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SUBCELLULAR DISTRIBUTION OF LOW- AND HIGH-MOLECULAR-WEIGHT ACID PHOSPHATASES

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Summary

Acid phosphatases (orthophosphoric-monoester phosphohydrolases (acid optimum), EC 3.1.3.2) of low and high molecular weight were separated by Sephadex G-75 filtration from extracts of rat brain, liver and kidney. The proportion of each phosphatase in the extract depends critically on the method employed for homogenate preparation, and no interconversion between high and low molecular weight forms was detected.

In extracts obtained from subcellular organelles only high-molecular-weight acid phosphatase was detected, which is of lysosomal origin. Low-molecular-weight acid phosphatase is restricted to the cell sap.

Low- and high-molecular-weight acid phosphatases were characterized by their elution volumes, specific inhibition and activity with two substrates. It is suggested that the distribution pattern found in rat tissues could be common to all eukaryotic cells.

Introduction

The presence of a high-molecular-weight ($M_r \geqslant 100\,000$) acid phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2) in lysosomes of different eukaryots has been clearly established by histochemical and separation techniques [1,2,3]. The isolation of a low-molecular-weight ($M_r \leqslant 30\,000$) acid phosphatase is more recent and stems from purification studies of acid phosphatases from human placenta [4], bovine liver [5] and brain [6]. Low-molecular-weight acid phosphatases have also been described in hamster epidermis [7], erythrocytes [8] *Escherichia coli* [9], *Xenopus levis* [10] rat mammary carcinoma [11] and horse liver [12]. Although molecular weights have not been determined, the properties of the acid phosphatases from rabbit leucocytes [13], bovine kidney [14], rat spinal cord [15] and testicles [16]

make it very probable that these enzymes are also low-molecular-weight acid phosphatases.

Unequivocal subcellular localization of low-molecular-weight acid phosphatase has been achieved in placenta, using a modified Gomori [17] technique. Hoffman and Di Pietro [3] demonstrated an extra-lysosomal acid phosphatase activity localized in the plasmalemma covering the microvilli of syncyotrophoblasts. This activity was shown to be inhibited by *p*-chloromercuribenzoate and little affected by fluoride, which abolished lysosomal phosphatase activity.

Low-molecular-weight acid phosphatase is inhibited by SH reagents [4,6,18] and unaffected by either fluoride or tartrate which are strong high-molecular-weight acid phosphatase inhibitors [2,4,19]. This differential inhibition can be used to characterize these two classes of enzymes.

Another distinctive feature is the high substrate specificity of low-molecular-weight acid phosphatase: only *p*-nitrophenylphosphate [4,6] riboflavin phosphate [5] and 17-β-estradiol 3-phosphate [20] are hydrolyzed efficiently. On the other hand, high-molecular-weight acid phosphatases show a low specificity for substrates [2]. The physiological function of low-molecular-weight acid phosphatase remains obscure although Di Pietro [20] has suggested that acid phosphatase III may play a role in placental steroid metabolism.

There is no clear information on any type of structural relationship between high- and low-molecular-weight acid phosphatases. The catalytic mechanism of both enzymes is very similar [18,21,22]. If this mechanism of phosphate ester hydrolysis has an evolutionary advantage [23] one would expect to find a relationship between high- and low-molecular-weight acid phosphatases other than the catalytic pathway.

Because of our general purpose of investigating the (possible) physiological significance of low-molecular-weight acid phosphatase we undertook this study to ascertain the distribution of the enzyme in different organs of rat. We have used Sephadex gel filtration for separation, and differential inhibition with two substrates as criteria for distinguishing at least two classes of acid phosphatases.

Experimental Procedure

p-Nitrophenylphosphate, α-glycerophosphate and *p*-chloromercuribenzoate were purchased from Sigma Chemical Co., Mo. (U.S.A.). Sephadex G-75, medium grade, was obtained from Pharmacia, ammonium sulfate was BDH enzyme grade. All other chemicals were analytical grade. Glass-double-distilled water was used throughout.

