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Purification and Heterogeneity of Inorganic Pyrophosphatase of Pig Scapula Cartilage

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Inorganic pyrophosphatase (pyrophosphate phosphohydrolase; EC 3.6.1.1) was purified from pig scapula cartilage by fractionation with *N*-cetylpyridinium chloride and $(NH_4)_2SO_4$, followed by ion-exchange and gel-filtration chromatography. Enzyme preparations of high purity were obtained, with specific activities (100–400 units/mg) higher than those previously described for mammalian pyrophosphatases. The enzyme activity could be separated into several subfractions on ion-exchange columns.

Inorganic pyrophosphatases (pyrophosphate phosphohydrolases; EC 3.6.1.1) are ubiquitous among living organisms. They have been studied in greatest detail in yeast and bacteria, from which they have been obtained in highly purified form (Tono & Kornberg, 1967; Butler, 1971; Josse & Wong, 1971). Pyrophosphatases have also been isolated from mammalian sources, but only at low purity (Pynes & Younathan, 1967; Irie *et al.*, 1970; Chen *et al.*, 1973). It is generally agreed that a major function of these enzymes is to pull pyrophosphorolytic reactions in the direction required for biosynthesis (Kornberg, 1962). Another role was proposed for such enzymes in cartilage by Alcock & Shils (1969) who found pyrophosphatase in large amounts in costal cartilage just before calcification. They suggested that the pyrophosphatase destroys PP_i, a known inhibitor of calcium phosphate crystal formation and which has been proposed to be a physiological inhibitor of calcification (Fleisch & Russell, 1970). The aim of this and the following paper (Felix & Fleisch, 1975) is to describe the purification and characterization of the enzyme from a calcifying cartilage to determine whether its properties are compatible with such a role in calcification.

Experimental

Materials

Pig scapula cartilage was obtained from a local slaughter house. A layer, 1–2mm thick, containing the calcifying and transforming cartilage, was dissected along the ossification line, as described by Kuettner *et al.* (1968). The tissue was kept frozen until extracted.

2-Mercaptoethanol (puriss. p.a.), *N*-cetylpyridinium chloride (purum) and bovine serum albumin (fraction V) were purchased from Fluka A.G., Buchs, Switzerland; DEAE-cellulose DE-52 was from Whatman, Maidstone, Kent, U.K.; Sephadex G-150 was from Pharmacia Fine Chemicals Inc., Uppsala, Sweden; Diaflo membranes UM-20E were from Amicon N.V., Oosterhout, The Netherlands; and collodion bags were from Schleicher and Schuell, D-3354 Dassel, W. Germany. The other reagents were analytical-grade and obtained from E. Merck A.G., Darmstadt, W. Germany.

Methods

Enzyme assays. Pyrophosphatase was assayed as a routine at 37°C for 15min in a reaction mixture (0.5ml) containing 0.1M-Tris–maleate, pH7.2, 2mM-PP_i and 10mM-$MgCl_2$. The reaction was terminated by the addition of 1ml of a solution containing 10% (w/v) trichloroacetic acid and 5mM-$CuSO_4$, and the P_i released was determined colorimetrically (Wöltgens & Ahsmann, 1970). The reaction was linear with respect to the amount of enzyme and time of incubation.

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase; EC 3.1.3.1) was determined by the method of Richterich (1968) with *p*-nitrophenyl phosphate as substrate.

One unit of pyrophosphatase or alkaline phosphatase activity corresponds to 1 μmol of substrate split/min under the assay conditions described.

Protein determination. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. Since 2-mercaptoethanol interferes, solutions to be assayed from the earlier purification steps were first dialysed against 0.01M-Tris–maleate, pH7.2. At the later steps the protein was precipitated with trichloroacetic acid and the protein in the pellet determined. In the column eluates protein was determined by measuring E_{280} on an LKB 8300 Uvicord II, or on a Beckman spectrophotometer DU model G 2400 for a more precise value.

Determination of proteoglycans. These were measured as uronic acid (Dische, 1947) on 0.5ml of a 1:2 diluted extract to give 7ml of final reaction mixture.

Polyacrylamide disc gel electrophoresis. The electrophoresis of protein solutions was performed by the method of Davis (1964). Protein was stained with Amido Black, and pyrophosphatase activity detected by the method of Tono & Kornberg (1967).

