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THE PYROPHOSPHATASE AND (Ca^{2+}-Mg^{2+})-ATPase ACTIVITY OF PURIFIED CALF BONE ALKALINE PHOSPHATASE

R. FELIX and H. FLEISCH

Department of Pathophysiology, University of Berne, Berne (Switzerland)

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SUMMARY

The pyrophosphatase and (Ca^{2+}–Mg^{2+})-ATPase (ATP phosphohydrolase, EC 3.6.1.3) activities of highly purified calf bone alkaline phosphatase were studied.

While Mg^{2+} inhibits pyrophosphatase appreciably at alkaline pH, this inhibition is negligible at neutral pH. Thus activity is found in the conditions used for measuring neutral inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1).

When assayed at the PP_i and Mg^{2+} concentrations found in plasma ($3.5 \cdot 10^{-6}$ M and 0.4 mM) the pH optimum was 7.0 in contrast to pH 8.5 at 2 mM PP_i. Ca^{2+} inhibited slightly, whereas orthophosphate inhibited very strongly, especially if HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid) buffer was used. Approximately 6 times less activity and a weaker inhibition by orthophosphate was found in the presence of Tris–maleate buffer. The diphosphonates EHDP (ethane-1-hydroxy-1,1-diphosphonate) and Cl_2MDP (dichloromethylene diphosphonate) also inhibited at 10^{-4} M. Parathyroid hormone and calcitonin were without effect.

At optimal Mg^{2+} concentrations Ca^{2+} did not activate but inhibited slightly the ATPase activity. At suboptimal Mg^{2+} concentrations, and in the absence of Mg^{2+}, Ca^{2+} stimulated the ATPase activity, but without reaching maximal activity. Thus bone alkaline phosphatase is not a "true" (Ca^{2+}–Mg^{2+})-ATPase.

INTRODUCTION

Based on the finding of raised urinary [1] and plasma [2] pyrophosphate levels in hypophosphatasia, a disease characterised by a deficiency in alkaline phosphatase, Russell [1] suggested that alkaline phosphatase possessed pyrophosphatase activity. Since then, several workers [3–11] have demonstrated that inorganic pyrophosphate (PP_i) can be split by alkaline phosphatases isolated from various tissues including bone [3, 8, 11].

This property of the enzyme might be of special biological importance in calcifying tissues as PP_i, at a concentration similar to that found in plasma [2], has the

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid; EHDP, ethane-1-hydroxy-1,1-diphosphonate; Cl_2MDP, dichloromethylene diphosphonate.

property of effectively inhibiting the growth of calcium phosphate crystals [12]. It has been suggested that PP_i might prevent calcification in soft tissue and that one of the functions of alkaline phosphatase in calcifying tissue could be to destroy this calcification inhibitor [13]. Thus the "calcification enzyme" [14] alkaline phosphatase would work through its pyrophosphatase activity.

Alkaline phosphatases have also been shown to hydrolyse ATP [10, 11, 15, 16]. The ATPase activity of intestinal alkaline phosphatase has been found to be calcium activated and it has been suggested that alkaline phosphatase might be involved in calcium transport [17, 18].

The aim of this study was (a) to investigate the pyrophosphatase activity of highly purified bone alkaline phosphatase in various conditions with special emphasis on conditions mimicking those present locally in vivo and (b) to assess if the ATPase activity was activated by calcium.

The effect of two diphosphonates on the activities of the enzyme were studied. These compounds have recently been shown to inhibit bone resorption [19, 20] and vitamin D induced soft tissue calcification [21]. When given in large amounts, some diphosphonates also inhibit mineralization occurring normally in hard tissues producing a morphological picture similar to that of vitamin D deficiency [22]. It has been suggested that an inhibition of the phosphatase might play a role in these effects since diphosphonates are closely related in structure to pyrophosphate [23].

The effect of parathyroid hormone and calcitonin were assessed, since these hormones have been reported [24] to have an effect on the pyrophosphatase activity of ascites tumour cells.

