

Biochemical and Immunohistochemical Evidence That in Cartilage an Alkaline Phosphatase Is a Ca^{2+} -binding Glycoprotein

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Abstract. A glycoprotein that exhibits alkaline phosphatase activity and binds Ca^{2+} with high affinity has been extracted and purified from cartilage matrix vesicles by fast protein liquid chromatography. Antibodies against this glycoprotein were used to analyze its distribution in chondrocytes and in the matrix of calcifying cartilage. Under the light microscope, using immunoperoxidase or immunofluorescence techniques, the glycoprotein is localized in chondrocytes of the resting zone. At this level, the extracellular matrix does not show any reaction. In the cartilage plate, between the proliferating and the hypertrophic region, a weak immune reactivity is seen in the cytoplasm, whereas in the intercolumnar matrix the collagen fibers appear clearly stained. Stained granular structures, distributed with a pattern similar to that of

matrix vesicles, are also visible. Calcified matrix is the most stained area. These results were confirmed under the electron microscope using both immunoperoxidase and protein A-gold techniques. In parallel studies, enzyme activity was also analyzed by histochemical methods. Whereas resting cartilage, the intercellular matrix of the resting zone, and calcified matrix do not exhibit any enzyme activity, the zones of maturing and hypertrophic chondrocytes are highly reactive. Some weak reactivity is also shown by chondrocytes of the resting zone. The observation that this glycoprotein (which binds Ca^{2+} and has alkaline phosphatase activity) is synthesized in chondrocytes and is exported to the extracellular matrix at the time when calcification begins, suggests that it plays a specific role in the process of calcification.

ALTHOUGH the molecular mechanism of tissue calcification still awaits full elucidation, undisputed evidence points to the involvement of alkaline phosphatase in this process. First, it has been repeatedly reported that the enzyme is present at significant levels in precalcifying matrices. Second, in cartilage and in other mineralizing tissues, an intense alkaline phosphatase activity is detected in matrix vesicles, where the earliest crystals of calcium phosphate are formed (5). In contrast, matrix vesicles of the elastic cartilage of the epiglottis, a tissue that does not calcify, show no alkaline phosphatase activity (24).

Studies on alkaline phosphatase extracted from various mineralizing tissues (8, 13, 14, 19, 20, 31) have shown that the purified enzymes are heterogeneous with respect to molecular weight, isoelectric point, substrate specificity, and stability. Thus, despite the great deal of data accumulated and of the discovery of its natural substrates, ATP and pyrophosphate (28), the function of the enzyme in calcification is not yet well understood.

To gain further evidence for its role in tissue mineralization, we have purified alkaline phosphatase from matrix vesicles of calf scapula epiphyseal cartilage and have then investigated its biochemical properties and the possible corre-

lation between the degree of cartilage calcification and the tissue distribution of the enzyme, as detected by immunohistochemical techniques. Epiphyseal cartilage is an ideal tissue for this purpose, since it contains a spectrum of regions ranging from noncalcifying (the resting cartilage) to the fully mineralized (the zone of provisional calcification), including the area where the matrix is prepared to be mineralized. Preliminary results of this work have been published elsewhere (10).

Materials and Methods

Calf scapulae, provided by an abattoir in Udine (Italy), were removed from the animals immediately after death and transferred in ice to the laboratory where they were immediately processed.

Preparation of Matrix Vesicles from Scapula Cartilage

Once cleaned of adherent tissues, the transforming and ossifying zones of the preosseous cartilage were collected. The procedure for the preparation of matrix vesicles was essentially as described by Ali et al. (1). Pieces of the tissue were digested in a solution (10 ml/g of tissue) containing 1,000 U of collagenase/ml (Worthington Biochemical Corp., Freehold, NJ), 120 mM NaCl, 10 mM KCl, 1,000 U of penicillin/ml, 1 mg of streptomycin/ml, and 20 mM Hepes buffer, pH 7.45. The digestion was carried out at 37°C

for 2 h in the presence of the following protease inhibitors: α -cysteine proteinase inhibitor (0.1 μ M), cystatin (0.1 μ M), and N-ethylmaleimide (0.1 mM). The digested mixture was centrifuged at 20,000 g for 10 min and the sediment was discarded. The supernate was then spun at 200,000 g for 20 min and the resulting precipitate was washed once with 10 mM Tris-buffered saline solution, pH 7.6.

Extraction and Purification of Alkaline Phosphatase from Matrix Vesicles

The matrix vesicles fraction was suspended in 0.1 M deoxycholate-10 mM Tris-HCl buffer (pH 7.6) containing 150 mM NaCl, and incubated for 30 min at 37°C before adding an equal volume of n-butanol, as described by Hsu et al. (18). The extraction was repeated twice. The combined extracts were centrifuged and the aqueous extract was extensively dialyzed against 20 mM Tris-HCl buffer, pH 7.5 (buffer A). A protein sample of \sim 4 mg in 2.5 ml of buffer A was then applied to a MONO Q HR 5/5 anion-exchange column of the fast protein liquid chromatography (FPLC)¹ apparatus (Pharmacia Fine Chemicals, Uppsala, Sweden), pre-equilibrated with the same buffer. The material was then eluted from the column by applying a stepwise gradient (up to 1.2 M) of KCl in buffer at a flow rate of 1 ml/min. Fractions (1.0 ml) of the eluate were collected and analyzed for enzymatic activity, Ca²⁺-binding, and protein content. The peak fractions were pooled and subjected to slab gel electrophoresis.

Alkaline Phosphatase Assay

The activity was assayed in 1-ml cuvettes by measuring the release of *p*-nitrophenol from *p*-nitrophenylphosphate (2 mM) at 37°C using a Pye Unicam SP8-400 recording spectrophotometer at a wavelength of 410 nm. The assay mixture (1.0 ml) contained 0.2 M diethanolamine-HCl, pH 10.0, and 2 mM substrate, 1 mM MgCl₂. Alternatively, the enzyme activity was determined with either 2 mM ATP as substrate in 0.2 M Tris-HCl buffer, pH 7.4 containing 1 mM MgCl₂, or with 2 mM pyrophosphate in 0.2 M diethanolamine-HCl, pH 8.5, containing 1 mM MgCl₂. In both cases, the time of incubation was 30 min at 37°C and the released phosphate was determined by the method of Baginski et al. (2).