Chromatography. Each DEAE-cellulose and Sephadex column was examined for uniform settling by running Malachite Green or Dextran Blue through the column. Equilibration of the cellulose with the buffer was tested by measuring the conductivity and pH of the effluent.

Results

Stability

Before purification, the stability of the enzyme was studied in order to determine the conditions for the purification procedure. Table 1 shows that the enzyme lost very little activity on standing at 4°C. However, it lost nearly all its activity if dialysed against the Tris–maleate buffer, pH7.2. This decrease was largely prevented if either $MgCl_2$ or 2-mercaptoethanol was added to the buffer. Interestingly, when dialysed against both compounds, enzyme activity was higher than in the fresh extract. When the enzyme was further purified the loss observed in the absence of either Mg^{2+} or 2-mercaptoethanol was smaller.

The effect of dialysis against different buffers at various pH values is shown in Fig. 1. Dialysis at low pH decreased the activity, except when piperazine–HCl was used as buffer.

When kept under 0.01M-$MgCl_2$ and 0.01M-2-mercaptoethanol with either 0.1M-Tris–HCl, pH7.5, or Tris–maleate, pH7.2, the enzyme at different steps of purification did not lose more than 10% of its activity during a period of 2 months at 4°C. Freezing and thawing once also did not cause an appreciable loss of activity.

Table 1. *Stabilization by Mg^{2+} and 2-mercaptoethanol*

Cartilage was extracted with 0.01M-Tris–maleate, pH7.2. The solution obtained (2.3 units/ml) was divided up and either left or dialysed against different buffer solutions for 3.5 days at 4°C. The enzyme activities are expressed as percentages of the value found directly after the extraction.

Dialysis	$MgCl_2$ (mM)	2-Mercaptoethanol (mM)	Relative activity (%)
No	0	0	95
Yes	0	0	4
Yes	10	0	83
Yes	0	10	88
Yes	10	10	125

