The Relationship Between Alkaline Phosphatase and Pyrophosphatase Activity in Quail Bone[1] (36880)

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Inorganic pyrophosphatase (EC 3.6.1.1, pyrophosphate phosphohydrolase) and alkaline phosphatase (EC 3.1.3.1, orthophosphoric monoester phosphohydrolase) activities are often closely associated in hard tissues (1–5). In bone the two activities appear to be important in the regulation of various calcification processes (5), and hydrolysis of pyrophosphate and phosphate esters which are known to inhibit hydroxyapatite deposition (6). An increase in the levels of the activities are often observed during vitamin D and calcium deficiency, and other conditions which have been reported to inhibit bone mineralization (5).

The interrelationship between alkaline phosphatase and pyrophosphatase in Japanese quail (cortunix) bone is reported here. Similar to bone inorganic pyrophosphatase and alkaline phosphatase in the hamster (1), rabbit (2), and human (3, 4), both activities in quail bone appear to be catalyzed by a single enzyme or at least by proteins possessing similar properties. The use of Japanese quail in studies involving calcification processes is becoming increasingly popular. The assay conditions and properties of the enzymatic activities described should be useful in further studies involving this species.

Materials and Methods. The two femur and tibia from 3 to 4 wk old quail were used to obtain enzyme extracts. Marrow was first removed by a stream of cold 0.025 *M* Tris–HCl buffer (pH 7.5) by means of syringe. Twelve to 15 g of bone could be obtained from 10 to 15 quails. The cleansed bone was then homogenized in a blender in a mixture of *n*-butanol and water (1:2, v/v) to give a 20% suspension (w/v). The suspension was stirred for 5 hr, centrifuged (25,000*g*; 20 min), and the *n*-butanol layer was discarded. The aqueous layer was dialyzed against 0.05 *M* Tris-HCl buffer (pH 7.5) overnight and assayed for alkaline phosphatase and inorganic pyrophosphatase.

Alkaline phosphatase activity was measured spectrophotometrically (410 nm) at pH 10.7 using *p*-nitrophenyl phosphate as substrate in the presence of magnesium ion (usually 4 m*M*). Inorganic pyrophosphatase activity was measured using the method of Wöltgens, Bonting and Bijuoit (1). Assay mixtures usually contained sodium pyrophosphate (2.5 m*M*) and magnesium ion (1 m*M*) in Tris buffer at pH 8.0. The pH values represent optima which were determined experimentally. One unit is that activity liberating either 1 μmole of *p*-nitrophenol or inorganic phosphate/min.

In initial fractionation attempts, ammonium sulfate was used, but was discarded because of repeated loss of activity. The procedure finally adopted involved first dialysis (see above) and concentration of the dialyzed solution by pressure dialysis using an ultramembrane filter with a 40,000 MW cut-off (7). The concentrated solution was applied directly to a column of DEAE-cellulose (30 × 1.5 cm) and eluted with 0.01 *M* Tris-HCl buffer (pH 7.7) containing NaCl to give a gradient from 0 to 1.0 *M* (see Fig. 1). Fractions containing the two activities were again concentrated by pressure dialysis and chromatographed on a column of Bio-gel P-300 (45 × 1.5 cm). The column had been previously calibrated with protein

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TABLE I. Fractionation of Alkaline Phosphatase and Inorganic Pyrophosphatase Activity.[a]

Step	Vol (ml)	Total protein (mg)	Sp act[b]	
			Alkaline phosphatase	Inorganic pyrophosphatase
1. Aqueous extract	42	63	0.072	—
2. Centrifugation (25,000g; 20 min)	41	31.6	0.057	69.2
3. Ultracentrifugation	14	22.4	0.116	—
4. DEAE-cellulose[c]	39	3.0	0.833	57.9

[a] Approximately 15 g of bone were used in a typical fractionation.
[b] Units per milligram protein.
[c] Fractions 21–28, see Fig. 1.

standards for molecular weight determination (8).

In addition, gel electrophoresis of fractions containing activity was performed using 7% acrylamide gels (pH 8.3) as described by Davies (9). Alkaline phosphatase was easily detected by incubating the gels in solutions containing Tris buffer (0.2 mM; pH 10.7), 1 mM p-nitrophenylphosphate and 0.5 mM magnesium ion. The presence of an intense yellow band from the production of p-nitrophenol indicated the location of the enzyme. Protein was detected by staining the gels with amido schwartz. Quantification of both