Protein concentrations were determined by the method of Bradford (6), using bovine immunoglobulin as standard.

SDS Slab Gel Electrophoresis and Electrophoretic Blotting

Proteins were separated by SDS gradient gel electrophoresis (12 h at 12 mA) in 10–20% polyacrylamide gels (9.5 \times 18 cm) using the discontinuous system of Laemmli (23). Staining was performed with Coomassie Blue G-250 for proteins and with the periodic acid/Schiff reagents (22) for glycoproteins. Cytochrome *c* (12,500), chymotrypsinogen A (25,000), ovalbumin (45,000), and bovine serum albumin (BSA) (68,000), all provided by Boehringer Mannheim GmbH (Mannheim, FRG), were used as protein standards for the calculations of apparent molecular weights. Electrophoretic blotting was carried out according to Towbin et al. (30).

Ca²⁺-binding Measurements

Ca²⁺-binding measurements were performed essentially by the technique of Gratzer and Beaven (16) using Arsenazo III (E. Merck, Darmstadt, FRG). The Ca²⁺ indicator was purified on a Chelex column (12). Measurements of free and total Ca²⁺ concentrations were obtained spectrophotometrically at 685–665 nm using a Phoenix dual wavelength recording spectrophotometer (Phoenix Precision Instrument Co., Philadelphia, PA) by a back-titration with 2 mM EDTA (pH 7.4) of a mixture containing in 3 ml \sim 100 μ g of protein, 0.16 mM Arsenazo III, 50 mM Tris-glycine buffer (pH 8.3), and sufficient free Ca²⁺ to obtain an absorbance of \sim 0.07. Aliquots of 5 nmol EDTA were repeatedly added to the mixture until titration was complete. Data were plotted according to Scatchard (26).

Analysis of Amino Acid and Carbohydrates

Amino acids were determined in the glycoprotein hydrolyzate (6 N HCl, 24 h at 105°C) with the Aminoacid Analyzer Technicon NC 2 (Technicon Instruments Corp., Tarrytown, NY). Sugars were determined in a sample hydrolyzed in 2 N HCl for 2 h. Derivatization was carried out with dansylhydrazine, and reversed-phase high performance liquid chromatography

was performed using a 250 \times 4.6-mm column of Ultrasphere-ODS (C-18) (5 μ m) (model 344, connected with a fluorimeter; Beckman Instruments, Inc., Palo Alto, CA). The areas of peaks were calculated using a Hewlett-Packard 3390 automatic integrator.

Production of Antibodies

200 μ g of protein, showing alkaline phosphatase activity and Ca²⁺ binding capacity, was subjected to SDS PAGE according to Laemmli (23). The gel was then stained with Coomassie Blue in water, and the protein corresponding to a 52,000-mol-wt band was extracted by grinding the gel in a Potter with 0.9% NaCl to a final volume of 1 ml. The homogenate was then added to 1 ml of complete Freund's adjuvant (Difco Laboratories Inc., Detroit, MI) and administered by intramuscular injection into the leg of rabbits. Bi-monthly booster injections of 0.2 mg of antigen in incomplete adjuvant were given until a satisfactory titer was obtained. Blood samples were taken from the marginal vein of the ear before each injection, and serum antibodies directed against the cartilage alkaline phosphatase were assayed by using the radial immunodiffusion method of Uchterlony (25).

Immunofluorescence Microscopy

Indirect immunofluorescence was performed on both undecalcified and decalcified (10% EDTA in pH 7.2 phosphate buffer for 10 min) 8- μ m thick frozen sections. After a short fixation in methanol, sections were rinsed in 0.05 M phosphate buffer normal saline (PBS), then incubated in a moist chamber for 30 min at room temperature with anti-alkaline phosphatase antiserum diluted 1:20 in PBS. After three washes in PBS, the sections were incubated for 20 min at room temperature with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:5 dilution in PBS). Sections were then washed three times (10 min each) with 0.05 M PBS, mounted in a glycerin-PBS mixture, and examined under a Leitz Orthoplan microscope equipped with an Hg light source, using a KP 490 excitation filter and a K 510 barrier filter. Controls included omission of anti-alkaline phosphatase antiserum and its replacement with rabbit nonimmune serum.

Immunoperoxidase Microscopy

Immunoperoxidase studies were performed both at the light and electron microscopic levels. For this purpose, blocks of tissue were frozen in isopentane, in liquid nitrogen, and sectioned at 10 μ m. Sections were fixed with 4% phosphate-buffered paraformaldehyde: fixed floating sections were thoroughly washed in PBS, treated with H₂O₂ to inhibit endogenous peroxidase, and finally incubated with anti-alkaline phosphatase antiserum (1:20) in a glass vial at room temperature for 45 min. After three washes in PBS (10 min each), the sections were maintained for 45 min in a new vial containing goat anti-rabbit IgG (1:5, DAKO Corp., Santa Barbara, CA), washed again (PBS, three times for 10 min each), and finally incubated with rabbit peroxidase-antiperoxidase (PAP) complexes (DAKO Corp.) in another vial for 45 min. After careful washing in PBS, the reaction for peroxidase was performed according to Graham and Karnovsky (15). Washed sections either were mounted on coverslips for light microscopy or postfixed in 1% OsO₄ in 10 mM phosphate buffer, pH 7.2, and embedded in Araldite for electron microscopy. Controls were performed as above.

Immunogold Electron Microscopy

Colloidal gold was prepared by reducing 50 ml of 0.01% HAuCl with 2.5 ml of 1% sodium citrate. Stabilization of 10 ml of colloidal gold was obtained by adding 38 μ g of protein A (0.06 ml of a stock solution containing 634 μ g/ml) after serial dilution tests. 1 mg of protein A in 0.1 ml of H₂O was added to 10 ml of stabilized colloidal gold, allowed to stand, then mixed with 1 ml of 1% polyethylene glycol. The suspension was centrifuged at 100,000 g for 1 h at 4°C. The supernatant containing free protein A was discarded and the pellet resuspended in 6 ml of PBS containing 0.2 mg polyethylene glycol/ml. Tissue samples were fixed in 4% phosphate-buffered paraformaldehyde and embedded in araldite without OsO₄ postfixation. Ultrathin sections were cut with a diamond knife and mounted on formvar-coated golden grids. After a rinse in PBS, the sections were exposed to a solution of 0.5% ovalbumin in PBS, then incubated with anti-alkaline phosphatase antiserum (1:10 in PBS) overnight at 4°C. After three washes in PBS (20 min each), the sections were incubated with protein A-gold for 1 h at room temperature, washed in PBS, and some sections were stained with either uranyl acetate and lead citrate or with uranyl acetate only. Controls included omission of the antiserum, use of nonimmune rabbit serum, and use of uncomplexed protein A before application of protein A-gold complexes.