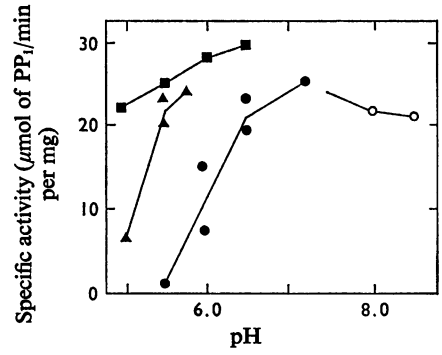


Fig. 1. *Stability of the pyrophosphatase at different pH values*

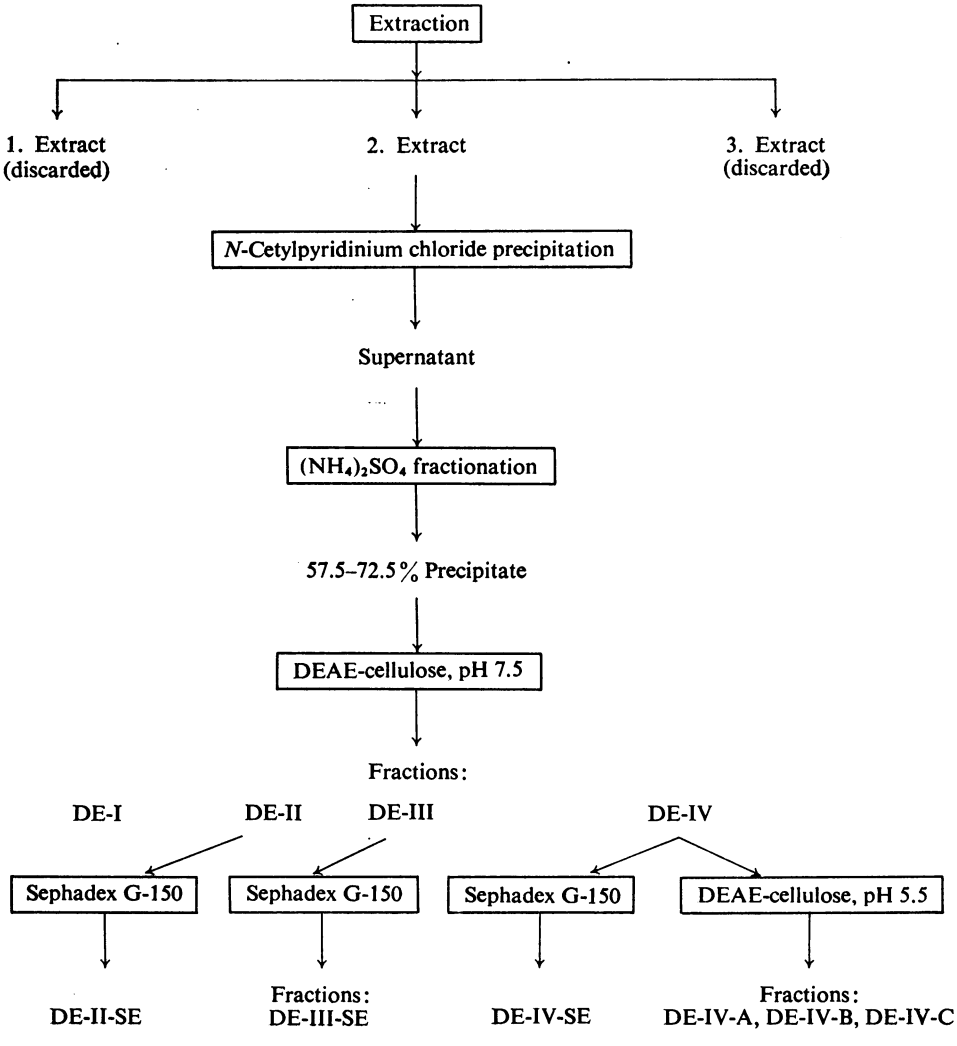
A part of the $(NH_4)_2SO_4$ fraction (57.5–72.5%) with a protein concentration of 0.5mg/ml was dialysed for 5 days against one of the following buffers, at various pH values: ▲, acetate buffer; ●, Tris–maleate; ○, Tris–HCl; ■, piperazine–HCl. All buffers were at a concentration of 0.1M and contained 0.01M-$MgCl_2$ and 0.01M-2-mercaptoethanol. In the case of Tris–maleate buffer the final concentration of Tris and maleate were 0.1M and the required pH was obtained by adjustment with NaOH. Enzyme activity was assayed as described in the text.

Purification

All steps were performed at 4°C or in an ice bath. They are summarized in Scheme 1 and Table 2.

Extraction. Cartilage was left for 30min in 5vol. of 0.1M-Tris–maleate (pH7.2)–0.01M-$MgCl_2$–0.01M-2-mercaptoethanol (buffer 1). Decantation yielded the first extract which contained some blood. The cartilage was then homogenized with 5vol. of buffer 1 in a Waring Blendor for 3min and stirred for 30min. Centrifugation at 22500$g_{av.}$ for 30min yielded the second extract. By further treatment with 2.5vol. of buffer 1 a third extract was obtained. Further extraction by adding 0.1% Triton X-100 to the buffer did not elute additional significant amounts of enzyme activity. Only the second extract with a high specific activity was further purified (see Table 2 and Scheme 1 for further details). A total of 1280g of cartilage cut from 81.3kg of scapula were extracted and yielded 20000 units at this first step.

N-Cetylpyridinium chloride precipitation. After centrifugation at 29500$g_{av.}$ for 2h proteoglycans were precipitated from the supernatant with *N*-cetylpyridinium chloride. This was necessary because addition of $(NH_4)_2SO_4$ did not result in any precipitation of protein without this step. As shown in Fig. 2, there was only a narrow range of *N*-cetylpyridinium chloride concentrations that precipitated proteoglycans, as determined by the uronic acid content of the supernatant, but which did not inactivate the enzyme. Within this range maximal specific activity



Scheme 1. Purification

was reached as shown in Fig. 2. Having defined the optimal conditions, precipitation was performed by pumping 353ml of 1% *N*-cetylpyridinium chloride, kept at room temperature, pH approx. 7.0, at a flow rate of 300ml/h into 829ml of extract stirred and cooled in an ice bath. Then 10min after the end of this addition the mixture was centrifuged at 20500$g_{av.}$ for 30min. The supernatant was concentrated by filtration through an Amicon membrane UM-20E and dialysed against buffer without change. Uronic acid was then no longer detectable.

