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SOLUBILIZATION OF MEMBRANE-BOUND MATRIX-INDUCED ALKALINE PHOSPHATASE WITH POLYOXYETHYLENE 9-LAURYL ETHER (POLIDOCANOL): PURIFICATION AND METALLOENZYME PROPERTIES

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Abstract—1. Matrix-induced alkaline phosphatase prepared from rat osseous plate was solubilized with polidocanol and purified on a Sephacryl S-300 column.

2. Purified solubilized alkaline phosphatase has a molecular weight of *ca* 115,000 and bind one magnesium and two zinc ions. At least 110 detergent molecules are bound to each enzyme molecule.

3. Solubilization and purification procedures did not destroy the ability of the enzyme to hydrolyze adenosine-5′-triphosphate, *p*-nitrophenylphosphate, pyrophosphate and bis *p*-nitrophenylphosphate.

4. Magnesium, manganese and cobalt ions are stimulators of PNPPase activity of solubilized enzyme whereas calcium and zinc ions are inhibitors.

INTRODUCTION

Alkaline phosphatase from bone and cartilage is a non-specific phosphomonohydrolase, associated with lipoprotein membranes (Arsenis *et al.*, 1975; Kahn *et al.*, 1978; Vaananen and Korhonen, 1979; Farley *et al.*, 1980; Skillen and Rahbani-Nobar, 1980; Warner *et al.*, 1983; Hsu *et al.*, 1985; Curti *et al.*, 1986).

In spite of intensive studies on its structural and kinetic characterization, the role of this intriguing enzyme in biological mineralization is still poorly understood (McComb *et al.*, 1979; Wuther, 1982; Wuthier and Register, 1985).

The techniques used to solubilize integral proteins from the membrane usually destroy the native membrane structure and care must be taken during the solubilization and purification procedures in order that the membrane-associated function of the protein could be preserved. In fact, the use of proteases and organic solvents for the preparation and purification of alkaline phosphatase from cartilage and bone led to conflicting results in the literature (Ali *et al.*, 1970; Arsenis *et al.*, 1975; Vaananen and Korhonen, 1979; Fortuna *et al.*, 1980; Cyboron and Wuthier, 1981). Furthermore, it has been suggested that the protease treatment may damage the noncatalytic moiety of the enzyme thus affecting characteristics which might be important for its physiological role (Cyboron and Wuthier, 1981).

Although the use of a detergent usually represents a complication in analyzing membrane proteins, they have been successfully used in recent years for solubilization, purification and reconstitution of membrane-bound enzymes (Hjelmeland and Chrambach, 1984; Koepsell, 1986; Jones *et al.*, 1987). Nevertheless for mammalian alkaline phosphatases there are few data concerning their solubilization by detergents (Ey and Ferber, 1977; Warner *et al.*, 1983; Pizauro *et al.*, 1987, 1988).

Matrix-induced alkaline phosphatase (MIAP) is a membrane-bound enzyme containing two apparently identical subunits of M_w 65,000 which bind one magnesium and two zinc ions and share a large number of properties with other alkaline phosphatases (Pizauro *et al.*, 1987; Ciancaglini *et al.*, 1989).

In this paper an improved method is described for solubilization of membrane-bound MIAP with polyoxyethylene 9-lauryl ether. Also the characterization of the metalloenzyme properties of the solubilized enzyme is described.

MATERIALS AND METHODS

Tris, fluorescamine, 2-amino-2-methyl-1-propanol, Fast Blue RR salt, Naphthyl phosphate and polyoxyethylene 9-lauryl ether were from Sigma Chemical Co.; *p*-nitrophenyl phosphate disodium salt was from Merck. Analytical grade salts were used without further purification as the source of metal ions.

Preparation of purified MIAP

Purified membrane bound MIAP was prepared as described previously by Curti *et al.* (1986).