1. *Abbreviation used in this paper:* PAP, peroxidase-antiperoxidase.

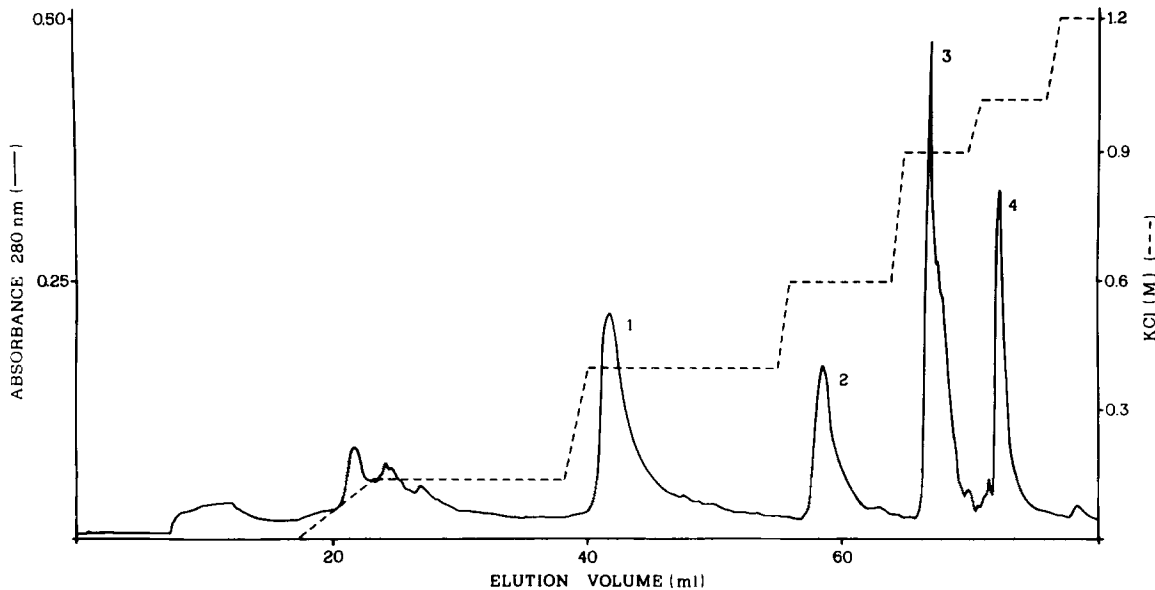


Figure 1. FPLC of deoxycholate-butanol extract of matrix vesicles. A sample of 4 mg protein was applied to a MONO Q anion-exchange column equilibrated with 20 mM Tris-HCl buffer, pH 7.5. The column was run at room temperature and the material was eluted by applying a stepwise gradient of KCl (up to 1.2 M) in buffer at a flow rate of 1 ml/min. The eluate was continuously monitored by measuring the absorbance at 280 nm.

Enzyme Histochemistry

Histochemical assays of alkaline phosphatase activity with naphthol-AS-phosphate and Fast Blue BB was performed on both frozen and glycol-methacrylate sections. These were prepared and stained as reported elsewhere (4).

Results

As shown in Fig. 1, the application of the aqueous phase of the deoxycholate-butanol extract of matrix vesicles to the FPLC column, followed by the elution with increasing concentrations of KCl, produces the separation of four peaks. Fractions of each peak were then analyzed for their enzymatic activities using *p*-nitro-phenyl-phosphate, ATP, and pyrophosphate as substrates (data are summarized in Table I) and for their Ca²⁺-binding activity. All peaks exhibited phos-

phatase activities. However, only fractions of peak 1 also showed Ca²⁺ binding with high affinity and for this reason they were further analyzed. Fractions were pooled, dialyzed against distilled water, and lyophilized. Proteins in this pool were analyzed by SDS gel electrophoresis. As shown in Fig. 2, the pool contained a single component, a glycoprotein with an apparent molecular weight of 52,000 (lanes *c* and *d*). The phosphatases present in peaks 2-4 (Fig. 1) have lower molecular weight (~30,000) and are not recognized by the antibodies raised against the phosphatase of peak 1 (see below). Their presence in the deoxycholate-butanol extracts very likely account for the relatively small increase in specific activity of the Ca²⁺-binding phosphatase, lower than that expected on the basis of protein recovery in peak 1. In this respect, the change in microenvironment around the enzyme (from the lipid-containing extract to the water so-

Table I. Purification of Alkaline Phosphatase from Cartilage Matrix Vesicles by FPLC

Purification steps	Total protein mg	Specific activities (μmol substrate/min per mg protein)*		
		pNPPase‡	ATPase§	PPase
Matrix vesicles	20.20 ± 1.35	14 ± 2	0.10 ± 0.02	0.4 ± 0.1
Deoxycholate-butanol extract	4.05 ± 0.32	63 ± 7	0.43 ± 0.08	1.8 ± 1.0
FPLC				
Peak 1	0.78 ± 0.15	116 ± 11	0.73 ± 0.07	3.4 ± 1.0
Peak 2	0.21 ± 0.10	74 ± 15	0.54 ± 0.15	2.1 ± 0.5
Peak 3	1.03 ± 0.20	95 ± 15	0.61 ± 0.10	2.7 ± 0.6
Peak 4	0.37 ± 0.07	113 ± 20	0.76 ± 0.11	3.1 ± 0.6
Residual fractions	0.78 ± 0.26	—	—	—
Total FPLC	3.17 ± 0.60			

* Mean values ± SD (from 6-14 experiments); assays in the presence of 1 mM Mg⁺⁺.

‡ *p*-Nitro-phenyl-phosphatase activity was measured in 0.2 M diethanolamine-HCl pH 10 containing 2 mM substrate.

§ ATPase activity was measured in 0.2 M Tris-HCl pH 7.4 containing 2 mM substrate.

|| Pyrophosphatase (PPase) activity was measured in 0.2 M diethanolamine-HCl pH 8.5 containing 2 mM substrate.

