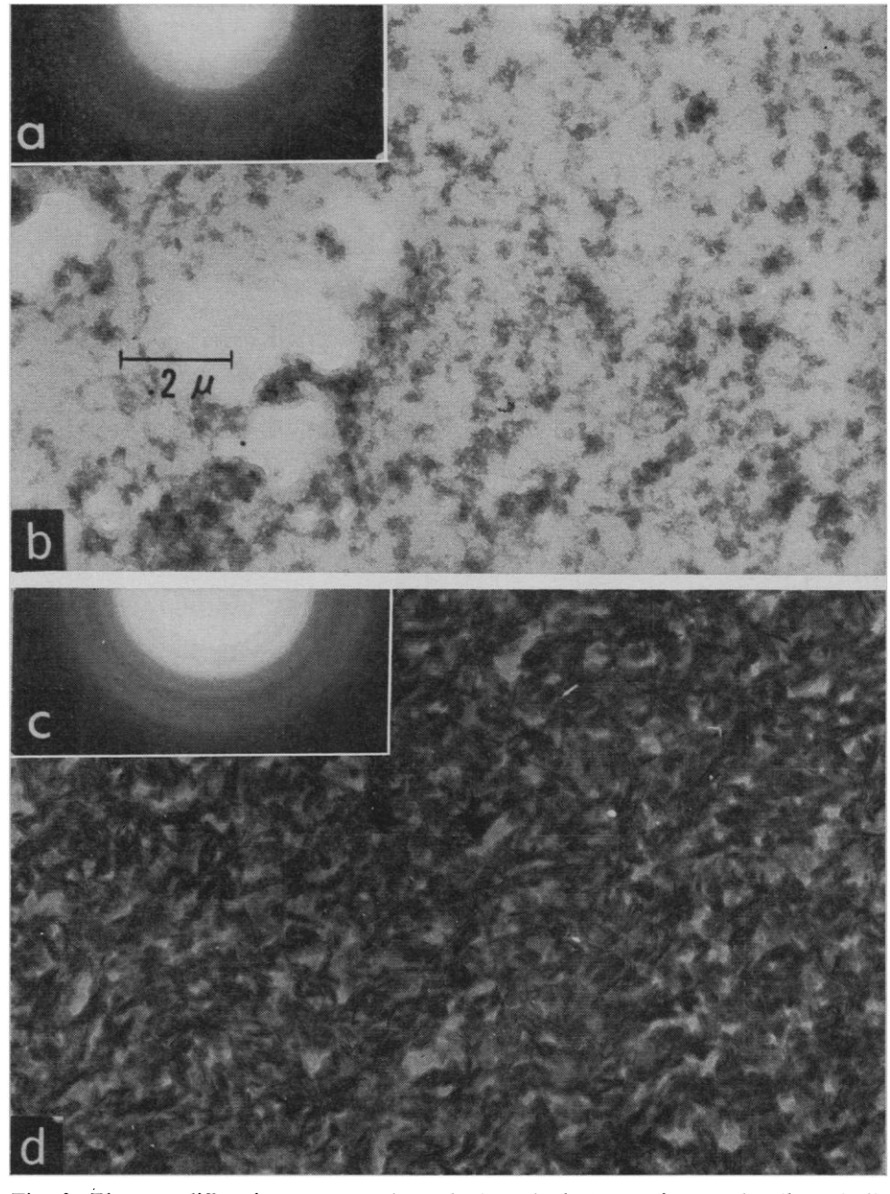


Fig. 2. Electron diffraction patterns (a and c) and electron micrographs (b and d) of freshly precipitated calcium orthophosphate. The precipitates were obtained at 22 hours from solutions of $CaCl_2$ and $NaH_2PO_4$ which, after mixing, were $4 \times 10^{-3}M$, $pH = 7.4$ and at 25°C; (a and b) with $2 \times 10^{-4}M$ $CH_3C(OH)(PO_3HNa)_2$; (c and d) without $CH_3C(OH)(PO_3HNa)_2$.

tetraacetate (EDTA) have no effect on either stage. Electron microscopy and electron diffraction showed that $CH_3C(OH)(PO_3HNa)_2$ (Fig. 2), $H_2C(PO_3HNa)_2$, and pyrophosphate inhibit the crystal growth of hydroxyapatite but that the monophosphonate and EDTA do not. The inhibitory effect of the diphosphonates on the crystal growth of hydroxyapatite is shown by the pattern obtained by electron diffraction and the gel-like appearance of the calcium phosphate in the electron microscope—both features typical of the amorphous form (*7, 8*).