Enzymatic activity measurements

Phosphodiesterase activity was assayed continuously, at 37°C, by following the liberation of *p*-nitrophenolate ion (E 1 M, pH 9.4 = 17,600 $M^{-1} \cdot cm^{-1}$) at 410 nm, in a Varian DMS-80 spectrophotometer equipped with thermostatted cell holders, using bis *p*-nitrophenyl-phosphate (BIS) as substrate. Standard conditions were 50 mM 2-amino-2-

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methyl-1-propanol (AMPOL) buffer, pH 9.4, containing 2 mM $MgCl_2$ in a final volume of 1.0 ml.

p-Nitrophenylphosphatase (PNPPase) activity was assayed, at 37°C, as described by Pizauro *et al.* (1987).

For inorganic pyrophosphate (PP) and ATP, phosphohydrolase activity was assayed by measuring the amount of inorganic phosphate liberated according to the procedure described by Heinonen and Lahti (1981), adjusting the assay medium to a final volume of 1.0 ml. The reaction was initiated by the addition of the enzyme, stopped with 0.5 ml of cold 30% TCA at appropriate time intervals and centrifuged at 4,000 g just before phosphate determination. Standard assay conditions were 50 mM Tris·HCl buffer, pH 8.0 containing 2 mM $MgCl_2$ and 50 mM AMPOL buffer pH 9.4 containing 2 mM $MgCl_2$ for pyrophosphatase and ATPase activities, respectively.

Determinations were carried out in duplicate and the initial velocities were constant for at least 90 min provided that < 5% of substrate was hydrolyzed. Controls without added enzyme were included in each experiment to allow for the nonenzymic hydrolysis of substrate. One enzyme unit (U) was defined as the amount of enzyme hydrolyzing 1.0 nmol of substrate per minute, at 37°C.

Determination of protein concentration

Protein concentrations were determined according to the procedure described by Hartree (1972) for the membrane-bound enzyme and according to Cadman *et al.* (1969) for the solubilized enzyme. In both cases, bovine serum albumin was used as standard.

Solubilization of MIAP with polyoxyethylene 9-lauryl ether

Samples containing 0.2 mg/ml of MIAP were solubilized with 1% polidocanol (final concentration) for 2 hr with constant stirring, at 25°C. After centrifugation at 30,000 *g* for 2 hr, the solubilized enzyme was concentrated on an YM-5 Amicon filter and dialyzed overnight, at 4°C, against 5 mM Tris:HCl buffer, pH 7.5, containing 2 mM $MgCl_2$, 150 mM NaCl and 0.01% polidocanol.

Chromatography on Sephacryl S-300 column

The solubilized enzyme was purified on a Sephacryl S-300 column (130 × 1.7 cm) equilibrated and eluted in the same buffer used for dialysis. The fractions showing PNPPase activity were pooled, concentrated on an YM-5 Amicon filter, dialyzed overnight, at 4°C and rechromatographed in the same conditions as described above. Finally, the purified polidocanol-solubilized alkaline phosphatase (PSAP) was dialyzed overnight, at 4°C, against 5 mM Tris·HCl buffer, pH 7.5 containing 0.01% polidocanol and used in all the experiments reported in this work.

Polyacrylamide gel electrophoresis

Electrophoresis was carried out in a 7% gels according to Davis (1964) using Coomassie Blue R for staining. Glycoproteins were detected according to Racusen (1979) and fluorescamine-labelled enzyme was prepared according to Bohlen *et al.* (1973). Phosphohydrolytic activity on the gel was detected in 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$, 0.12% 1-naphthyl phosphate and 0.12% Fast Blue RR, at 37°C.

Molecular weight determination

The molecular weight of the solubilized enzyme was estimated on a Sephacryl S-300 column (130 × 1.7 cm) equilibrated and eluted with 5 mM Tris·HCl buffer, pH 7.5, containing 2 mM $MgCl_2$, 150 mM NaCl and 0.01% polidocanol according to the procedure described by Pizauro *et al.* (1987). Myosin, phosphorylase b, liver alcohol dehydrogenase, bovine serum albumin, egg-albumin, carbonic anhydrase and myoglobin were used as molecular markers.