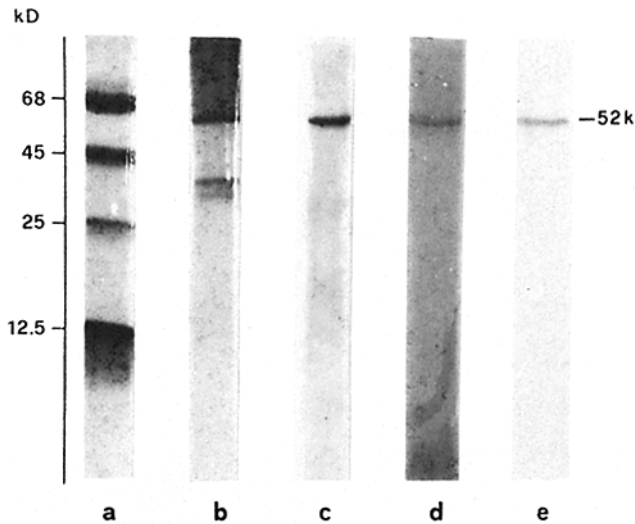


Figure 2. SDS PAGE of protein during alkaline phosphatase purification. Proteins were analyzed by SDS gradient gel electrophoresis in 10–20% polyacrylamide gels (9.5×18 cm) using the discontinuous system of Laemmli (23). Samples and amount loaded are: lane *a*, standards (BSA, ovalbumin, chymotrypsinogen A, cytochrome *c*, 10 µg each); lane *b*, deoxycholate-butanol extract of matrix vesicles, 30 µg protein; lanes *c–e*, 5 µg protein of peak 1. Samples were subjected to electrophoresis for 12 h at 12 mA and stained with Coomassie Blue G-250 (lanes *a–c*); glycoprotein were evidenced by periodic acid/Shiff reaction according to Konat et al. (22) (lane *d*). Proteins of lane *e* were transferred to nitrocellulose, reacted with rabbit antiserum raised against phosphatase, and stained with peroxidase-conjugated swine immunoglobulins to rabbit IgG.

lution) might also be critical. In fact a six- to sevenfold increment of the specific activity of peak 1 phosphatase was observed upon addition of phosphatidylcholine (0.5 mg/ml) to its assay mixture.

The Ca^{2+} -binding properties of the phosphatase of peak 1 were then analyzed (Fig. 3). The enzyme binds Ca^{2+} with high affinity (K_d of 0.31 µM), and the number of binding sites are 25 ± 3 per mole of protein (mean value of four experiments \pm SEM). The deoxycholate extract and peak 1 bind 270 ± 10 and 490 ± 50 nmol of Ca^{2+} per milligram of protein, respectively, with a purification ratio of 1.8, the same observed for the phosphatase activity.

To evaluate possible antigenic similarities of the four phosphatases, fractions of peaks 1–4 were subjected to SDS PAGE under reducing conditions, followed by electroblotting and staining of the nitrocellulose fingerprints with the antiserum to the peak 1 phosphatase (30). These experiments showed that the antiserum reacted intensely only with the reduced molecule of 52,000 mol wt (Fig. 2, lane *e*) and was not reacting with the proteins of the other three peaks (data not