Acid phosphatase activity was measured as described elsewhere [6] using *p*-nitrophenylphosphate or α-glycerophosphate as substrates.

Protein was measured by a modification of the Lowry method [24] with crystalline bovine serum albumin as standard.

Rat tissue extracts were prepared by homogenization in 4 vols. (w/v) of cold 0.25 M sucrose containing 10^{-3} M EDTA, 10^{-3} M Tris · HCl pH 7.5 (medium I) with organs extensively perfused in situ with the same medium. Mild conditions of homogenization were achieved using a loose-fitting, Potter type glass homogenizer (clearance 0.23 mm), equipped with a Teflon pestle, driven at 300

rev./min with four strokes. Drastic conditions were obtained homogenizing tissues in 4 vols. of medium I in a Omni-mixer (Sorvall) operated at top speed for 3 min followed by sonication for 2 min (4 times 30 s) in a Branson sonicator at full output. During sonication, homogenate temperature was maintained below 8°C. Subcellular fractions were obtained by the method of the Duve et al. [25] using perfused rat organs. Brain mitochondrial fraction was prepared according to the method of Jansen et al. [26]. Rat liver nuclear fraction was prepared by the method of Chauveau et al. [27] as modified by Wang [28]. Acid phosphatase from all particulate fractions was extracted in potassium acetate, 0.1 M, pH 5.0, containing 10^{-3} M EDTA (Buffer I) by the same procedure described above for drastic homogenization of whole tissues.

All extracts obtained as described above were centrifuged at 20 000 × *g* for 30 min in a Sorvall RC2-B centrifuge; the supernatants were concentrated by the addition of solid ammonium sulfate up to 70% saturation. The precipitated protein was collected and dissolved in a minimum volume of buffer I (usually 1—2 ml). This solution was dialyzed against 2000 vols. of the same buffer for 3 × 30 min, and then centrifuged at 10 000 × *g* for 10 min. The yield of this concentration step was routinely 100% expressed as total acid phosphatase activity present in the extract. The clear supernatant (containing between 1 and 4 units) was passed through a K 16/40 Pharmacia column (16 × 400 mm) filled with Sephadex G-75 equilibrated with buffer I. The flow rate of the column was usually 38 ml/h. Fractions of 2 ml were collected and assayed for absorbance at 280 nm and acid phosphatase activity.

Results

Elution profiles obtained by filtration in Sephadex of rat brain total extracts obtained in different conditions show sharp differences in the low- to high-molecular-weight acid phosphatase ratios. Essentially identical results are obtained with liver extracts. These ratios, when mild conditions of homogenization were employed, are 20 and 3 for brain and liver extracts respectively. A five-fold increase in high-molecular-weight acid phosphatase concentration in these extracts is observed when drastic homogenization is used (Table I) and this reflects the contribution of lysosomal acid phosphatase to the extract [29].

TABLE I

LOW- TO HIGH-MOLECULAR-WEIGHT ACID PHOSPHATASE RATIOS IN DIFFERENT EXTRACTS

Details of extraction procedure are given under Experimental Procedure. Results are presented as average ± S.E. of 4 experiments for brain and liver extracts and 3 for kidney extracts. Ratios were calculated using the areas of the peaks obtained in elution profiles by planimetry.

Homogenization conditions	Organ		
	Brain	Liver	Kidney
Mild	20.8 ± 2.09	3.2 ± 0.21	2.6 ± 0.26
Drastic	3.91 ± 0.12	0.63 ± 0.10	0.83 ± 0.12