MATERIALS

Calf femora and humeri were obtained from a local source. If not used immediately they were frozen at −20 °C. Diaflo membranes UM 10 or UM 20 E Amicon N.V., Oosterhout, Holland, or a collodion bag of Schleicher and Schull, 3354 Dassel, W-Germany, were used to concentrate protein solutions. Diethylaminoethyl cellulose DE 52 of Whatman, Maidstone, England, was used for ion-exchange chromatography, Sephadex G-200, Uppsala, Sweden, for gel filtration. Specialised reagents used were obtained as follows: bovine serum albumin from Fluka AG, Buchs SG, Switzerland; HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) from Sigma, St. Louis, Mo 63118, U.S.A.; $Na_4{}^{32}P_2O_7$, specific activity 456 mCi/mole from New England Nuclear, Dreieichenhain, W-Germany (stored frozen and diluted with cold pyrophosphate to give the desired specific activity immediately before use); sodium ATP from Calbiochem, Los Angeles, Calif. 90045, U.S.A.; disodiumethane-1-hydroxy-1,1-diphosphonate (EHDP) and disodium dichloromethylene diphosphonate (Cl_2MDP) from the Procter and Gamble Company, Cincinnati, Ohio 45239, U.S.A.; bovine parathyroid hormone (250 units/mg) from Wilson Laboratories, Chicago, Ill. 60609, U.S.A.; and salmon calcitonin (1.8 MRC units/mg) was a gift from Dr. H. Copp, Vancouver. All other reagents were of analytical grade from E. Merck AG, Darmstadt, W-Germany. Parathyroid hormone was dissolved with ten times its weight of bovine serum albumin in a stock solution containing 100 ml 0.001 M HCl, 2.0 g glucose, 500 mg glutathione. The calcitonin was dissolved in 0.16 M acetic acid, pH 5.4, containing 0.1 % bovine serum albumin.

METHODS

Enzyme purification

The enzyme was extracted from the spongiosa of the condyles and the heads of calf femora and humeri with 5 vol. of cold water and with 5 vol. of cold *n*-butanol and tissue fragments were removed by centrifugation. The butanol and aqueous phases were left to separate overnight. The aqueous phase was dialysed against 0.01 M Tris–HCl, pH 8.5, and concentrated on an Amicon filter (12.4 mg protein per ml). Saturated $(NH_4)_2SO_4$ solution, pH 8.6, was added with stirring. The enzyme precipitated between 60–80% $(NH_4)_2SO_4$. After centrifugation the pellet was dissolved in, and dialysed against, 0.005 M Tris–HCl, pH 7.5 and applied to a DEAE-cellulose column, equilibrated with the same buffer. After the non-adsorbed protein had been eluted, a linear gradient 0–0.2 M in NaCl was started. *p*-Nitrophenyl phosphatase and pyrophosphatase activity came out in the same peak. The active fractions were concentrated, dialysed and applied to a Sephadex G-200 column, equilibrated with 0.01 M Tris–HCl, pH 8.5, 0.1 M NaCl. *p*-Nitrophenyl phosphatase and pyrophosphatase activity again appeared in the same peak just in front of a large protein peak. The first fractions with the highest specific activity and well separated from this protein were collected and used for this study. The purification is summarized in Table I.

TABLE I

PURIFICATION OF BONE ALKALINE PHOSPHATASE

	Total activity (units)	Specific activity (units/mg)	Purification ratio	% of initial enzyme recovered
Concentrated extract	8000	0.57	1	100
60–80% $(NH_4)_2SO_4$ precipitation	6100	1.40	2.5	76
DE-52 cellulose	4240	6.5–8.8	13.5	53
Sephadex G-200	2400	330	580	30

The enzyme preparation was further characterized. The molecular weight was 216 000, a value similar to the one found for human bone alkaline phosphatase [11]. Contamination of the pyrophosphatases other than alkaline phosphatase was shown to be negligible as the *p*-nitrophenyl phosphatase, pyrophosphatase and Mg^{2+}-ATPase activities were reduced at very similar rate during heating at 60 °C.

Enzyme assays

Alkaline phosphatase was determined according to Richterich [25] in a medium containing 0.05 M ammediol–HCl (= 2-amino-2-methyl-1,3-propanediol–HCl), pH 10.3, 35 mM Na_2CO_3, 1 mM $MgCl_2$, 3.8 mM p-nitrophenyl phosphate. The final volume was 0.5 ml. After incubation for 10 min at 37 °C with 10 μl of enzyme, the reaction was stopped with 2 ml 0.1 M NaOH and the absorption measured at 405 nm. 1 unit corresponds to 1 μmole *p*-nitrophenyl phosphate hydrolysed per min.