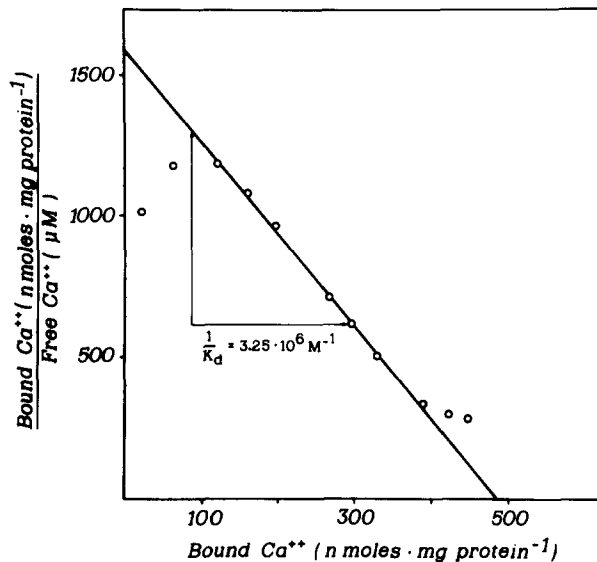


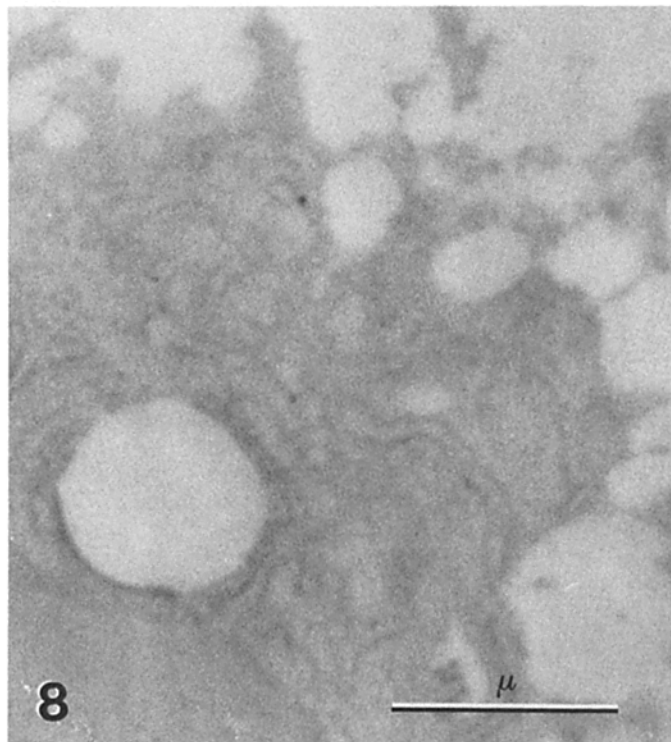
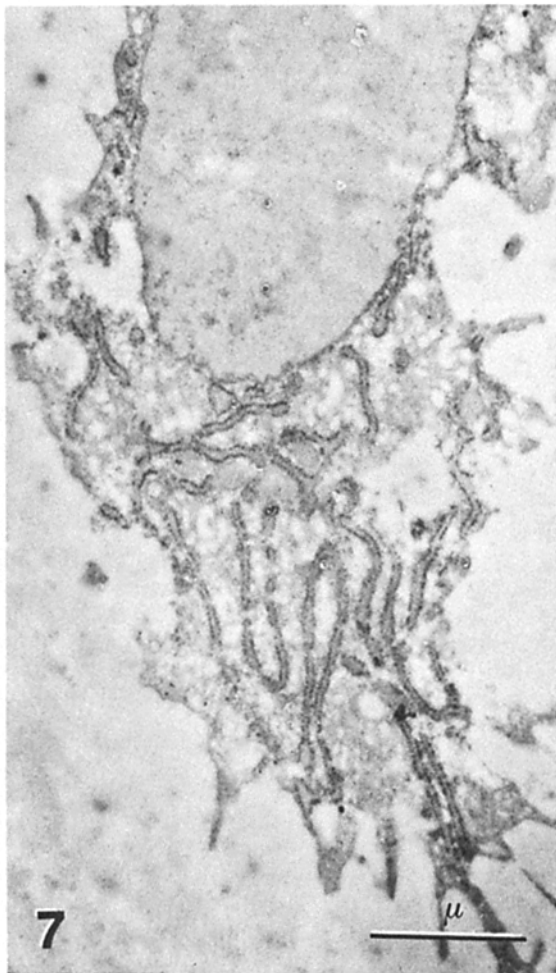
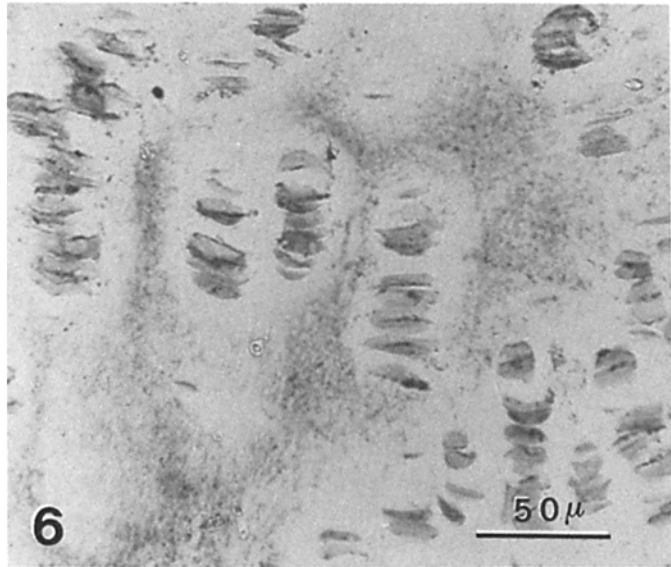
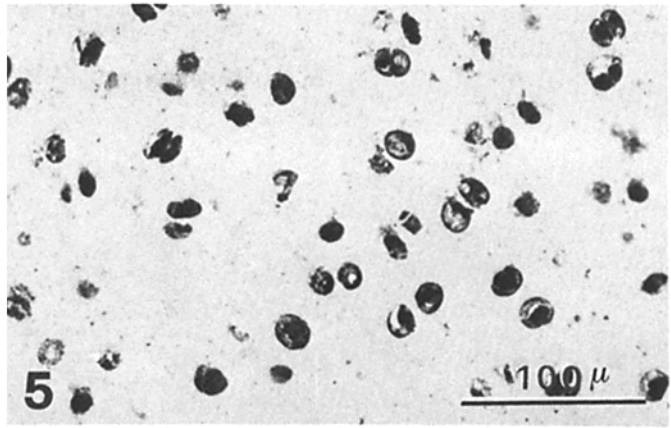
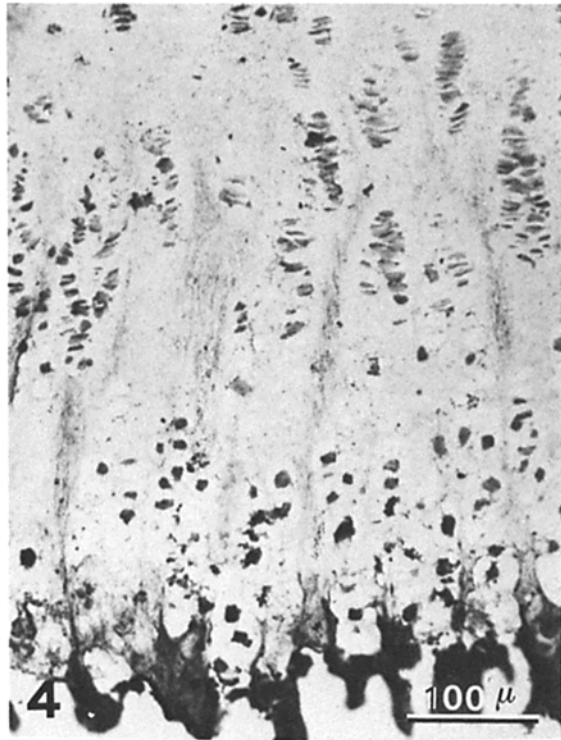
Figure 3. Measurement of the Ca^{2+} -binding activity of the M_r 52,000 glycoprotein. Samples (100 µg) of the purified glycoprotein were added to a mixture containing 0.16 mM Arsenazo III, 50 mM Tris-glycine buffer (pH 8.3), and free Ca^{2+} . Aliquots of 5 nmol EDTA were added until titration was complete as described in Material and Methods. Measurements of free Ca^{2+} concentration were obtained spectrophotometrically. Data are plotted according to Scatchard (26).