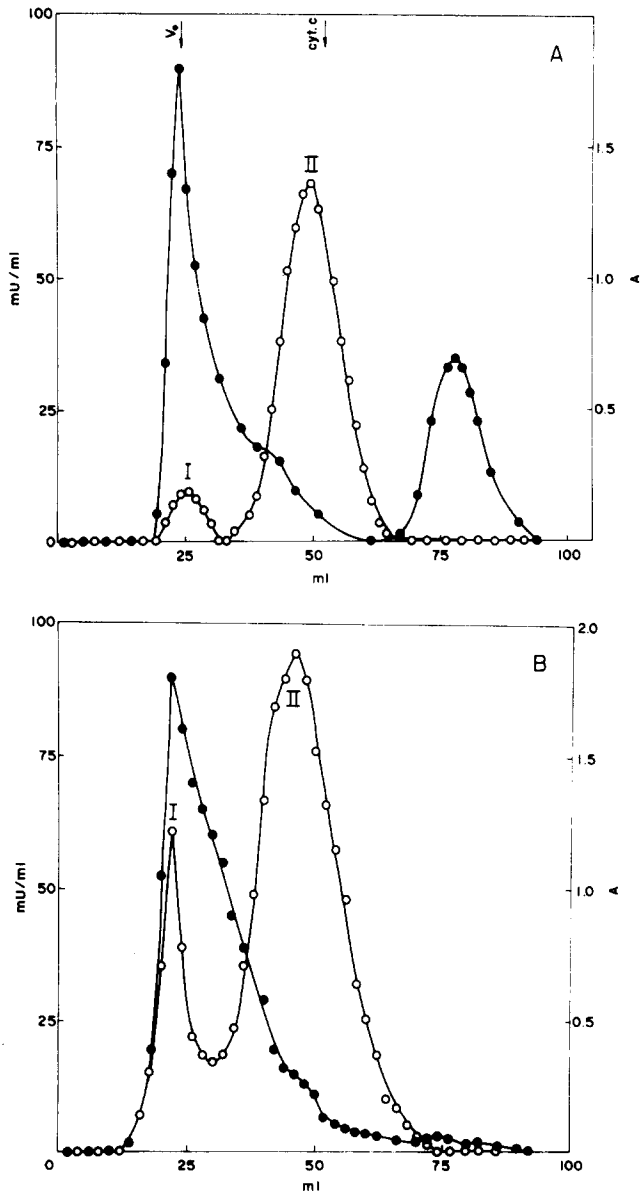

Fig. 1. Elution profiles of rat brain extracts in different conditions. ○——○, Acid phosphatase activity with *p*-nitrophenylphosphate; ●——●, absorbance at 280 nm. A, 900 munits of an extract obtained under mild conditions of homogenization was passed through a Sephadex G-75 column. B, 1600 munits of an extract obtained under drastic conditions of homogenization. For details refer to Experimental Procedure. Arrows indicate the void volume of the column (V_0) and elution of cytochrome *c* (cyt. *c*) in the same column. I, high-molecular-weight acid phosphatase and II, low-molecular weight acid phosphatase.

The separation between high- and low-molecular-weight acid phosphatases on Sephadex columns is clear, as evidenced by the activity profile of brain extract obtained under mild conditions of homogenization (Fig. 1). When a brain extract obtained under the same mild conditions is incubated for up to