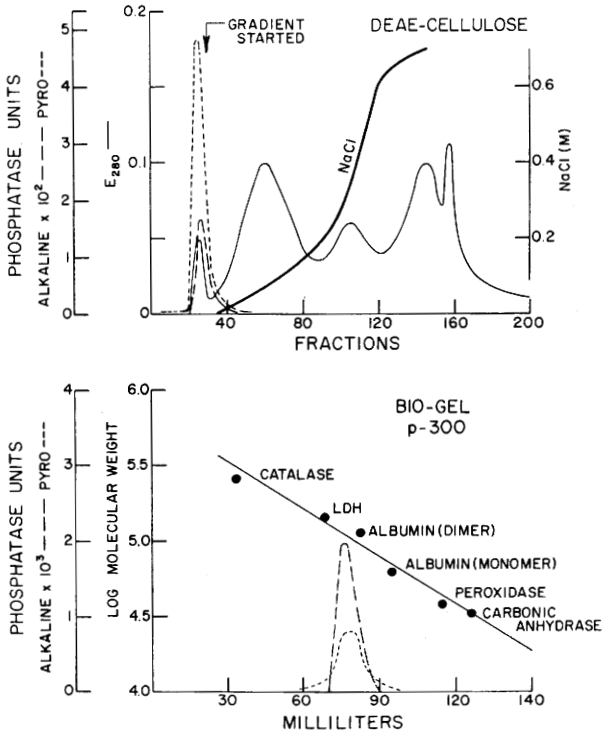

FIG. 1. Ion-exchange chromatography (DEAE-cellulose, pH 7.7) and gel filtration (Bio-gel P-300) of quail bone extracts. For DEAE-cellulose chromatography 5 ml fractions were collected from a 30 × 1.5 cm column with a flow rate of 1 ml/min. For gel filtration, a column (45 × 1.5 cm) was used and protein was eluted with 0.1 M Tris buffer (pH 7.5) containing 0.5 M NaCl with a constant flow rate of 1 ml/5 min. The column had been previously standardized using the proteins indicated.

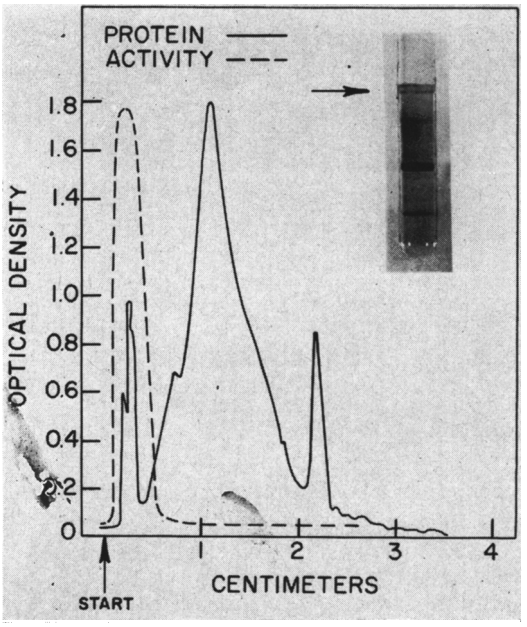

FIG. 2. Acrylamide gel electrophoresis of the extract and active fractions obtained from DEAE-cellulose chromatography. The presence of alkaline phosphatase (- - -) in the gel was detected after a 20 min incubation in the presence of 1 m*M* *p*-nitrophenylphosphate. The gel was scanned at 410 nm using a Gilford spectrophotometer with a gel scanning attachment. Protein (—) was measured by staining with amido schwartz and scanning at 600 nm. The scan represents activity and proteins in the crude extract. The gel depicted represents a fraction obtained after ion-exchange chromatography. The arrow indicates the portion of the gel where alkaline phosphatase activity was observed.

protein and activity was achieved by a densitometer adapted for scanning acrylamide gels.

Results. Fractionation of bone extracts by means of DEAE-cellulose chromatography resulted in a 12-fold purification of alkaline phosphatase activity (Table I, Fig. 1). Considerable losses in inorganic pyrophosphatase activity, however, occurred with each attempt at purification and with storage. Approximately 50% of the pyrophosphatase activity in fractions obtained after DEAE-cellulose chromatography was lost per month even when stored at −10°. Alkaline phosphatase activity also declined after chromatography on DEAE-cellulose, but activity remained stable for longer periods (50% loss after 2 mo).

Both enzymatic activities were closely associated throughout attempts at fractionation, and could not be separated by ion-exchange chromatography or by gel filtration (Fig. 1). On the calibrated columns of Biogel P-300, the activities eluted in a manner similar to proteins of 100,000 to 130,000 MW. When active fractions obtained from the extract and after DEAE-cellulose chromatography were electrophoresed on polyacrylamide gels, a broad single band of alkaline phosphatase activity could be detected (Fig. 2). This activity could be easily eluted from the gel slices by mild homogenization in buffer. Unfortunately no method was available for the direct determination of inorganic pyrophosphatase activity in the gels. Protein eluted from gel slices, however, did contain some pyrophosphatase activity.