Pyrophosphatase activity was usually determined in a medium containing 2 mM sodium pyrophosphate (PP_i), 0.1 M ammediol–HCl, pH 8.85, 1.6 mM $MgCl_2$ in

a final volume of 1 ml. Any modifications of this medium are described in the text. After incubation for 15 min at 37 °C the reaction was stopped with 1 ml of cold 20% trichloroacetic acid. The phosphate released was determined according to the method of Baginski et al. [26]. After addition of the ammonium molybdate solution it was found necessary to wait at least 2.5 min before adding the citrate–arsenite solution. Absorbance was determined at 700 nm after standing 25 min at room temperature. Under the conditions used the amount of phosphate released was proportional to the amount of enzyme added. PP_i at the concentrations used did not interfere with the P_i determination. 1 unit corresponds to 1 μmole PP_i split per min.

Pyrophosphatase activity was also determined under conditions similar to those existing in plasma ultrafiltrate, i.e. at a substrate concentration of $3.5 \cdot 10^{-6}$ M pyrophosphate [2] and a Mg^{2+} concentration of 0.4 mM. In this case ^{32}P labelled pyrophosphate was used. The final volume of the incubation medium was 1 ml. Further details of the medium are described in the text. After the enzyme reaction phosphate and pyrophosphate were separated by the method of Sugino and Miyoshi [27] modified according to Fernley and Bisaz [5]. 1 ml solution was put in 10 ml 0.7 M NH_4OH solution and the radioactivity measured by Cerenkov radiation [28] in a Packard Tri-Carb liquid scintillation spectrometer, Model 2524. In later experiments phosphate and pyrophosphate were separated with a isobutanol–petrolether mixture and an acid ammonium molybdate solution [29].

ATPase activity was measured in the presence of an ATP concentration of 5 mM. The final volume of the incubation medium was 0.5 ml. Buffer and metal ion concentrations are indicated in the description of the experiments concerned. Incubation was carried out for 15 min at 37 °C and the phosphate (P_i) released was determined by the method of Wöltgens et al. [30] or Baginski et al. [26]. The amount of phosphate released was proportional to the amount of enzyme added under the conditions used.

Chemical analyses

Protein was determined by ultraviolet absorption [31] up to the stage of $(NH_4)_2SO_4$ fractionation and, at later steps in the purification, by the method of Lowry et al. [32]. Column eluates were monitored automatically by a LKB 8300 A Uvicord II, and the ultraviolet absorption of critical fractions was also measured in a Beckman spectrophotometer DU, model G 2400.

RESULTS

Dependence of the pyrophosphatase activity on pH and magnesium concentration at 2 mM PP_i

Fig. 1 shows the effects of varying magnesium concentration on the pyrophosphatase activity over the pH range 7.0–10.0. The same optimal conditions were found as for other alkaline phosphatase preparations. Around neutral pH higher concentrations of magnesium were no longer inhibitory. Since this might have been due to the binding of magnesium by maleate, the experiment at pH 7.5 was repeated using Tris–HCl buffer. Although the activity found was approximately double that found with Tris–maleate the inhibition of magnesium was again nearly absent. Thus it appeared that alkaline phosphatase would have some pyrophosphatase activity under the con-

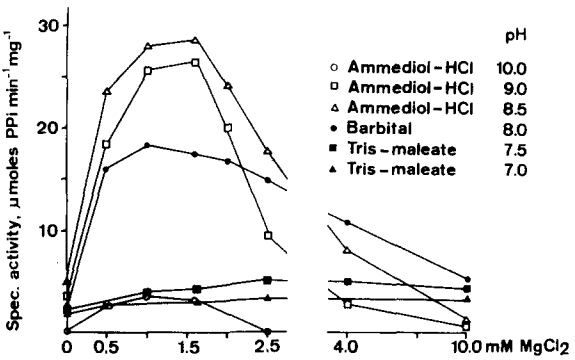

Fig. 1. Influence of Mg^{2+} and pH on pyrophosphatase activity of alkaline phosphatase. Pyrophosphate concentration 2 mM, buffer concentration 0.1 M, except barbital, which was 0.05 M.

ditions used for determining inorganic pyrophosphatase (EC 3.6.1.1) [33] that is at 2 mM pyrophosphate, 10 mM $MgCl_2$, 0.1 M Tris–maleate, pH 7.2. This activity was assessed and found to be about 14 and 2% respectively of the values obtained under optimal conditions for the alkaline pyrophosphatase and alkaline *p*-nitrophenyl phosphatase activity.