$(NH_4)_2SO_4$ fractionation. Increasing amounts of solid $(NH_4)_2SO_4$ were then added to the enzyme solution containing 1mg of protein/ml. After being left for 2h the solution was centrifuged at 30300$g_{av.}$ for 30min. The enzyme precipitated between 52.5 and 72.5%-satd. $(NH_4)_2SO_4$. This precipitate was dissolved in a small amount of buffer 1 and dialysed against buffer 1.

*DEAE-cellulose chromatography at pH*7.5. The enzyme solution was then applied to a DEAE-cellulose column equilibrated at pH7.5 (details are given in the legend of Fig. 3). The enzyme activity was separated into seven peaks. The eluate was combined

Table 2. *Purification*

For details see the text. The results are given as the mean ± S.E.M. (*n*) when $n > 2$. Where *n* is less than or equal to 2 the individual values are given.

	Total activity (units)	Overall yield (%)		Specific activity (units/mg)	Purification
Extraction					
1. Extract	5960 ± 650 (4)			1.20 ± 0.12 (8)	
2. Extract	20000 ± 650 (4)	100		2.62 ± 0.11 (8)	1
3. Extract	6450 ± 840 (4)			1.49 ± 0.16 (8)	
N-Cetylpyridinium chloride precipitation, supernatant	16580 ± 240 (9)	82.8 ± 1.2 (9)		8.32 ± 0.27 (9)	3.2
$(NH_4)_2SO_4$ fractionation, 57.5–72.5% -satd. pellet	13800 ± 440 (4)	68.4 ± 2.2 (4)		16.4 ± 1.3 (4)	6.3
DEAE-cellulose, pH 7.5					
Fraction DE-II	2040 ± 340 (4)	10.2 ± 1.2 (4)	42.8 ± 2.2 (4)	69.2 ± 8.5 (4)	26.4
Fraction DE-III	3720 ± 200 (4)	18.6 ± 1.0 (4)		80.7 ± 3.9 (4)	30.7
Fraction DE-IV	2820 ± 180 (4)	14.1 ± 0.9 (4)		151.0 ± 23.0 (4)	57.6
DEAE-cellulose, pH 5.5					
Fraction DE-IV-A		2.8		167	61
Fraction DE-IV-B		3.1	8.1	127	47
Fraction DE-IV-C		2.2		397	147
Sephadex G-150					
DE-II-SE	1240 ± 120 (3)	6.2 ± 0.6 (3)		104.2 ± 14.9 (3)	39.7
DE-III-SE	3300/3260	16.5/16.3		94/101	37.6
DE-IV-SE	2100/1560	10.5/ 7.8		142/195	64.3

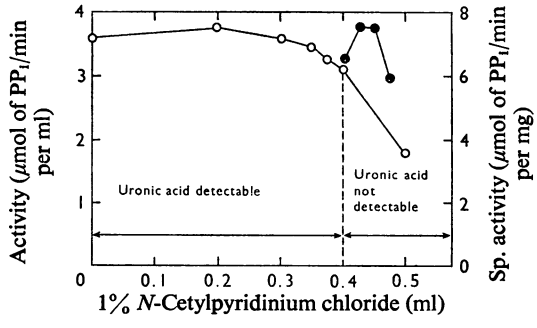


Fig. 2. *Precipitation by N-cetylpyridinium chloride*

Various amounts of 1% *N*-cetylpyridinium chloride were added to a 1 ml extract. After being left for 10 min the solution was centrifuged at $2000g_{av.}$ for 15 min and the activity in the supernatant was determined and expressed with regard to the original volume (○). In the relevant range where no more uronic acid was detectable, the specific activity was also determined (●).

into four fractions as described in Fig. 3 and Scheme 1. The highest specific activity was obtained in fraction DE-IV (Table 2). Although alkaline phosphatase behaved differently from the inorganic pyrophosphatase, a complete separation was not possible on this column.

DEAE-cellulose chromatography at pH 5.5. A portion of fraction DE-IV was put on a DEAE-cellulose column equilibrated at pH 5.5 (Fig. 4). The enzyme activity again separated into several peaks, of which fraction DE-IV-C showed the highest specific activity (400 units/mg; Table 2). This fraction gave, after disc electrophoresis and staining with Amido Black, a diffuse broad band containing two sharp lines close together; fractions DE-IV-A and DE-IV-B gave three to five bands.

