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STUDIES ON MATRIX VESICLES ISOLATED FROM CHICK EPIPHYSEAL CARTILAGE

ASSOCIATION OF PYROPHOSPHATASE AND ATPase ACTIVITIES WITH ALKALINE PHOSPHATASE

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Summary

Fractions composed primarily of cells (Fraction I), membrane fragments (Fraction II) and matrix vesicles (Fraction III) were isolated from chick epiphyseal cartilage. The characteristics of the alkaline phosphatase (EC 3.1.3.1), pyrophosphatase (EC 3.6.1.1) and ATPase (EC 3.6.1.3) activities in the matrix-vesicle fraction were studied in detail.

Mg^{2+} was not absolutely essential to any of the activities, but at low levels was stimulatory in all cases. Higher concentrations inhibited both pyrophosphatase and ATPase activities. Both the stimulatory and inhibitory effects were pH-dependent.

Ca^{2+} stimulated all activities weakly in the absence of Mg^{2+}. However, when Mg^{2+} was present, Ca^{2+} was slightly inhibitory. Thus, none of the activities appear to have a requirement for Ca^{2+}, and hence would not seem to be involved with active Ca^{2+} transport in the typical manner.

The distribution of alkaline phosphatase, pyrophosphatase, and Mg^{2+}-ATPase activities among the various cartilage fractions was identical, and concentrated primarily in the matrix vesicles. Conversely, the highest level of (Na^+ + K^+)-ATPase activity was found in the cell fraction. All activities showed nearly identical sensitivities to levamisole ($4 \cdot 10^{-3}$ M) which caused nearly complete inhibition of alkaline phosphatase and pyrophosphatase. About 10—15% of the ATPase activity was levamisole-insensitive. The data are consistent with the concept that the Mg^{2+}-ATPase and pyrophosphatase activities of matrix vesicles stem from one enzyme, namely, alkaline phosphatase.

Introduction

The first detectable deposition of mineral in many calcifying tissues occurs in extracellular, membrane-bound vesicles [1—3]. These vesicles are

known to possess alkaline phosphatase, pyrophosphatase, and ATPase activities [4,5]. However, no extensive studies on the nature of these vesicle-associated activities have yet been reported. Thus, their relationship to vesicular function in the mineralization process is not clear.

Alkaline phosphatase has long been associated with calcifying tissues [6], and can utilize both PP_i and ATP as substrates [7—9]. Its physiological role has been postulated to be the hydrolysis of PP_i, an inhibitor of hydroxyapatite crystal growth [10]. Thus, it is possible that all three of the vesicle-associated enzymatic activities might stem from this single enzyme. However, the existence of other pyrophosphatases or ATPase enzymes in the vesicles has not been ruled out. A pyrophosphatase distinct from alkaline phosphatase has been found in calcifying rat costal cartilage [11].

In addition, it has been proposed that matrix vesicles might contain an enzymatic Ca^{2+} pump [12]. The observed stimulation of Ca^{2+} uptake in the presence of PP_i [13] and ATP [14] by in vitro systems is consistent with this hypothesis. If such a pump exists, though, it would most likely be coupled to an enzyme other than alkaline phosphatase. Unlike the intestinal enzyme [15,16], bone alkaline phosphatase does not possess the Ca^{2+}-sensitive activities necessary for a Ca^{2+} pump [9].

Therefore this investigation had two purposes: first, to determine whether the pyrophosphatase and ATPase activities of matrix vesicles resulted from alkaline phosphatase alone, or if other more specific enzymes were present; and second, to assess if any of these activities were stimulated by Ca^{2+}. In order to determine whether or not the three enzyme activities stemmed from a single enzyme, the effects of pH, Mg^{2+}, Ca^{2+} and levamisole were studied, as well as the distribution of these activities in fractions of epiphyseal cartilage. The results obtained are consistent with the idea that one enzyme, alkaline phosphatase, is responsible for the three activities observed in the isolated matrix vesicles.