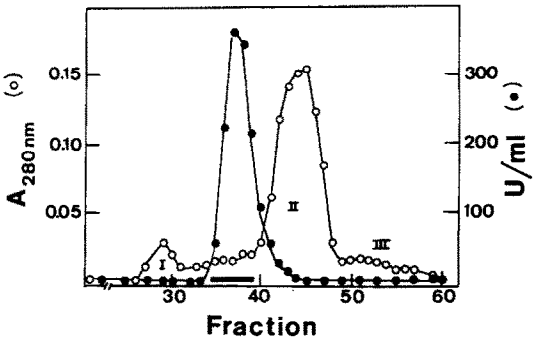

Fig. 1. Chromatography of PSAP on Sephacryl S-300 column (130 × 7 cm) equilibrated and eluted with 5 mM Tris·HCl buffer, pH 7.5, containing 2 mM $MgCl_2$, 150 mM NaCl and 0.1% and 0.01% polidocanol. Polled fractions (——) correspond to SAP. Volume of each fraction: 3.2 ml. Linear flow rate: 5.7 cm·hr^{-1}.

Determination of metal ions

The determination of magnesium and zinc ions was performed in an Atom Spek atomic absorption spectrometer using as standard 0.2 ppm zinc chloride and 0.4 ppm magnesium chloride solutions previously standardized against EDTA.

Estimation of kinetic parameters

Kinetic parameters obtained from substrate hydrolysis were fitted on an IBM/PC microcomputer according to the Hill equation (Hill, 1910) using the procedure described by Atkins (1973). V, K_d, K_m and n which appear in this paper as computed values stand for maximal velocity, half-maximum effect, apparent dissociation constant and Hill coefficient, respectively.

RESULTS

Chromatography of polidocanol-solubilized enzyme on Sephacryl S-300 column (Fig. 1) always resulted in a chromatographic pattern consisting of three protein peaks in which all PNPPase activity (not coincident with any of them) was eluted between fractions 35–40 (SAP fraction). When the SAP fraction was rechromatographed under the same conditions only two protein peaks were observed (Fig. 2) and the second one, which constituted 6% of total

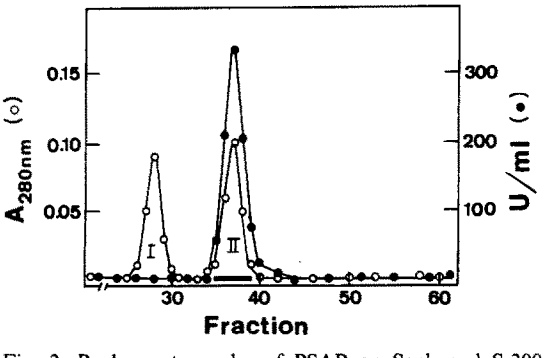

Fig. 2. Rechromatography of PSAP on Sephacryl S-300 column (130 × 1.7 cm) equilibrated and eluted with 5 mM Tris·HCl buffer, pH 7.5, containing 2 mM $MgCl_2$, 150 mM NaCl and 0.01% polidocanol. Polled fractions (——) correspond to PSAP. Volume of each fraction: 3.2 ml. Linear flow rate: 5.7 cm·hr^{-1}.

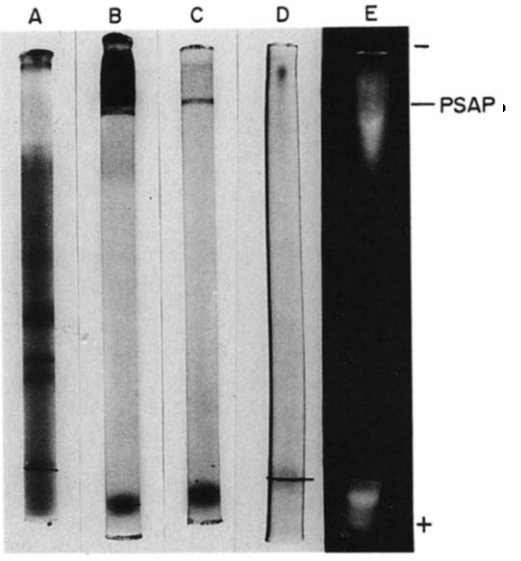

Fig. 3. Polyacrylamide gel electrophoresis of PSAP. (A) Polidocanol extract stained with coomassie R. (B) Phosphophydrolytic activity of polidocanol extract. (C) Phosphohydrolytic activity of PSAP. (D) PSAP stained for glycoprotein. (E) Fluorescamine-labelled PSAP.