In order to determine whether the separation of fraction DE-IV into subfractions at pH 5.5 (Fig. 4) was induced by piperazine buffer, portions of fraction DE-IV were dialysed against the piperazine–HCl buffer at pH 5.5, containing 0.01 M-$MgCl_2$ and 0.01 M-2-mercaptoethanol. After further dialysis against 0.005 M-Tris–HCl (pH 7.5) – 0.01 M-$MgCl_2$ – 0.01 M-2-mercaptoethanol the fraction was again put on a DEAE-cellulose column equilibrated with the latter buffer at pH 7.5. Enzyme activity was eluted in the same position as the original fraction DE-IV showing that piperazine itself does not convert the enzyme into subfractions, at least not in an irreversible manner.

Sephadex G-150 chromatography. The fractions DE-II, DE-III and DE-IV from the DEAE-cellulose column at pH 7.5 (Fig. 3) were each run on a Sephadex G-150 column. As seen from Fig. 5 in each case the pyrophosphatase could be separated clearly from the

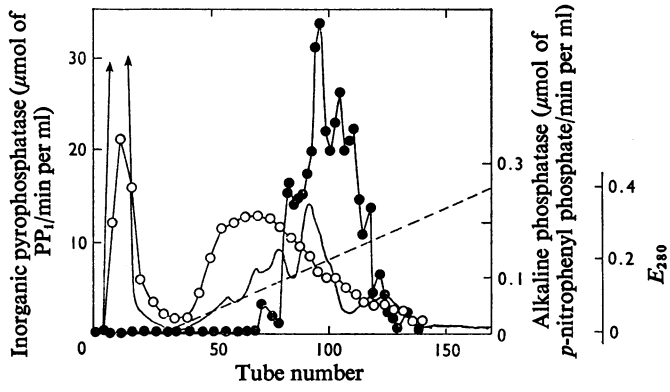


Fig. 3. *Purification of the enzyme on DEAE-cellulose column, pH 7.5*

The column (30cm × 1.5cm) was equilibrated with a solution containing 0.005M-Tris–HCl, pH 7.5, 0.01M-$MgCl_2$ and 0.01M-2-mercaptoethanol; 27ml of the enzyme solution containing 126mg of protein was applied. The column was then eluted with the buffer until no more protein was detected. Subsequently a linear NaCl gradient (total 600ml) containing 0.005M-Tris–HCl, pH 7.5, 0.01M-$MgCl_2$, 0.01M-2-mercaptoethanol and 0–0.18M-NaCl was started (----); flow rate 30ml/h; volume per tube 4ml. Pyrophosphatase activity (units/ml) (●); alkaline phosphatase (units/ml) (○); u.v. record at 280nm, E_{280} (——). For further analysis the tubes were pooled in four fractions as follows: fraction DE-I, tubes 70–80; fraction DE-II, tubes 81–90; fraction DE-III, tubes 91–101; fraction DE-IV, tubes 102–111.