Materials

Chicks (Broiler strain no. 663) were obtained from Hubbard Hatcheries, Walpole, N.H. Purified bovine testicular hyaluronidase (300 U.S.P. units per mg, ICN Nutritional Biochemicals, Cleveland, Ohio 44218) and clostridial col-

TABLE I

COMPOSITION OF SYNTHETIC CARTILAGE FLUID

In addition, synthetic cartilage fluid contained 5.74 mM D-glucose as well as 1000 units of pencillin and 1000 ug of streptomycin per ml. The pH was 7.45. Concentrations of ions were based on the composition of ultrafiltrates of fluid expressed from slices of chick epiphyseal cartilage (Wuthier, R.E., unpublished).

Cations	Concentration (mM)	Anions	Concentration (mM)
Na^+	93.9	Cl^-	98.5
K^+	20.9	HCO_3^-	18.0
Mg^{2+}	0.94	$H_2PO_4^-$	0.66
Ca^{2+}	1.48	HPO_4^{2-}	1.24

lagenase (CLS II, crude, Worthington Biochemical Corp., Freehold, N.J. 07728) were dissolved to make 1% and 0.2% solutions, respectively, in synthetic cartilage fluid (see Table I), a balanced salts solution made up to mimic the ionic composition of chick cartilage ultrafiltrates (Wuthier, R.E., unpublished). The collagenase solution was centrifuged for 40 min at 81 500 × *g* (average centrifugal force), prior to use, and the pellet discarded. Disodium *p*-nitrophenyl phosphate, *p*-nitrophenol standard, and disodium adenosine 5′-triphosphate were obtained from Sigma Chemical Corp., St. Louis, Mo. 63160. AMP, (2-amino-2-methyl-1-propanol) was purchased from P-L Biochemicals, Milwaukee, Wisc. 53205. Levamisole was graciously provided by Dr Julian Jaffe. All other chemicals were reagent grade.

Methods

Preparation of matrix vesicles

Chickens aged 6—8 weeks were killed by exsanguination and the leg bones rapidly freed of muscle and chilled on ice. Slices of epiphyseal cartilage (proliferating, hypertrophic, and calcifying zones) were obtained as previously described [17] and collected in cold synthetic cartilage fluid buffer. The slices were washed several times in cold synthetic cartilage fluid to remove blood, then blotted and weighed. The slices were next digested for 30 min at 37° C in the hyaluronidase solution (10 ml per g tissue). The supernatant was decanted and the slices further digested for 90—120 min in the crude collagenase solution as above.

The digest was then filtered through a nylon mesh, chilled on ice, and subjected to a differential centrifugation scheme modified from that of Ali et al. [4]. In this scheme, all centrifugal forces noted are average values, computed for the midpoint of the tube. Fraction I (10 min, 150 × *g*) consisted of whole cells (chondrocytes and erythrocytes) and some large mineralized aggregates. Fraction II (15 min, 13 000 × *g*) contained large membrane elements and some mitochondria. The former were either sheets or large vesicles, and some appeared to contain ribosomes. Fraction III (30 min, 81 500 × *g*) contained small vesicles of varying size, a few containing ribosomes. In general, the morphology of the fraction was similar to that reported by Ali et al. [4]. The final supernatant, containing the crude collagenase and digested matrix material, was not extensively studied. The content of the sedimented fractions was analyzed by light and electron microscopy, and assayed for alkaline phosphatase activity.

The vesicle pellet (Fraction III) was washed once and then resuspended in 50 mM Tris · HCl buffer, pH 7.5. The resuspension was either assayed directly or stored at −20° C. Vesicles stored in this way retained their enzymatic activity for weeks, although repeated freezing and thawing diminished their activity.