The two activities were markedly affected by the presence of magnesium ion (Fig. 3a and b). For maximal stimulation a molar ratio of 0.3–0.5 (*p*-nitrophenylphosphate to magnesium) was required for alkaline phosphatase and a ratio of 2.5 (pyrophosphate to magnesium) was required for pyrophosphatase. Inhibition usually occurred at ratios less than 2.5 for pyrophosphatase. For alkaline phosphatase the effect of magnesium activation, however, was only significant at the pH optima and was not observed when the hydrolysis of *p*-nitrophenylphosphate was measured below pH 8. In this regard the effects of pyrophosphate addition upon the hydrolysis of *p*-nitrophenylphosphate were easily determined, and it could be demonstrated that in the region of physiological pH pyrophosphate competitively inhibited alkaline phosphatase activity (Fig. 3c). Inhibition due to fluoride was also observed (Fig. 3d). Fluoride inhibition could be demonstrated with pyrophosphatase but only at the optimal ratio of substrate to magnesium and never greater than 30%. In contrast, alkaline phosphatase activity was inhibited approximately 35% when 4 m*M* fluoride, and no magnesium, was added to assays. This effect was not observed when magnesium was added in excess of 2 m*M*.

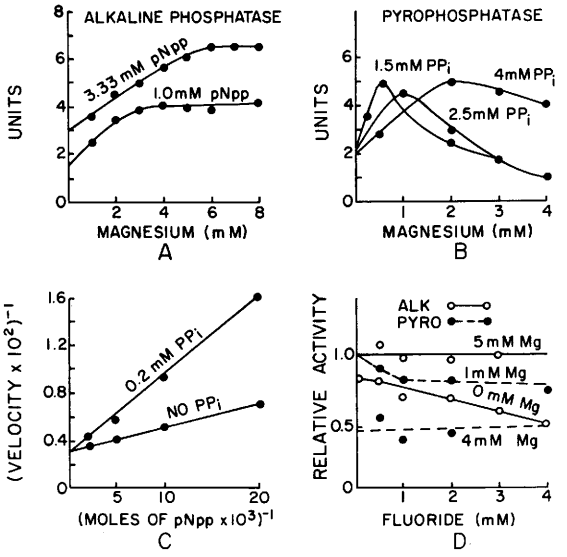

FIG. 3. Properties of pyro- and alkaline phosphatase. (A) Stimulation of alkaline phosphatase activity (pH 10.7, 0.05 *M* Tris buffer) by magnesium. (B) Stimulation of pyrophosphatase activity (pH 8.0, 0.05 *M* Tris buffer) by magnesium. (C) Competitive effects of pyrophosphate upon the hydrolysis of *p*-nitrophenylphosphate (pH 7.5). (D) Effect of Mg and fluoride on pyro- and alkaline phosphatase activity. Values for pyro- and alkaline phosphatase activities are expressed relative to those obtained when 2.5 m*M* of pyrophosphate and 1 m*M* magnesium (pH 8.0) and 1 m*M* *p*-nitrophenylphosphate and 5 m*M* of magnesium (pH 10.7) were added to reaction mixtures, respectively.

Discussion. The association of alkaline phosphatase activity with calcification was first suggested by Robinson in 1923 (10). Only recently has it become clear that this rather nonspecific phosphoester hydrolase is probably a pyrophosphate phosphohydrolase (1–5). That the two activities in quail bone may also be viewed similarly is based on the following evidence.

The two activities could not be separated after ion-exhange chromatography and gel filtration. Furthermore, alkaline phosphatase activity when subjected to gel electrophoresis always appeared as a single band of protein. The failure to separate the two types of enzymatic activities and the inability to demonstrate more than one active form of alkaline phosphatase constitutes strong evidence that the two activities are properties of the same enzyme. In keeping with this view is the fact that pyrophosphate inhibited competitively the hydrolysis of *p*-nitrophenylphosphate, which may be interpreted as a single enzyme acting simultaneously on two substrates (11). With respect to the effects of magnesium activation and pH optima, there were some differences between the two activities. However, these differences were interpreted as being due to changes in the structural characteristic of the substrates or changes at the enzyme active center. Such differences in the properties of the two enzymatic activities would not necessarily imply that there are two different species of enzyme (11).

When fluoride is added to otherwise normal diets for the quail, both alkaline and pyrophosphatase activities in bone tissue are elevated (12). Similar observations have been made using chicks (13). This is in contrast to the effects of fluoride *in vitro* in which inhibition is usually observed (5, 13). Although the quail bone activities were not extremely sensitive to fluoride, some inhibition *in vitro* could be demonstrated.

Stimulation of activity by magnesium ion addition is a general property of bone phosphatase activity in most animals and man (1–4). In quail bone magnesium also acts as an activator. Likewise with respect to the other properties (pH optima, molecular size,

etc.), the two enzymatic activities in quail bone behave similarly to those reported in most other species (1–5). It is felt the data presented in this report corroborate with these previous findings and strongly suggest that so called alkaline phosphatase and near-neutral pyrophosphatase activities in bone may be catalyzed by a single enzyme.

Summary. The pyro- and alkaline phosphatase activities of quail bone appear to be catalyzed by a single enzyme. The activities could not be separated after ion-exchange chromatography or gel filtration. The pH optima for pyro- and alkaline phosphatase were 8.0 and 10.7, respectively. Alkaline phosphatase activity was stimulated by magnesium. Optimal stimulation of pyrophosphatase activity occurred at a pyrophosphate to magnesium ratio of 2.5. Both enzymatic activities were not markedly affected by high concentrations of fluoride.

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