Table 1. Purification of PSAP on Sephacryl S-300 column

Step	Protein (mg)	Enzyme (U)	U/mg	Yield (%)
MIAP	8.0	5424	678	100
Polidocanol extract	3.7	6179	1687	114
SAP fraction	0.93	3858	4129	71
PSAP fraction	0.49	2797	5728	51

and the second one, which constituted 6% of total solubilized protein, was coincident with that of alkaline phosphatase activity. Homogeneous PSAP was eluted as a single symmetrical peak coincident with PNPPase activity. PSAP had a sp. act. of 5728 U/mg, the yield was 51% and a 8.5-fold purification was obtained. Moreover, PSAP (70 μg/ml) was stable for 30 days when stored in 5 mM Tris·HCl buffer, pH 7.5, containing 2 mM $MgCl_2$ and 0.01% polidocanol, at 4°C. A typical purification procedure is summarized in Table 1.

Polyacrylamide gel electrophoresis showed that PSAP was isolated as an electrophoretically pure, although diffuse, form (Fig. 3C and 3E). The broad diffuse band observed for fluorescamine-labelled enzyme could be attributed to its glycoproteic nature (Fig. 3D).

The molecular weight of PSAP, estimated by filtration on Sephacryl S-300 was 115,000. At least 110 molecules of the detergent are bound to each enzyme molecule (results not shown).

The substrate specificity of PSAP was checked using PNPP, ATP, BIS and PP and the results obtained are shown in Table 2. It is evident that the solubilization and purification procedures did not destroy the ability of the enzyme to hydrolyze these substrates. On the other hand, solubilization changed the characteristics of the pyrophosphate saturation curve which showed only a single class of binding sites for this substrate. Whether this change was due to release of latent enzyme from membrane or to the binding of detergent to functional sites of the enzyme molecule, remains to be investigated.

Atomic absorption spectrometry revealed that each subunit of PSAP binds at least one magnesium and two zinc ions (results not shown).

As seen in Fig. 4 divalent metal ions were required for maximal activity of PSAP. The stimulation of the hydrolytic activity of the enzyme by magnesium (Fig. 4A), manganese (Fig. 4B) or cobalt ions (Fig. 4C) was very similar. Best stimulation, of *ca* 40–60% was observed for magnesium and manganese ions in contrast to the poorer stimulation of 16% observed for cobalt ions. Nevertheless, cobalt ions showed better half maximum effects ($K_d = 2.7\ \mu$M) when compared with those of manganese and magnesium

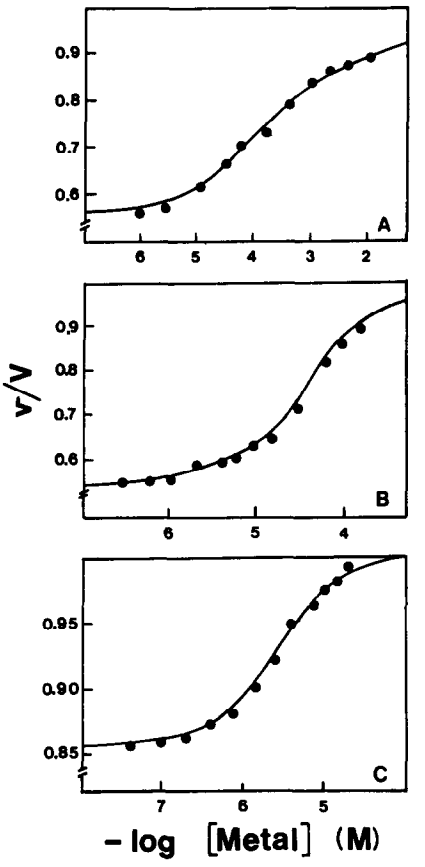

Fig. 4. Stimulation of phosphohydrolytic activity as PSAP by divalent metal ions: (A) magnesium; (B) manganese; (C) cobalt. Protein concentration used was 3 μg. Enzymatic activity was assayed using PNPP as substrate as described in Materials and Methods.

ions. The kinetic parameters calculated for the stimulation of PSAP by these ions are summarized in Table 3.