Enzyme assays

All enzyme assays were carried out at 37° C for 5—10 min (alkaline phosphatase) or 10—20 min (pyrophosphatase and ATPase). Reaction mixtures were made to 0.5 ml, and the reactions initiated by the addition of 10—20 μl of resuspension. In all cases, the velocity was proportional to the amount of resuspension added. All pH vs rate profile experiments, regardless of substrate,

were performed using 20 mM Tris · AMP buffer adjusted to the desired pH with HCl.

Alkaline phosphatase was determined in the presence of 20 mM Tris · AMP, pH 9.9, 100 mM NaCl, 5 mM KCl, and 2 mM disodium *p*-nitrophenyl phosphate. The reaction was stopped by the addition of 5 ml of 0.02 M NaOH and the absorbance read at 410 nm.

Pyrophosphatase was determined in the presence of 20 mM Tris · HCl, pH 7.5, 100 mM NaCl, 5 mM KCl, and 1 mM $Na_4P_2O_7$. The reaction was stopped by the addition of 0.5 ml 10% trichloroacetic acid containing 10 mM $CuSO_4$ [18], and inorganic phosphate determined [19].

ATPase was determined in the presence of 20 mM Tris · HCl, pH 7.5 or 8.9, 100 mM NaCl, 5 mM KCl, and 1 mM disodium ATP. The reaction was stopped by the addition of 0.5 ml 10% trichloroacetic acid and inorganic phosphate determined [19].

Protein was determined by the method of Lowry et al. [20].

Results

Enzyme-activity distribution in cartilage fractions

Table II shows the pattern of enzyme and protein distribution among the three cartilage fractions isolated. While the vesicle fraction (Fraction III) was least rich in protein, over half of the alkaline phosphatase and pyrophosphatase activities were found there. A majority of the two ATPase activities remained in the cellular fraction (Fraction I). However, the relative specific activities of the Mg^{2+}-ATPase, alkaline phosphatase, and pyrophosphatase activities increased progressively from Fractions I to III, while that of the $(Na^+ + K^+)$-ATPase diminished. Enzyme activities in the final supernatant fraction (not shown) were negligible.

Effects of Mg^{2+} and pH

The alkaline phosphatase activity of isolated matrix vesicles behaved like

TABLE II

PROTEIN AND ENZYMATIC CONTENT OF CARTILAGE FRACTIONS

ATPase activity was measured at pH 7.5 in the presence of 0.5 mM ATP, 100 mM NaCl, 5 mM KCl, 0.55 mM $MgCl_2$, and 0.05 mM EDTA, both with and without 0.4 mM ouabain. $(Na^+ + K^+)$-ATPase, defined as the ouabain-inhibitable portion, was determined by difference. Other enzymes were assayed as described in Methods. Fraction I = chondrocytes; Fraction II = miscellaneous membranes; Fraction III = matrix vesicles. % of total = percentage of total recovered in the three fractions. Relative spec. act. = (specific activity in fraction)/(specific activity in fraction I).

Fraction	$(Na^+ + K^+)$-ATPase		Mg^{2+}-ATPase		Pyrophosphatase		Alkaline phosphatase		Protein
	% of total	Relative spec act.	% of total	Relative spec act.	% of total	Relative spec act.	% of total	Relative spec act.	(% of total)
I	85	1.00	60	1.00	34	1.00	32	1.00	76
II	10	0.62	14	1.2	13	1.8	13	2.1	16
III	5	0.58	26	4.2	53	14.0	55	16.0	8