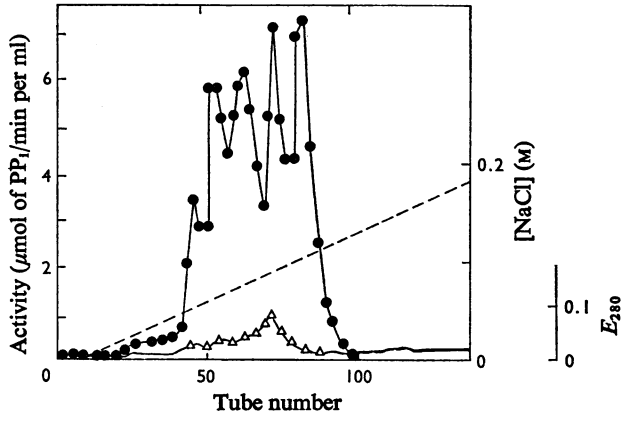


Fig. 4. *Purification of fraction DE-IV on DEAE-cellulose column, pH 5.5*

The column (30cm × 1.5cm) was equilibrated with a solution containing 0.005M-piperazine–HCl, pH 5.5, 0.01M-$MgCl_2$ and 0.01M-2-mercaptoethanol. After being dialysed against this buffer, 10ml of protein solution (0.8mg/ml) was applied. It was eluted with a gradient (total 600ml) containing 0.005M-piperazine–HCl, pH 5.5, 0.01M-$MgCl_2$, 0.01M-2-mercaptoethanol and 0–0.18M-NaCl (----). Flow rate 30ml/h; volume per tube 4ml. Pyrophosphatase activity (units/ml) (●); u.v. record at 280nm (——); some fractions were measured on a Beckman spectrophotometer: E_{280} (△). The tubes were pooled as follows: fraction DE-IV-A, tubes 44–64; fraction DE-IV-B, tubes 65–79; fraction DE-IV-C, tubes 80–92.

remainder of the alkaline phosphatase, which was eluted first. Fractions DE-II-SE and DE-III-SE were further separated from contaminating protein. The enzyme activity for fractions DE-III-SE and DE-IV-SE also coincided with a protein peak. After disc electrophoresis the following protein bands were observed. For fraction DE-II-SE, three bands with R_F values 0.27, 0.36 and 0.45, for fraction DE-III-SE three bands with R_F values 0.34, 0.38 and 0.44 and for fraction DE-IV-SE two bands with R_F values 0.38 and 0.46. Pyrophosphatase activity was found in broad bands; for fraction DE-II-SE the R_F value was 0.32–0.54, for fraction DE-III-SE 0.21–0.53 and for fraction DE-IV-SE 0.33–0.56.

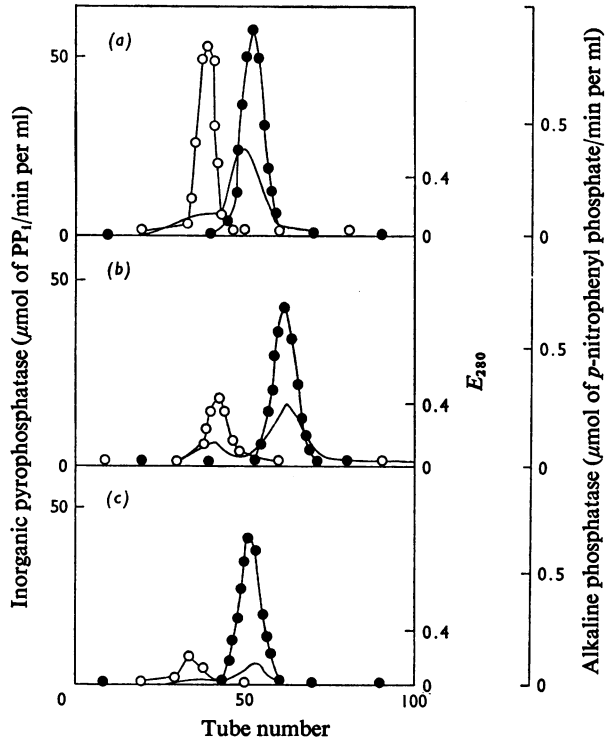


Fig. 5. *Purification of fraction DE-II, DE-III and DE-IV on a Sephadex G-150 column*

The columns were equilibrated with a solution containing 0.01 M-Tris–HCl, pH 7.5, 0.01 M-$MgCl_2$, 0.01 M-2-mercaptoethanol and 0.1 M-NaCl. (*a*) Fraction DE-II. Column size, 2 cm × 73 cm; tube volume, 2.4–2.6 ml; flow rate, 13 ml/h. (*b*) Fraction DE-III. Column size, 2.5 cm × 75 cm; tube volume, 2.2 ml; flow rate, 10–13 ml/h. (*c*) Fraction DE-IV. Column size, 2 cm × 73 cm; tube volume, 2.5 ml; flow rate, 15 ml/h. ●, Pyrophosphatase activity (units/ml); ○, alkaline phosphatase (units/ml); ——, u.v. record at 280 nm (E_{280}).

Heterogeneity of enzyme activity

To investigate whether the various peaks obtained on the DEAE-cellulose column at pH 7.5 as shown in Fig. 3 were due to some artifact arising from the column itself, specific fractions were run again on the same column. As shown in Fig. 6 the various fractions retained their original behaviour and appeared as individual peaks in the same position as before. This suggests that the peaks represent individual isoenzymes.