Figure 5 shows the effect of increasing concentration of calcium and zinc ions on PNPPase activity of solubilized enzyme. Excess zinc ions were potent inhibitors of PSAP even in the presence of 2 mM $MgCl_2$, resulting in a total loss of activity of PSAP. These results suggested that the inhibitory effect observed was probably due to the displacement of magnesium by calcium (Fig. 5A) or zinc ions (Fig. 5B). Calcium ions revealed a half maximum effect ($K_d = 5.7\ \mu$M) very similar to that obtained for zinc alone ($K_d = 6.7\ \mu$M) or in the presence of 2 mM $MgCl_2$ (K_d 5.6 μM).

Table 2. Kinetic parameters for the hydrolysis of physiological and pseudosubstrates by both MIAP and PSAP

		PSAP			MIAP		
Substrate	pH	K_m (mM)	V (U/mg)	n	K_m (mM)	V (U/mg)	n
PNPP	9.4	0.04	1058	1.1	0.10	344	1.0
ATP	9.4	0.09	228	0.7	0.15	592	0.9
BIS	9.4	2.24	451	1.1	3.95	169	1.3
PP	8.0	1.11	250	1.8	0.04[1]	37[1]	1.4[1]
					1.40[2]	65[2]	2.2[2]

[1] High affinity binding sites for PP.
[2] Low affinity binding sites for PP.

Table 3. Stimulation of PNPPase activity of PSAP by divalent metal ions, at pH 9.4

Metal	Mg (II)	Mn (II)	Co (II)
V (U/mg)	915	1501	913
K_d (μM)	267	45.7	2.7
n	0.7	1.0	1.2
Stimulation (%)	52	57	16

DISCUSSION

Due to the complexity of the interaction between molecules of different chemical structures such as proteins, lipids and detergents, the success of a solubilization and purification procedures is often determined by the choice of a suitable detergent.

In spite of the fact that we did not check for selective extraction of membrane components, polidocanol was very effective in solubilizing MIAP (see Table 1). High yields of solubilized MIAP were also reported elsewhere for a series of detergents (Pizauro *et al.*, 1987).

Among the commonly-used detergents, Triton X-100 has been widely used for solubilization of alkaline phosphatases obtained from different sources (Doellgast and Fishman, 1974; Ey and Ferber, 1977; Pizauro *et al.*, 1987). However, high concentrations (3–5%) of this detergent are required for the solubilization of membrane proteins (Hjelmeland and Chrambach, 1984). The low concentration of polidocanol used (0.01%) seems appropriate for studying the metalloenzyme characteristics of PSAP since it is well known that in the solubilized state, the presence of detergent may affect the concentration of substrate or ligands available to the protein.

Although it has been reported that alkaline phosphatase obtained from chicken and bovine epiphyseal cartilage represents $<1\%$ of the total proteins of microsomes (Cyboron and Wuthier, 1981; Hsu *et al.*, 1985) our results showed that PSAP corresponds to 6% of the total extractable matrix proteins (see Table 1). Furthermore, considering that only a 8-fold purification was required to obtain a homogeneous preparation it seems that a considerable proportion of matrix phosphatase activity was MIAP. It should be noted that, like other mammalian alkaline phosphatases (McComb *et al.*, 1979), PSAP is also a glycoprotein (see Fig. 3D).

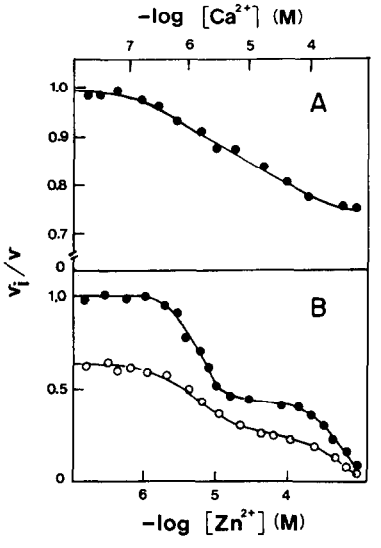

Fig. 5. Inhibition of phosphohydrolytic activity of PSAP by: (A) calcium ions; (B) zinc ions in the presence of 2 mM $MgCl_2$ (●) no $MgCl_2$ (○). Protein concentration used was 3 μg. Enzymatic activity was assayed using PNPP as substrate as described in Materials and methods.