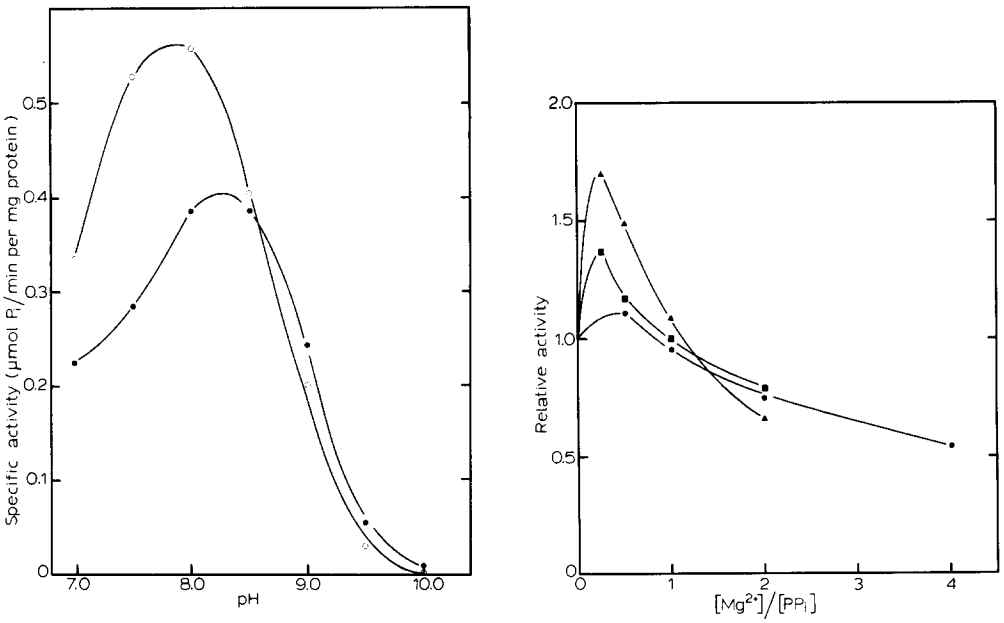


Fig. 1. Effect of $[Mg^{2+}]$ on the pH rate profile of matrix-vesicle pyrophosphatase activity. The incubation medium contained 100 mM NaCl, 5 mM KCl, 1 mM $Na_4P_2O_7$ and 20 mM Tris · AMP buffer, with or without added $MgCl_2$. ●———●, no $MgCl_2$; ○———○, 0.2 mM $MgCl_2$.

Fig. 2. Effect of $[Mg^{2+}]/[PP_i]$ ratio on matrix-vesicle pyrophosphatase activity. The incubation medium contained 100 mM NaCl, 5 mM KCl, 20 mM Tris · HCl buffer, pH 7.5, and varying concentrations of $MgCl_2$ and $Na_4P_2O_7$. ●———●, 0.5 mM $Na_4P_2O_7$; ■———■, 1 mM $Na_4P_2O_7$; ▲———▲, 2.0 mM $Na_4P_2O_7$.

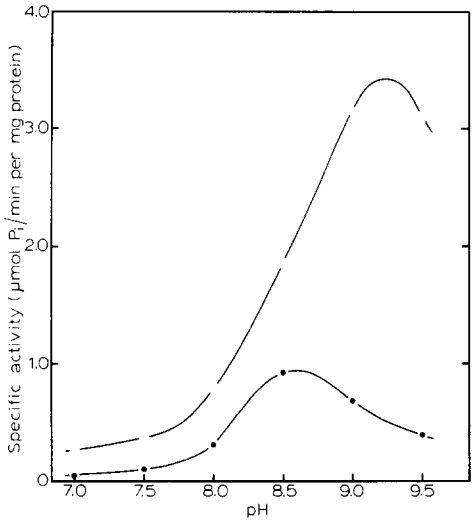
other known alkaline phosphatases with respect to pH and Mg^{2+} [21—24]. The pH optimum varied with substrate concentration, becoming more basic with increasing substrate. At 2 mM substrate, the pH optimum was 9.9. While Mg^{2+} was not absolutely essential for activity, small amounts (relative to the substrate concentration) were strongly stimulatory. The stimulation followed apparent saturation kinetics, with a K_m for Mg^{2+} of 0.12 mM.