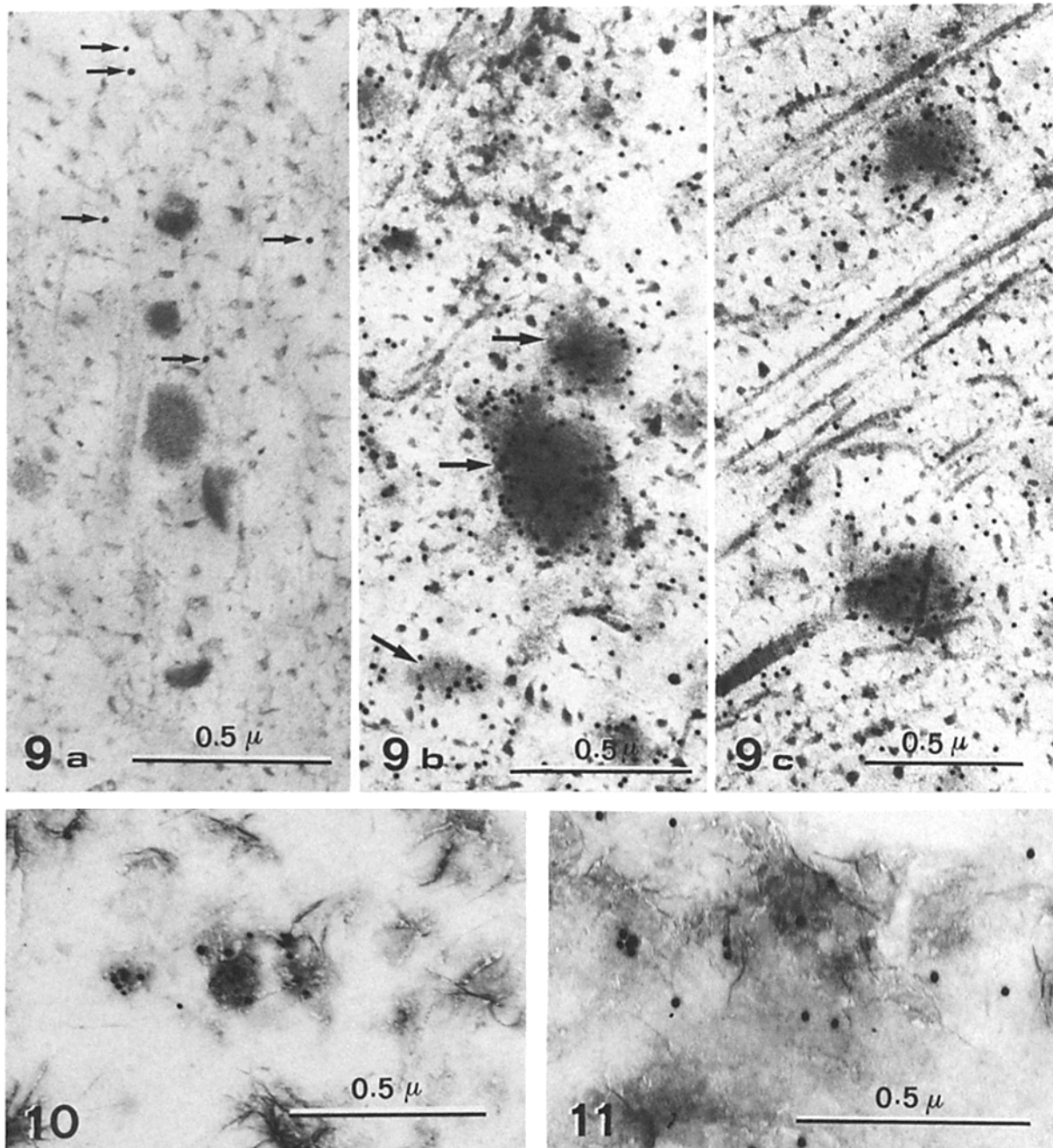
shown). The specificity of antiserum was also documented by the formation of immunocomplexes whose removal, by addition of protein A and centrifugation, caused the same extent of decrease of total phosphatase activity and of the number of Ca^{2+} -binding sites (from 50 to 75%).

The Ca^{2+} -binding alkaline phosphatase was shown to have a different localization in the different zones of the cartilage by immunohistochemical methods (peroxidase-antiperoxidase and immunofluorescence techniques). In resting cartilage, both methods showed that the enzyme has an intracellular localization, the intercellular matrix being completely negative (Figs. 4 and 5). Under the electron microscope, by both PAP and protein A-gold methods, a reactivity was evident only for perinuclear cisterna and cisternae of granular endoplasmic reticulum, where the strongest reaction was found in ribosomes (Figs. 7 and 8).

In the zones of seriated cartilage (maturing, hypertrophic, and degenerating), the chondrocytes were still positive (Fig. 4). Furthermore, a positive reaction was found in the matrix between chondrocyte columns, related to thin filaments and small granular structures. In oblique (Fig. 6) and transverse sections, these granular structures appeared to be distributed around chondrocytes, a localization similar to that of matrix vesicles. Immunostaining was very intense at the level of the calcifying and calcified cartilage (Fig. 4).

Figures 4–8. (Fig. 4) Immunoperoxidase staining of epiphyseal cartilage reacted with anti-alkaline phosphatase; resting zone (*top*) and calcified matrix (*bottom*). Note that the PAP reaction is positive in chondrocytes, intercellular longitudinal bundles where granules and fibrils are detectable and, above all, in calcified matrix. Matrix of the resting zone is negative. (Fig. 5) Immunoperoxidase staining of the resting cartilage; detail. Note the intense PAP reaction in chondrocytes and the negative reaction in intercellular matrix. (Fig. 6) Immunoperoxidase staining of the maturing zone of epiphyseal cartilage; section cut obliquely through chondrocyte columns; halos of positive granules surround the chondrocytes. (Fig. 7) Immunocytochemical localization of alkaline phosphatase in a resting chondrocyte using the PAP method on frozen 8-µm thick sections, subsequently postfixed in OsO_4 and embedded in araldite. Ultrathin sections examined without further staining. Note the intense reaction in perinuclear cisterna and endoplasmic reticulum. (Fig. 8) Immunocytochemical localization of alkaline phosphatase in resting cartilage; control section. Detail of a chondrocyte (compare with Fig. 7).