Pyrophosphate increases the minimum product (Ca × P) required to induce precipitation of calcium phosphate from solution under defined conditions (*1*). The two diphosphonates have similar, striking effects at concentrations as low as $10^{-7}M$; the monophosphonate has only a small effect.

Aortic calcification was induced in rats by treating them by mouth daily with 75,000 I.U. of vitamin $D_3$ per kilogram of body weight for 5 days (*4, 5*). The phosphonates—$CH_3C(OH)(PO_3HNa)_2$, $H_2C(PO_3HNa)_2$, or $CH_3(CH_2)_4PO_3HNa$ (10 mg of P per kilogram of body weight)—were given subcutaneously or by mouth daily from 2 days before vitamin $D_3$ was administered and continued until 7 days after administration of the vitamin D; the animals were then killed. Calcification of the aortas was assessed by measuring calcium content of aortas ashed at 600°C. When given orally or subcutaneously, the two diphosphonates completely prevent calcification (Table 1); the monophosphonate does not prevent calcification by either route.

Our data show that diphosphonates have effects similar to those of pyrophosphate and condensed phosphates on the inhibition of crystal growth of hydroxyapatite in vitro (*1, 9, 10*) and on the prevention of aortic calcification in rats given large doses of vitamin $D_3$ (*5*). In addition, they possess properties not shared with the condensed phosphates. Thus, not only do they prevent aortic calcification when given by mouth rather than subcutaneously, but they also prevent kidney calcification (*10*) when given by either route.

Studies with other phosphonate compounds have shown that there is a good correlation between the ability of any single compound to prevent hydroxyapatite crystal growth in vitro and its ability to prevent calcification in vivo (*10*). This strengthens the view that the biological activity of these compounds and of pyrophosphate is a consequence of their observed action on crystal growth. The most likely mechanism of action of the diphosphonates both in vivo and in vitro is strong chemisorption on hydroxyapatite, as demonstrated with $^{14}C$-ethanehydroxydiphosphonate on synthetic apatite (*8*). The compounds that have so far proved most active have all contained the P–C–P bond; $CH_3(CH_2)_4PO_3HNa$, which contains a single C–P bond, is ineffective. The fact that EDTA has no effect shows that the inhibition of crystal growth is not determined solely by the chelation properties of the P–C–P materials and of EDTA itself. Since the molecular configuration of the P–C–P bond is close to that of the P–O–P bond, the diphosphonates, pyrophosphate, and polyphosphates may all act on crystal growth by a similar chemisorption mechanism. The observation that the diphosphonates, unlike the polyphosphates, are effective when given orally and are able to inhibit kidney calcification may be attributed to their resistance to either chemical or enzymatic hydrolysis.

The diphosphonates might provide a convenient model for investigating the action of pyrophosphate in calcium homeostasis. Since the diphosphonates are of low toxicity, they might also be used against diseases in which calcium salts deposit in soft tissues. The administration of $CH_3C(OH)(PO_3HNa)_2$ to two patients in the acute phase of myositis ossificans (*11*) has been associated with an arrest in the progress of the disease.

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## Ant Alarm Pheromone Activity: Correlation with Molecular Shape by Scanning Computer

Abstract. *The ant* Iridomyrmex pruinosus *utilizes 2-heptanone as an alarm pheromone. The activities of 49 ketones and 35 nonketones as alarm pheromones for this species were determined. The molecular shapes of these compounds were assessed by submitting silhouette photographs of their molecular models to a pattern recognition machine. A highly significant correlation exists between molecular shape and alarm activity.*

The alarm pheromones employed by certain species of ants provide extraordinarily favorable tools with which to examine the relationships between chemical constitution and biological activity (*1*). One of us (M.S.B.) has collected a large amount of data on biological activity, using chemical relatives of 2-heptanone, the alarm pheromone of *Iridomyrmex pruinosus* (*2*). The results showed clearly that the behavioral response, while far from exclusive to 2-heptanone, is nevertheless specific to a restricted range of compounds. Among current theories of olfaction, the stereochemical theory seemed to hold considerable promise of being able to accommodate the experimental data (*3*). Accordingly, the compounds employed in the survey were subjected to a stereochemical analysis of their molecular shapes by another author (J.E.A.). These assessments of chemical constitution were rendered impartial and quantitative by submitting molecular model silhouette photographs to the other collaborators (G.P. and E.W.) for scanning by the PAPA pattern recognition machine (*4*).

Highly significant correlations were