The effect of Mg^{2+} on vesicle pyrophosphatase activity was pH-dependent (Fig. 1). The addition of 0.2 mM Mg^{2+} shifted the pH optimum from 8.3 to 7.8, and stimulated activity in the lower pH range. However, this concentration of Mg^{2+} became inhibitory at higher pH. Higher concentrations of Mg^{2+} inhibited the pyrophosphatase activity even at pH 7.5. The degree of inhibition appeared related to the $[Mg^{2+}]/[PP_i]$ ratio (Fig. 2).

Matrix-vesicle ATPase activity was low in the absence of Mg^{2+} (Fig. 3). The addition of 0.25 mM Mg^{2+} sharply stimulated the activity and shifted the pH optimum from 8.5 to near 9.0. As with pyrophosphatase, low levels of Mg^{2+} were sufficient for maximum stimulation, while higher levels became inhibitory (Fig. 4). Both stimulatory and inhibitory effects were much less pronounced at pH 7.5 than at pH 8.9.

Effects of Ca^{2+}

To determine whether any of the vesicle activities (especially pyrophos-



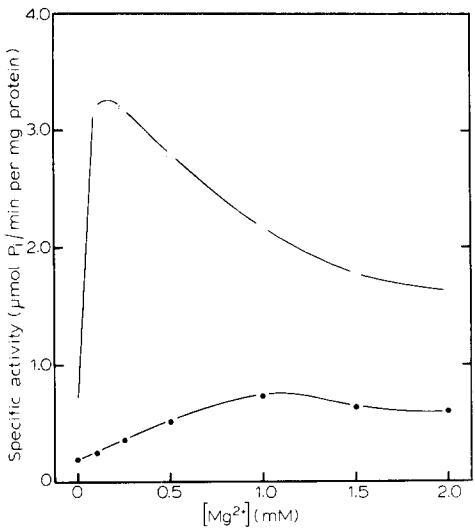


Fig. 3. Effect of [Mg^{2+}] on the pH rate profile of matrix-vesicle ATPase activity. The incubation medium contained 100 mM NaCl, 5 mM KCl, 1 mM ATP and 20 mM Tris · AMP buffer, with or without added $MgCl_2$. ●——●, no $MgCl_2$; ○——○, 0.25 mM $MgCl_2$.

Fig. 4. Effect of pH and increasing [Mg^{2+}] on matrix-vesicle ATPase activity. The incubation medium contained 100 mM NaCl, 5 mM KCl, 1 mM ATP, 20 mM Tris · HCl buffer, and varying concentrations of $MgCl_2$. ●——●, pH 7.5; ○——○, pH 8.9.

phatase and ATPase) might function as a Ca^{2+} pump, the effects of Ca^{2+} in the presence and absence of Mg^{2+} were studied (Table III). In the absence of Mg^{2+}, increasing amounts of Ca^{2+} weakly mimicked the Mg^{2+} effect. Alkaline phosphatase activity was slightly stimulated by Ca^{2+} while pyrophosphatase and ATPase were first stimulated, then inhibited by higher concentrations. The Ca^{2+} effect on the latter two activities was especially weak, causing at most a 14% increase over control values. In the presence of 1 mM Mg^{2+}, alkaline phos-

TABLE III

EFFECTS OF Ca^{2+} AND Mg^{2+} ON MATRIX-VESICLE ENZYME ACTIVITIES

Specific Activity = μmol product/min per mg protein. Relative Activity = Activity in presence of Ca^{2+}/Activity in absence of Ca^{2+}.