Pyrophosphatase activity at physiological substrate concentration

Fig. 2 shows the variation in activity with change in pH at a pyrophosphate concentration of $3.5 \cdot 10^{-6}$ M and at 0.4 mM Mg^{2+}. The curve differed substantially from that obtained if optimal pyrophosphate concentrations were used, having a max-

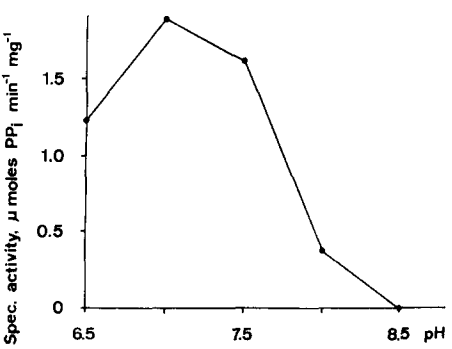

Fig. 2. Effect of pH on the pyrophosphatase activity at a pyrophosphate concentration of $3.5 \cdot 10^{-6}$ M, 0.4 mM $MgCl_2$, 0.05 M HEPES.

imum in the physiological range instead of around pH 8.5. In the experiment shown 0.05 M HEPES buffer was used whereas if Tris–maleate buffer was employed a similar curve was obtained but the activity was roughly 6 times less. This result cannot be explained solely on the basis of binding of magnesium by Tris–maleate, as use of HEPES alone (no addition of magnesium) resulted in activity 3 times that found in Tris–maleate plus 0.4 mM $MgCl_2$ suggesting that Tris–maleate is itself inhibitory.

As seen in Table II calcium had a slight inhibitory effect when activity was measured at a physiological pyrophosphate concentration. At a calcium concentration approximately equivalent to that of Ca^{2+} in the plasma, slightly less than 10% inhibition was observed. This inhibitory effect increased with increasing calcium concentration.

TABLE II

EFFECT OF Ca^{2+} AT $3.5 \cdot 10^{-6}$ M PYROPHOSPHATE

Incubation medium: $3.5 \cdot 10^{-6}$ M pyrophosphate; 0.4 mM $MgCl_2$; 0–3 mM $CaCl_2$; 0.05 M HEPES, pH = 7.4. Alkaline phosphatase was incubated for 10 min.

Ca^{2+} (mM)	Spec. act. (μmoles PP_i/min per mg)	Inhibition (%)
0	2.09	0
0.8	2.05	2
1.2	1.91	9
1.6	1.77	15
2.0	1.64	22
3.0	1.40	33

Orthophosphate, on the other hand, was a very strong inhibitor (Table III). This effect was tested in the presence of either 0.05 M HEPES or 0.05 M Tris–maleate buffer. With HEPES as buffer and with 0.4–3.0 mM orthophosphate activity was less than 2% that in the absence of orthophosphate. In Tris–maleate buffer the inhibitory effect of orthophosphate was much weaker.

At 10^{-4} M the diphosphonates EHDP and Cl_2MDP were inhibitory, while in contrast, at 10^{-5} M, activity was slightly increased, as shown in Table IV.

Neither calcitonin nor parathyroid hormone influenced the activity (Table V). The apparent stimulation found at high doses originated from the solvent in which the hormones were dissolved.

TABLE III

EFFECT OF ORTHOPHOSPHATE AT $3.5 \cdot 10^{-6}$ M PYROPHOSPHATE

Incubation medium: $3.5 \cdot 10^{-6}$ M pyrophosphate, 0.4 mM $MgCl_2$, 1.0 mM $CaCl_2$, 0.05 M buffer, pH 7.4. Alkaline phosphatase was incubated in either HEPES or Tris–maleate buffer for 10 min.

Buffer	Enzyme added (μl)	P_i (mM)	Activity, (nmoles PP_i/10 min)	Relative activity (%)
HEPES	10	0	0.85	100
	10	0.4–3.0	0–0.01	0–1
	50	1.0	0.049	1.2
	100	1.0	0.091	1.1
Tris–maleate	70	0	1.09	100
	70	0.4	0.42	39
	70	0.8	0.22	21
	70	1.2	0.18	12
	70	2.0	0.12	11
	70	3.0	0.08	7

TABLE IV

EFFECT OF EHDP AND Cl_2MDP AT $3.5 \cdot 10^{-6}$ M PYROPHOSPHATE

Incubation medium: $3.5 \cdot 10^{-6}$ M pyrophosphate, 0.4 mM $MgCl_2$; EHDP or Cl_2MDP; 0.05 M HEPES, pH 7.4. Alkaline phosphatase was incubated for 10 min. ($n = 3$ or 4).