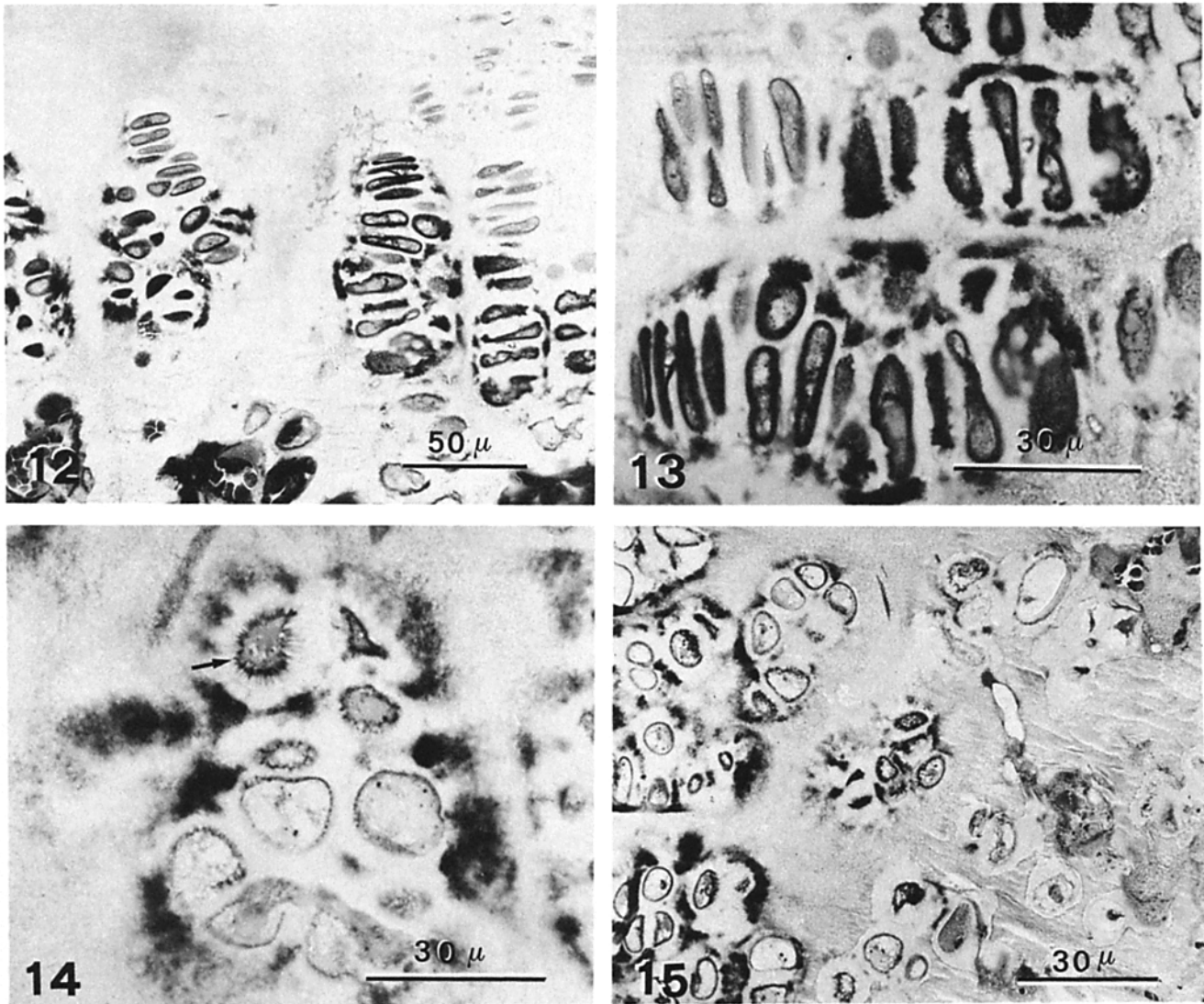


Figures 9–11. (Fig. 9) Immunogold staining of alkaline phosphatase in calcifying cartilage: samples embedded in araldite without OsO_4 postfixation. Tissue was fixed in paraformaldehyde. Uranyl acetate and lead citrate staining. (a) Control section; arrows point to a few background gold particles, easily distinguishable from proteoglycan granules. Note negativity of matrix vesicles. (b) Specific immunostaining of matrix vesicles of different width (arrows). (c) Specific immunostaining of two matrix vesicles. (Fig. 10) Immunogold staining of alkaline phosphatase in three partially calcified matrix vesicles; detail. Tissue preparation as in Fig. 9. (Fig. 11) Immunogold staining of alkaline phosphatase in an area of initial cartilage calcification. Note colloidal gold particles in calcification nodules. Tissue preparation as in Figs. 9 and 10.

Under the electron microscope, besides intracellular reaction of granular endoplasmic reticulum, positive reaction was found in the intercellular matrix, where collagen fibrils and, to a greater extent, matrix vesicles were labeled by the peroxidase and gold reactions (Fig. 9). The gold particles were often placed over the outer membrane of the matrix vesicles. Calcifying matrix vesicles, i.e., matrix vesicles containing early crystals, were also reactive (Fig. 10).

In the areas of initial calcification, positive reaction was found over and at the periphery of calcification nodules (Fig. 11). The fully calcified matrix was also labeled by the gold particles that, however, were mostly placed at its periphery. None of the control sections gave a positive reaction (Fig. 9 a).

In parallel with the immunochemical study of the distribution of the alkaline phosphatase, the enzyme activity was also



Figures 12-15. (Fig. 12) Histochemical demonstration of alkaline phosphatase activity in epiphyseal cartilage; note weak reaction of resting (*upper*) and degenerating (*bottom*) chondrocytes, strong reaction of maturing chondrocytes (*center*), and extracellular reaction at their sites. Naphthol-AS-phosphate and Fast Blue BB. (Fig. 13) Histochemical demonstration of alkaline phosphatase activity in epiphyseal cartilage; detail of Fig. 12. Note weak reactivity of resting chondrocytes (*upper left*) and strong reactivity of maturing chondrocytes. The enzyme is also active extracellularly, at both sites of these chondrocytes, where matrix vesicles are usually located. Naphthol-AS-phosphate and Fast Blue BB. (Fig. 14) Histochemical demonstration of alkaline phosphatase activity in epiphyseal cartilage; cross-section of chondrocyte columns. Note alkaline phosphatase activity in the membrane of the chondrocytes and their processes (*arrow*) and reactivity of pericellular areas, corresponding to those where matrix vesicles are usually located. Naphthol-AS-phosphate and Fast Blue BB. (Fig. 15) Histochemical demonstration of alkaline phosphatase activity in epiphyseal cartilage; detail of calcified cartilage. Alkaline phosphatase activity, visible in noncalcified matrix (*left*), is completely absent in the calcified cartilage (*center and right*). Naphthol-AS-phosphate and Fast Blue BB.

analyzed by histochemical methods. No reaction was found in the resting cartilage (Fig. 12). The reaction became strongly positive in the zones of maturing and hypertrophic chondrocytes (Figs. 12 and 13). This was chiefly seen on peripheral membrane of chondrocytes and their cytoplasmic processes (Figs. 13 and 14). Moreover, at the level of these zones, there was an evident extracellular reaction, which occurred in the same pericellular areas, where matrix vesicles are usually located (Figs. 13 and 14).