Mg^{2+} (mM)	Ca^{2+} (mM)	Alkaline Phosphatase (pH 9.9)		Pyrophosphatase (pH 7.5)		ATPase (pH 7.5)	
		Specifec activity	Relative Act.	Spec. Act.	Relative Act.	Spec. Act.	Relative act.
—	—	67	1.00	0.42	1.00	0.50	1.00
—	0.5	78	1.16	0.48	1.14	0.57	1.14
—	1.0	85	1.27	0.45	1.07	0.54	1.08
—	1.5	86	1.28	0.42	1.00	0.53	1.06
1.0	—	142	1.00	0.91	1.00	0.62	1.00
1.0	0.5	132	0.93	0.74	0.81	0.59	0.95
1.0	1.0	132	0.93	0.77	0.85	0.60	0.97
1.0	1.5	132	0.93	0.77	0.85	0.62	1.00

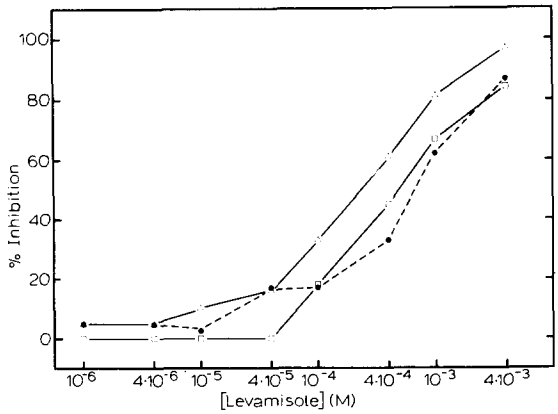


Fig. 5. Effect of Levamisole on matrix-vesicle enzyme activities. All incubation media contained 100 mM NaCl, 5 mM KCl, and the following additions: alkaline phosphatase (△———△): 20 mM Tris · AMP buffer, pH 9.9, 1 mM $MgCl_2$ and 2 mM Na_2 Nph-*P*; pyrophosphatase (●- - - - -●): 20 mM Tris · HCl buffer, pH 7.5, 0.25 mM $MgCl_2$ and 1 mM $Na_4P_2O_7$; ATPase (□———□): 20 mM Tris · HCl buffer, pH 8.9, 0.5 mM $MgCl_2$ and 1 mM ATP.

phatase and pyrophosphatase were both slightly inhibited by Ca^{2+}, while the ATPase activity was hardly affected.

Effects of levamisole

Levamisole (S (−)tetramisole) is a drug which stereospecifically inhibits a number of alkaline phosphatases, including the enzyme from canine bone [25]. Its action appears restricted to non-specific phosphatases rather than those enzymes hydrolysing only a single substrate, such as 5′-nucleotidase [26].

All enzyme activities were assayed under the conditions which had previously yielded maximal activity. Levamisole in millimolar concentrations strongly inhibited all activities (Fig. 5). Alkaline phosphatase was inhibited by more than 96% by $4 \cdot 10^{-3}$ M levamisole. The ATPase dose vs response curve was parallel to that for alkaline phosphatase, but was consistently about 10—15% less sensitive. The drug vs response of pyrophosphatase varied slightly from the other two activities, although the overall effect (i.e. nearly complete inhibition) was similar in all cases.

All the vesicle enzyme activities were approximately ten times less sensitive to levamisole than the sensitive canine phosphatases [25]. On the other hand, the levamisole-insensitive alkaline phosphatases (*Escherichia coli*, canine intestine) were unaffected by levels of the drug which nearly totally inhibited all three enzyme activities in the isolated matrix vesicles.

Discussion

This study sought to analyze certain properties of the three major enzymatic phosphatase activities of cartilage matrix vesicles; in particular, whether the three activities stemmed from one or more enzymes, and whether any of the activities were stimulated by Ca^{2+}. It is clear from the results obtained that the alkaline phosphatase, pyrophosphatase, and ATPase activities have a num-

ber of common properties. These include the stimulation of all activities by low Mg^{2+} levels, the complex response to Ca^{2+}, and the nearly identical sensitivity to levamisole. These common characteristics strongly suggest that, with the exception of a small ATPase component, all the activities are due to the alkaline phosphatase enzyme. The complex Ca^{2+} effects seen further indicate that none of the activities possesses the Ca^{2+} sensitivity necessary to be considered a Ca^{2+}-transport enzyme.