EHDP (M)	Cl_2MDP (M)	Specific activity (μmoles PP_i/min per mg $\pm$ S.E.)	Relative activity (% $\pm$ S.E.)
0	0	1.96 $\pm$ 0.03	100 $\pm$ 1.5
10^{-5}	—	2.17 $\pm$ 0.03	111 $\pm$ 1.5
10^{-4}	—	1.22 $\pm$ 0.04	62 $\pm$ 2.1
—	10^{-5}	2.04 $\pm$ 0.08	104 $\pm$ 3.9
—	10^{-4}	0.79 $\pm$ 0.01	40 $\pm$ 0.5

TABLE V

EFFECT OF PARATHYROID HORMONE AND CALCITONIN ON PYROPHOSPHATASE ACTIVITY OF ALKALINE PHOSPHATASE

Control: 0.05 M Tris–maleate, pH 7.4, 0.4 mM $MgCl_2$, $3.5 \cdot 10^{-6}$ M pyrophosphate. The hormones were added dissolved to the incubation medium (see Materials). The two different amounts of parathyroid hormone were added in the same volume of solvent, the different amounts of calcitonin were added in different volumes of solution of the same concentration. (The assay solvent — calcitonin was done with the higher volume of solvent); ($n = 3$).

	Relative activity $\pm$ S.E. (%)
Control	100 $\pm$ 4
Solvent + parathyroid hormone 2 units/ml	87 $\pm$ 1
Solvent + parathyroid hormone 16 units/ml	82 $\pm$ 1
Solvent — parathyroid hormone	80 $\pm$ 1
Solvent + calcitonin 40 munits/ml	96.5 $\pm$ 6
Solvent + calcitonin 160 munits/ml	122 $\pm$ 2
Solvent — calcitonin	122 $\pm$ 1

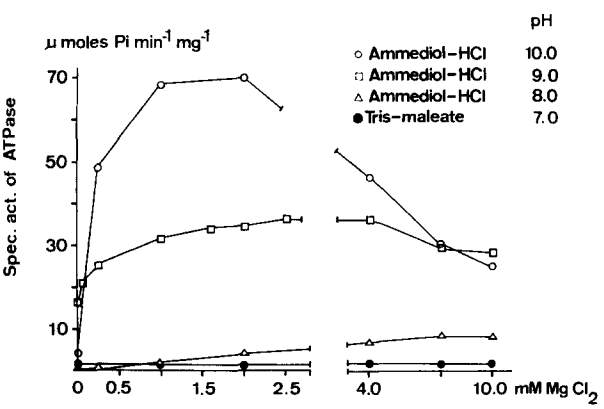

Fig. 3. Influence of Mg^{2+} and pH on the ATPase activity of alkaline phosphatase. 5 mM ATP, 0.1 M buffer concentration.

TABLE VI

EFFECT OF Ca^{2+} ON ATPase ACTIVITY OF ALKALINE PHOSPHATASE IN THE PRESENCE OF Mg^{2+} AND 5 mM ATP

pH	Mg^{2+} (mM)	Ca^{2+} (mM)	Relative activity (%)
10.0*	2	—	100
	2	2.5	78
	2	5.0	88
	2	10.0	78
	0.1	—	48
	0.1	2.5	57
	0.1	5.0	69
9.0*	2.5	—	100
	2.5	2.5	93
	2.5	5.0	91
	2.5	10.0	99
	0.1	—	27
	0.1	2.5	45
	0.1	5.0	52
8.0**	7.5	—	100
	7.5	2.5	97
	7.5	5.0	90
	7.5	10.0	86
	2.0	—	44
	2.0	2.5	55
	2.0	5.0	60

* 0.1 M ammediol–HCl.
** 0.1 M Tris–maleate.

ATPase activity

Fig. 3 shows the ATPase activity as a function of the magnesium concentration at different pH values. The highest activity was found at pH 10.0 and between 1.0 and 2.0 mM Mg^{2+}. At lower pH the effect of Mg^{2+} was small.

As seen from Table VI, when studied at optimal magnesium concentrations, Ca^{2+} did not activate at any pH, but tended rather to inhibit the enzyme. Thus the enzyme was not a true (Ca^{2+}–Mg^{2+})-ATPase. If however the magnesium concentra-

TABLE VII

EFFECT OF Ca^{2+} ON ATPase ACTIVITY OF ALKALINE PHOSPHATASE IN THE ABSENCE OF Mg^{2+}

Incubation medium: 5 mM ATP; 0.1 M ammediol–HCl, pH 9.0.