Interestingly enough, the solubilization and purification procedures did not destroy the ability of PSAP to hydrolyze PNPP, ATP, BIS and PP (see Table 2). The possibility that these activities were associated with different enzyme molecules present in the membrane bilayer is unlikely since PSAP was obtained as a homogeneous protein (see Fig. 3C and 3E). Although several authors reported that these activities stem from one enzyme (Majeska and Wuthier, 1975; Felix and Fleisch, 1976; Fortuna *et al.*, 1980; Cyboron and Wuthier, 1981; Hsu *et al.*, 1985; Pizauro *et al.*, 1987), contradictory results have also been reported (Skillen and Rahbani-Nobar, 1980).

According to Farley *et al.* (1980) it is unlikely that cAMP could act as a modulator of alkaline phosphatase activity "*in vivo*". Our results showed that both MIAP and PSAP have phosphodiesterase activity *in vitro*, suggesting new perspectives to clarify the physiological role of this enzyme during the calcification process. In fact, MIAP can also hydrolyze cAMP ($K_m = 0.3$ mM), a physiological substrate, at pH 7.5 (A. A. Rezende, unpublished results).

In spite of the fact that magnesium ions inhibit the placental and epiphyseal cartilage enzymes (Harkness, 1968; Fortuna *et al.*, 1980; Cyboron *et al.*, 1982), the slight stimulatory effect exerted by these ions on the activity of PSAP (see Fig. 4A) was qualitatively similar to those reported for other mammalian alkaline phosphatases (Hiwada and Wachsmuth, 1974; Ohkubo *et al.*, 1974; Cathala *et al.*, 1975; Jahan and Butterworth, 1986; Navaratnam and Stinson, 1986). The difference in the extent of activation may be attributed to the varying effectiveness of the purification procedure used to remove these ions from the enzyme molecule. Furthermore, the binding of magnesium ions to the magnesium binding site does not affect K_m value for PNPP, indicating that saturation of these sites does not interfere with the recognition of PNPP by the substrate binding site (Ciancaglini *et al.*, 1989).

Cobalt and manganese ions can be substituted for magnesium ions to stimulate PSAP (see Fig. 4B and 4C). A similar result was reported for alkaline phosphatases from kidney and brain (Brunel and Cathala, 1973; Hiwada and Wachsmuth, 1974; Cathala *et al.*, 1975). However contradictory results have been reported for calf thymus and bovine epiphyseal cartilage enzymes (Ey and Ferber, 1977; Fortuna *et al.*, 1980).

Analysis of several purified phosphatases has established that zinc is a functional component of the enzyme (McComb *et al.*, 1979). Indeed this ion is also a functional component of MIAP (Ciancaglini *et al.*, 1989) and it is not removed from the enzyme molecule during the solubilization and purification procedures. However, excess zinc is a potent inhibitor of PSAP even in the presence of 2 mM $MgCl_2$ (see

Fig. 5A). Our results suggest that this inhibition was caused by zinc ions replacing magnesium at magnesium binding sites as was suggested by others (Brunel and Cathala, 1973; Hiwada and Wachsmuth, 1974; Cathala *et al.*, 1975; Ey and Ferber, 1977; Fortuna *et al.*, 1980; Jahan and Butterworth, 1986). The inhibition observed for calcium ions also suggests a displacement of magnesium ions from its binding sites.

In conclusion, our results suggest that MIAP, is better considered as a multifunctional metalloenzyme rather than an alkaline phosphatase of broad specificity, as suggested by others (McComb *et al.*, 1979).

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REFERENCES

Ali S. Y., Sajdera S. W. and Anderson H. C. (1970) Isolation and characterization of calcifying matrix vesicle from epiphyseal cartilage. *Proc. natn. Acad. Sci. U.S.A.* **67,** 1513–1520.