In the hypertrophic and degenerating zones, where the early calcification nodules can be found, the chondrocyte membrane, as well as cytoplasmic processes, stain positively

and the reaction was also positive along a pericellular halo, roughly corresponding to the area of matrix vesicles (Fig. 14). On the contrary, no enzymatic activity was detectable in the calcified matrix (Fig. 15).

Discussion

The rate of hydrolysis of phosphate esters by alkaline phosphatase at physiological pH is considered by some investigators to be too low for being relevant to the process of mineralization. Other investigators are more inclined to consider the enzyme as a phosphate transporter (14). However, eluci-

dition of the role of alkaline phosphatase in the mechanism of calcification requires an analysis that goes beyond the catalytic properties of the protein. In this regard, it is interesting that production of inorganic phosphate by the phosphatase can be associated with high capacity binding of Ca^{2+} to the enzyme molecule, as shown here. This is not the only Ca^{2+} -binding protein discovered in a mineralizing tissue. A dentine phosphoprotein has been extensively studied from this point of view (35). Also interesting is that this protein, like the phosphatase here described, shows many high-affinity binding sites (35), very likely for the specific sequence of oxygen- and phosphate-containing amino acids, suitable for the coordination of Ca^{2+} , as shown by nuclear magnetic resonance analysis (7). This feature seems to be unique for Ca^{2+} -binding proteins, as most of them have few binding sites. This points to a specific role of this class of proteins in calcifying tissues.

The alkaline phosphatase purified from matrix vesicles of epiphyseal cartilage is not different from the enzyme previously purified from the whole tissue (28). The two glycoproteins have the same fundamental biochemical features: amino acid and carbohydrate composition (data not shown) (9, 11), Ca^{2+} affinity (32), substrate specificity (28), and molecular weight (32), with the present enzyme being purified to the monomer condition and the previous one as a tetramer. Also the alkaline phosphatase purified from microsomes of chicken epiphyseal cartilage (8) has practically the same molecular weight (53,000) and similar catalytic and structural properties.

The amino acid composition of another alkaline phosphatase, very recently purified from matrix vesicles of fetal bovine epiphyseal cartilage (19), is similar to that of the enzyme here described (11), although the molecular weight reported for the former is higher (81,000). Unfortunately no data were given on the amount of sugars bound to the enzyme of fetal epiphyseal cartilage, which might be higher in fetal glycoproteins than in those of grown up animals. An alkaline phosphatase was recently purified also from teeth with a molecular weight of 50,200 and an isoelectric point of 3.7 (13), very close to the pI of our phosphatase, which is 4.15 (29).

Some degradation of the enzyme may have occurred, however, during the course of the isolation, during either the crude collagenase digestion step or the detergent extraction step. Furthermore matrix vesicles contain a metallo-proteinase (21), which may also contribute to a partial degradation of the phosphatase. It appears therefore that different laboratories have purified similar if not identical phosphatases. Unfortunately Ca^{2+} binding was measured only in our glycoprotein or in nonhomogeneous preparations (17, 34).

The role of the Ca^{2+} -binding phosphatase in the preosseous cartilage and its participation in the process of calcification are illustrated by the results of immunostainings, both at the light and electron microscope.

These results, in fact, show that in all cartilage zones cytoplasm react positively. At the light microscope, immune reactivity is present between the territorial and interterritorial matrix and discrete focal sites appear around mature and hypertrophic chondrocytes. Longitudinal septa are also positive. Moreover, the calcified matrix is strongly reactive. Electron microscopy confirms this distribution and shows reactivity of matrix vesicles, of calcification nodules, and of calcified matrix, especially at the periphery. The immuno-

gold reaction observed under the electron microscope is unevenly distributed and less marked than that obtained with the PAP method. This may be simply because samples are treated differently. It is surprising but interesting to note that the enzyme activity distribution in epiphyseal cartilage only in part coincides with that of the enzyme molecule, as detected by immune reactivity. In fact, an alkaline phosphatase activity is detected by histochemical techniques in the plasma membrane and cytoplasmic processes of maturing and, to a lesser extent, degenerating chondrocytes and their territorial matrix, including matrix vesicles. On the contrary, in the calcified matrix the enzyme protein, although present, does not show any activity.

The fact that the enzyme is active in the extracellular matrix of the maturing and hypertrophic regions, where calcification starts, and inactive, although present, in calcified matrix, suggests that the enzyme molecules are inhibited after calcification. The mechanism of this inhibition is unknown. It might be suggested that the molecules are incorporated in, and consequently masked by, inorganic substance.

In conclusion, the results reported in this paper strongly indicate that the cartilage phosphatase has the property of a Ca^{2+} -binding protein. By following its way to the calcification area, from the chondrocytes where the molecule is synthesized, the glycoprotein appears extruded from cells partly via matrix vesicles and partly into the surrounding environment. The enzyme appears to belong then to the same series of nucleating agents as dentine phosphoprotein, another Ca^{2+} -binding protein (35), with the important difference that the latter is not extruded with matrix vesicles. We have already shown that at least in vitro (33) cartilage phosphatase interacts with proteoglycan subunits and with type II collagen. This protein thus possesses all the features one would expect for an agent that catalyzes calcium phosphate formation and orients its deposition (Bangs et al. [3]). The crucial moment in the process of calcification is the passage of the glycoprotein from membranes of cells to the extracellular territory. On the basis of the present data and on those obtained with a study on the control of Ca^{2+} movements in chondrocytes (36), the triggering event appears to be the rise of Ca^{2+} concentration in cells. In epiphyseal cartilage this event seems to be promoted by a lack of oxygen (27). In other calcifying tissues the mechanism of Ca^{2+} elevation is still not known. At any rate, the transient Ca^{2+} rise very likely triggers the release of both matrix vesicles, with their Ca^{2+} -binding phosphatase, and hydrolytic enzymes which, by dissociating proteoglycans, greatly increase the availability of free Ca^{2+} in the extracellular matrix.

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