The distribution patterns of alkaline phosphatase and pyrophosphatase in the cartilage fractions were nearly identical, while the Mg^{2+}-ATPase pattern differed. Although the relative specific activity of Mg^{2+}-ATPase (in contrast to the $(Na^+ + K^+)$-ATPase) increased as the fractions became more enriched in matrix vesicles, most of the activity remained in the cellular fraction (Fraction I). This most likely resulted from the presence of other Mg^{2+}-ATPase activities in the cellular fraction. Many tissues possess Mg^{2+}-ATPase activities more active than their $(Na^+ + K^+)$-ATPase Na^+ pump enzymes [27]. These Mg^{2+}-ATPase activities are presumably not related to the alkaline phosphatase enzyme, since cells nearly devoid of alkaline phosphatase [24] have substantial Mg^{2+}-ATPase activities [27].

Not all of the vesicle ATPase activity is associated with the alkaline phosphatase enzyme. A minor amount of $(Na^+ + K^+)$-ATPase activity was found in the vesicle fraction (Fraction III). In addition, the levamisole response of vesicle ATPase indicates that 10—15% of the ATPase activity is not due to alkaline phosphatase. Since both Na^+ and K^+ were present in the assay media, it is impossible to determine the relative amounts of $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase activities in this "contaminant" element. It is possible that some $(Na^+ + K^+)$-ATPase is associated with the vesicles, rather than being an exogenous contaminant. Some of the enzyme may segregate with the vesicles as they bud from the chondrocytes.

The pyrophosphatase activity showed a response to levamisole which differed slightly from that of the other two activities. This is probably due to the low pH at which the pyrophosphate was assayed. Levamisole is insensitive to changes in pH in the range 8.6 to 9.9 [25]. However, the fact that all three activities were nearly completely inhibited by the same concentration of levamisole shows that probably all of the pyrophosphatase and most of the ATPase stems from alkaline phosphatase.

The matrix-vesicle pyrophosphatase and ATPase activities generally behave like those activities associated with known alkaline phosphatases [7—9]. In particular, the stimulation by low levels and inhibition by higher levels of Mg^{2+} is typical, as is the pH dependence of both stimulatory and inhibitory effects. The lack of strong Ca^{2+} stimulation, indeed its inhibitory effect in the presence of Mg^{2+}, indicates that these phosphatase activities are not associated with Ca^{2+} transport in the usual sense. This finding is similar to that observed for the pyrophosphatase and ATPase activities associated with purified calf bone alkaline phosphatase [9].

Two conspicuous differences between the chicken matrix vesicle and calf bone activities [9] were observed, however. First, the pH optima of the vesicle activities were lower than those reported for the bone enzyme. Second, inhibition of bone enzyme pyrophosphatase activity by Mg^{2+} at pH 7.5 was very

weak, while inhibition of the vesicle activity was substantial. Both of these differences are related in part to the dependence of pH optimum of alkaline phosphatase on substrate concentration [24]. Felix and Fleisch [9] noted a shift in pyrophosphate pH optimum to 7.0 when the substrate concentration was $3.5 \cdot 10^{-6}$ M. They normally used 2 mM PP_i and 5 mM ATP for assay, whereas our vesicle enzyme activities were assayed using 1 mM substrate. Other differences in the assay conditions were considerable, most notably (1) the presence of Na^+ and K^+ in all the vesicle enzyme assays, and (2) the use of vesicles for assay rather than a purified enzyme. These differences may be significant in view of the sensitivity of alkaline phosphatase to the ionic environment [24].

The results presented above reaffirm the importance of alkaline phosphatase in the calcification process, since that enzyme appears to be the primary enzymatic component of matrix vesicles. Its lack of Ca^{2+} sensitivity suggests that its in vivo function is related to its action as a hydrolase rather than a Ca^{2+} pump. Still the mechanism of vesicular Ca^{2+} accumulation is unclear. These data are not inconsistent with widely differing models [12,28] proposed for initial mineralization.

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