Arsenis C., Rudolph J. and Hackett M. H. (1975) Resolution, purification and characterization of the orthophosphate releasing activity from fracture callus calcifying cartilage. *Biochim. biophys. Acta* **391,** 301–315.

Atkins G. L. (1973) A simple digital-computer program for estimating the parameters of the Hill equation. *Eur. J. Biochem.* **33,** 175–180.

Bohlen P., Stein S., Dairman W. and Undenfriend S. (1973) Fluorometric assay of proteins in the nanogram range. *Archs Biochem. Biophys.* **155,** 213–220.

Brunel C. and Cathala G. (1973) Activation and inhibition processes of alkaline phosphatase from bovine brain by metal ions (Mg^{2+} and Zn^{2+}). *Biochim. biophys. Acta* **309,** 104–115.

Cadman E., Bostwick J. R. and Eichberg J. (1979) Determination of protein by a modified Lowry procedure in the presence of some commonly used detergents. *Analyt. Biochem.* **96,** 21–23.

Cathala G., Brunel C., Chappelet-Tordo D. and Lazdunski M. (1975) Bovine kidney alkaline phosphatase. Catalytic properties, subunit interactions in the catalytic process, and mechanism of Mg^{2+} stimulation. *J. biol. Chem.* **250,** 6046–6053.

Ciancaglini P., Pizauro J. M., Grecchi M. J., Curti C. and Leone F. A. (1989) Effect of Zn(II) and Mg(II) on phosphohydrolytic activity of rat matrix-induced alkaline phosphatase. *Cell. molec. Biol.* (submitted).

Curti C., Pizauro J. M., Rossinholi G., Vugman I., Mello de Oliveira J. A. and Leone F. A. (1986) Isolation and kinetic properties of an alkaline phosphatase from rat bone matrix-induced cartilage. *Cell. molec. Biol.* **32,** 55–62.

Cyboron G. W. and Wuthier R. E. (1981) Purification and initial characterization of intrinsic membrane bound alkaline phosphatase from chicken epiphyseal cartilage. *J. biol. Chem.* **256,** 7262–7268.

Cyboron G. W., Vejins M. S. and Wuthier R. E. (1982) Activity of epiphyseal cartilage membrane alkaline phosphatase and the effects of its inhibitors at physiological pH. *J. biol. Chem.* **257,** 4141–4146.

Davis B. J. (1964) Disc electrophoresis. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121,** 404–427.

Doellgast G. J. and Fishman W. E. (1974) Purification of human placental alkaline phosphatase: salt effects in affinity chromatography. *Biochem. J.* **141,** 103–112.

Ey P. L. and Ferber E. (1977) Calf thymus alkaline phosphatase. II. Interactions with detergents. *Biochim. biophys. Acta* **480,** 163–177.

Farley J. R., Ivey J. L. and Baylink D. J. (1980) Human skeletal alkaline phosphatase. Kinetic studies including pH dependence and inhibition by theophylline. *J. biol. Chem.* **255,** 4680–4689.

Felix R. and Fleisch H. (1976) Pyrophosphatase and ATPase of isolated cartilage matrix-vesicles. *Calc. Tissue Res.* **22,** 1–7.

Fortuna R., Anderson H. C., Carty R. and Sajdera S. W. (1980) Enzymatic characterization of the matrix vesicle alkaline phosphatase isolated from bovine fetal epiphyseal cartilage. *Calc. Tissue Int.* **30,** 217–225.

Harkness D. R. (1968) Studies on human placental alkaline phosphatase. II. Kinetic properties and studies on the apoenzyme. *Archs Biochem. Biophys.* **126,** 513–523.

Hartree E. F. (1972) Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Analyt. Biochem.* **48,** 422–427.

Heinonen S. K. and Lahti R. J. (1981) A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay or inorganic pyrophosphatase. *Analyt. Biochem.* **113,** 313–317.

Hill A. V. (1910) the possible effects of the aggregation of the molecules of hemoglobin on its dissociation curves. *J. Physiol. (Lond,)* **40,** IV–Vii.