Mg^{2+} (mM)	Ca^{2+} (mM)	Relative activity (%)
4	—	100
—	0.5	25
—	1.0	31
—	2.0	32
—	4.0	42
—	8.0	50

tion was not optimal, calcium was able to stimulate, although the activity did not reach the levels measured at optimal magnesium concentrations. If calcium was added in the absence of magnesium, stimulation again occurred (Table VII).

DISCUSSION

In the study a highly purified alkaline phosphatase was used. The behaviour of the alkaline phosphatase and pyrophosphatase activities during the various isolation steps as well as the heat inactivation study suggest that this pyrophosphatase activity originates from the alkaline phosphatase. Such an identity has been shown for various alkaline phosphatases.

As known optimal pyrophosphatase activity at 2 mM PP_i substrate concentration was found at alkaline pH. This was inhibited by high concentrations of magnesium. Interestingly this inhibition disappeared around neutral pH. This is of importance in the assay of inorganic pyrophosphatase (EC 3.6.1.1) which is usually carried out at neutral pH and with 10 mM Mg^{2+} [33]. Under these conditions the alkaline phosphatase preparation in the present study still exhibited about 14% of its optimal pyrophosphatase activity. Therefore if inorganic pyrophosphatase is to be measured in a homogenate, one must allow for the contribution to the activity of alkaline phosphatase, if the latter enzyme is present in large excess.

The finding that the characteristics of the pyrophosphatase activity of the alkaline phosphatase changed when it was assayed under more physiological conditions, i.e. $3.5 \cdot 10^{-6}$ M pyrophosphate, 0.4 mM magnesium, may be relevant to the functioning of the enzyme in vivo. The pH optimum of the pyrophosphatase under these conditions was 7.0, as has been found for partially purified chick intestinal phosphatase by Tenenhouse and Rasmussen [24]. The decrease in activity at higher pH might be due to a lack of substrate as in the alkaline region most of the PP_i will be in the form $Mg_2P_2O_7$, which is not considered a substrate [9, 34]. On the other hand, at neutral pH, the majority exists as $HP_2O_7^{3-}$, $MgP_2O_7^{2-}$ and $MgHP_2O_7^-$ which might all be substrates. This explanation agrees well with the finding that at neutral pH Mg^{2+} showed no inhibition (Fig. 1). Whereas calcium only slightly influenced pyrophosphatase activity in these "physiological" conditions, orthophosphate inhibited very strongly at concentrations present in extracellular fluid, especially if HEPES buffer was used. It is conceivable that orthophosphate regulates the activity in vivo. It is interesting to note that while about 6 times less activity was found in the presence of Tris–maleate buffer compared with HEPES buffer the effect of orthophosphate in the former buffer was also weaker than in the latter system. Thus it might be possible that natural inhibitors similar to maleate exist in vivo and alter the sensitivity of the enzyme to orthophosphate.

EHDP and Cl_2MDP inhibited at 10^{-4} M, but not at 10^{-5} M. The inhibition at 10^{-4} M cannot be due to a binding of Mg^{2+} since at this low concentration of diphosphonate binding is negligible. It cannot be ascertained whether this inhibition could explain the action of the diphosphonates in vivo. It should be noted however that only EHDP blocks bone mineralization in vivo while both diphosphonates have a similar effect on the enzyme. Parathyroid hormone and calcitonin had no effect in our conditions in agreement with the fact that hormones do not usually act directly on enzymes. It appears that the change of the pyrophosphatase of bone obtained by

administration of calcitonin [35, 36] and parathyroid hormone [35] to rats has been probably not due to a direct effect on enzyme activity. On the other hand, calcitonin has been found to have an effect on the pyrophosphatase of ascites tumour cells [24], decreasing the inhibition due to calcium.

Much attention has been devoted lately to the possible role of alkaline phosphatase in the transport of calcium because of its probable identity with Ca^{2+}-ATPase. In this study calcium did not stimulate the ATPase activity but rather inhibited at optimal magnesium concentration. Calcium could, however, substitute partially for magnesium if no magnesium or less than the optimal amount was present. This in fact is the case in the extracellular fluid where about twice as much Ca^{2+} as Mg^{2+} is found. Under these conditions bone alkaline phosphatase may function as a (Ca^{2+}–Mg^{2+})-ATPase. However, our enzyme was not a true (Ca^{2+}–Mg^{2+})-ATPase in the sense that ATPase is stimulated by Ca^{2+} at maximal magnesium concentrations. Thus bone alkaline phosphatase is different from intestinal alkaline phosphatase [17, 18] which possesses true (Ca^{2+}–Mg^{2+})-ATPase activity.

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