Discussion

As found for other pyrophosphatases (Pynes & Younathan, 1967; Irie *et al.*, 1970) the enzyme isolated from cartilage was stabilized both by Mg^{2+} and the thiol-group-protecting agent 2-mercaptoethanol. These two agents were therefore utilized to increase stability throughout the purification.

The purification obtained in this study was highest after DEAE-chromatography at pH 5.5 (Table 2, Fig. 4). The specific activity was about half that of the purest crystallized preparation obtained by Kunitz (1952) from baker's yeast. The preparations obtained after Sephadex chromatography, although less pure, had the highest activity reported for mammalian inorganic pyrophosphatases (Pynes & Younathan, 1967; Irie *et al.*, 1970; Chen *et al.*, 1973) by a factor of 5–8-fold. The inorganic pyrophosphatase was partly separated from alkaline phosphatase by DEAE-cellulose chromatography (Fig. 3) and completely by Sephadex gel filtration (Fig. 5). Disc gel electrophoresis showed more than one protein band. Staining for pyrophosphatase gave a diffuse band so that it was not possible to distinguish whether single or multiple bands were associated with the pyrophosphatase activity.

The distribution of the pyrophosphatase activity suggests that the enzyme is heterogeneous, since rechromatography did not show that these peaks were due to a column artifact or to an effect of the buffer. The various fractions obtained probably

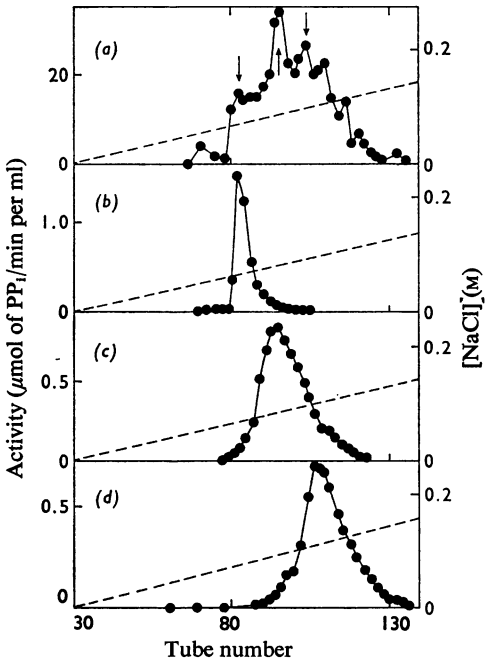


Fig. 6. *Rechromatography of the various fractions obtained in Fig. 3*

Column and conditions are the same as in Fig. 3. (*a*) Chromatography as presented in Fig. 3; (*b*) rechromatography of tube 84; (*c*) rechromatography of tube 97 (*d*) rechromatography of tube 106. ●, Pyrophosphatase activity (units/ml); ----, NaCl gradient (0–0.18 M-NaCl). Arrows indicate the tubes 84, 97 and 106.

represent isoenzymes rather than different enzymes because they have similar molecular weights, pH optima, magnesium activation and substrate specificity (Felix & Fleisch, 1975). It is possible that some of the observed heterogeneity can be attributed to enzymes originating from different subcellular fractions. Indeed, although the major part of inorganic pyrophosphatase activity has usually been found in the cytosol (Irie *et al.*, 1970; Pynes & Younathan, 1964; Nordlie & Lardy, 1961) pyrophosphatase activity has also been demonstrated in rat liver nuclei (Kesselring & Siebert, 1967) and in mitochondria (Irie *et al.*, 1970; Schick & Butler, 1969), the mitochondrial enzyme being heterogeneous (Irie *et al.*, 1970). Further to this, Buruiana & Hadarag (1968) demonstrated several isoenzymes of pyrophosphatase from the brain of different animals. Another possibility is that the heterogeneity of the enzyme preparation may originate during extraction from proteolytic enzymes present in the cartilage extract. Thus Eifler *et al.* (1972) found three peaks after DEAE-cellulose chromatography of inorganic pyrophosphatase of baker's yeast, despite the fact that *in vivo* only one enzyme species exists. The others were produced by the proteolytic action of yeast carboxypeptidase during the extraction.

Further characterization of these three highly purified pyrophosphatases DE-II-SE, DE-III-SE and DE-IV-SE is described in the following paper.

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