Hiwada K. and Wachsmuth E. D. (1974) Catalytic properties of alkaline phosphatase from pig kidney. *Biochem. J.* **141,** 283–291.

Hjelmeland L. M. and Chrambach A. (1984) Solubilization of functional membrane proteins. In *Methods in Enzymology*, Vol. 104, pp. 305–318. Academic Press, New York.

Hsu H. H. T., Munoz P. A., Barr J., Oppliger I., Morris D. C., Vaananen H. K., Tarkenton N. and Anderson H. C. (1985) Purification and partial characterization of alkaline phosphatase of matrix vesicles from fetal bovine epiphyseal cartilage. *J. biol. Chem.* **260,** 1826–1831.

Jahan M. and Butterworth P. J. (1986) Alkaline phosphatase of chick kidney. *Enzyme* **35,** 61–69.

Jones O. T., Earnest J. P. and McNamee M. G. (1987) Solubilization and reconstitution of membrane proteins. In *Biological Membranes* (Edited by Findlay J. B. C. and Evans W. H.), pp. 139–177. I.R.L. Press, Oxford.

Kahn S. E., Jafri A. M., Lewis N. J. and Arsenis C. (1978) Purification of alkaline phosphatase from extracellular vesicles of fracture callus cartilage. *Calc. Tissue Res.* **25,** 85–92.

Koepsell H. (1986) Methodological aspects of purification and reconstitution of transport proteins from mammalian plasma membranes. *Rev. Physiol. Biochem. Pharmac.* **104,** 65–137.

Majeska R. J. and Wuthier R. E. (1975) Studies on matrix vesicles isolated from chick epiphyseal cartilage. Association of pyrophosphatase and ATPase activities with alkaline phosphatase. *Biochim. biophys. Acta* **391,** 51–60.

McComb R. B., Bowers G. N. Jr and Posen S. (1979) Alkaline phosphatase. Plenum Press, New York.

Navaratnam N. and Stinson R. A. (1986) Modulation of activity of human alkaline phosphatases by Mg^{2+} and thiol compounds. *Biochim. biophys. Acta* **869,** 99–105.

Ohkubo A., Langerman N. and Kaplan M. M. (1974) Rat liver alkaline phosphatase. Purification and properties. *J. biol. Chem.* **249,** 7174–7180.

Pizauro J. M., Curti C., Ciancaglini P. and Leone F. A. (1987) Triton X-100 solubilized bone matrix-induced alkaline phosphatase. *Comp. Biochem. Physiol.* **87B,** 921–926.

Pizauro J. M., Curti C., Ciancaglini P. and Leone F. A. (1988) Kinetic properties of Triton X-100 solubilized bone matrix-induced alkaline phosphatase. *Cell. molec. Biol.* **34,** 553–562.

Racusen D. (1979) Glycoprotein detection in polyacrylamide gel with thymol and sulfuric acid. *Analyt. Biochem.* **99,** 474–476.

Skillen A. W. and Rahbani-Nobar M. (1980) Alkaline phosphatase and ATPase activities of rat bone: separation and characterization. *Calc. Tissue Int.* **30,** 67–71.

Vaananen H. K. and Korhonen L. K. (1979) Matrix vesicles in chicken epiphyseal cartilage. Separation from lysosomes and distribution of inorganic pyrophosphatase activity. *Calc. Tissue Int.* **28,** 65–72.

Warner G. P. Hubbard H. L., Loyd G. C. and Wuthier R. E. (1983) Inorganic phosphorus-32 and calcium-45 metabolism by matrix vesicles enriched microsomes prepared from chicken epiphyseal cartilage by isoosmotic Percoll density gradient fractionation. *Calc. Tissue Int.* **35,** 327–338.

Wuthier R. E. (1982) A review of the primary mechanism of endochondral calcification with special emphasis on role of cells, mitochondria and matrix vesicles. *Clin. Orth.* **169,** 219–242.

Wuthier R. E. and Register T. C. (1985) Role of alkaline phosphatase, a polyfunctional enzyme, in mineralizing tissues. In *The Chemistry and Biology of Mineralized Tissues* (Edited by Butler W. T.), pp. 113–124. EBSCO Media Inc., Birmingham.