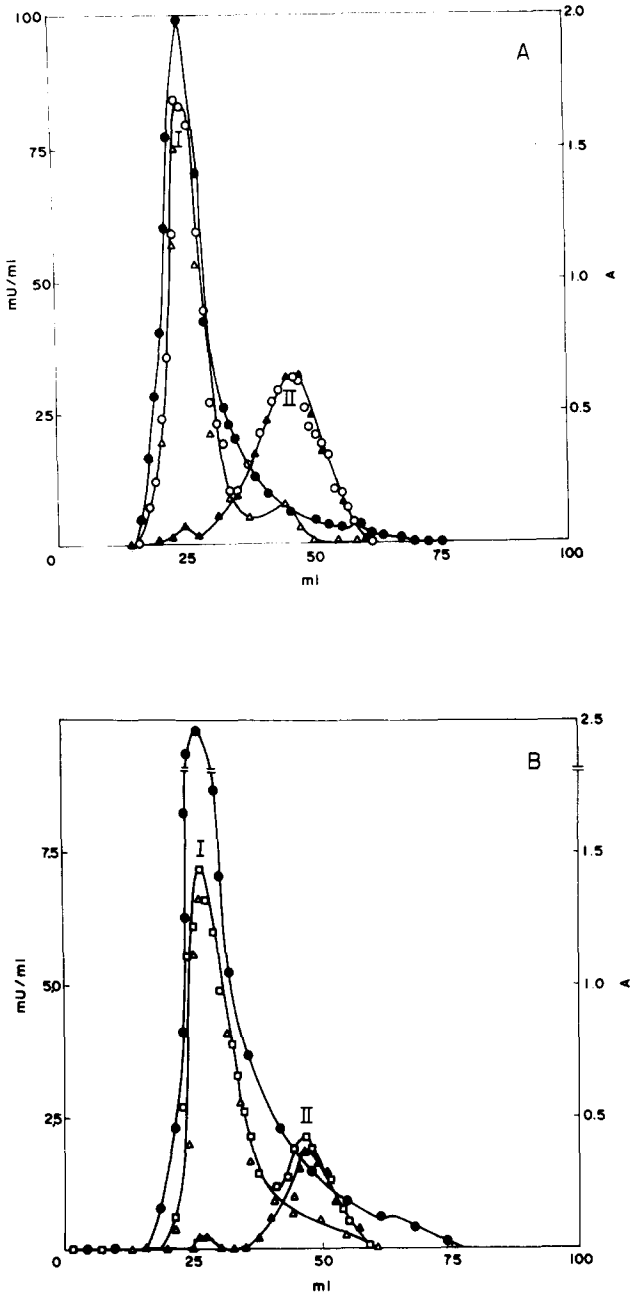

Fig. 2. Elution profiles of rat liver extracts. ○———○, acid phosphatase activity with *p*-nitrophenylphosphate as substrate; □———□, acid phosphatase activity with α-glycerophosphate as substrate; △———△, activity with $2 \cdot 10^{-6}$ M *p*-chloromercuribenzoate in the assay; ▲———▲, activity with $5 \cdot 10^{-3}$ M tartrate in the assay; ●———●, absorbance at 280 nm. For the assay conditions see Experimental Procedure. A, 1160 munits of extract was passed through a Sephadex G-75 column. B, 2100 munits of extract, activities in the effluent were measured with α-glycerophosphate as substrate. I, high-molecular-weight acid phosphatase and II, low-molecular-weight acid phosphatase.

30 min at 30°C the elution profile, both in peak position and profile and total acid phosphatase activity remains identical to that shown in Fig. 1. This indicates strongly that neither degradation nor interconversion between high- and low-molecular-weight acid phosphatase are important under our conditions.

Differential inhibition clearly separates low- from high-molecular-weight acid phosphatase activity. 10^{-4} M tartrate inhibits more than 80% the activity of high-molecular-weight acid phosphatase with no effect upon the low-molecular-weight acid phosphatase activity. *p*-Chloromercuribenzoate in concentrations up to 10^{-5} M abolishes low-molecular-weight acid phosphatase activity with no effect on that of the high-molecular-weight acid phosphatase. The same inhibition pattern is observed with both *p*-nitrophenylphosphate or α-glycerophosphate as substrates, although with the later the activity is only 10% of the activity when *p*-nitrophenylphosphate is used as substrate [6] (note the differences in scales in Fig. 2).

Differential inhibition patterns for liver extracts (Fig. 2) are identical to those obtained from brain and kidney extracts.

Total acid phosphatase activity was measured in the various fractions obtained by subcellular fractionation of rat liver cells and it appears that acid phosphatase is concentrated in the mitochondria-lysosomal and cell-sap fractions. It is noteworthy that the nucleus shows only negligible activity (Table II) which is compatible with a 1% contamination of the nuclear fraction by other fractions [28].

Acid phosphatase extracted from particulate fractions (see experimental procedure) were passed through Sephadex columns and only one peak of activity, which was eluted in the void volume, was detected (Fig. 3). The pooled fractions of those peaks were fully inhibited by tartrate and insensitive to *p*-chloro-

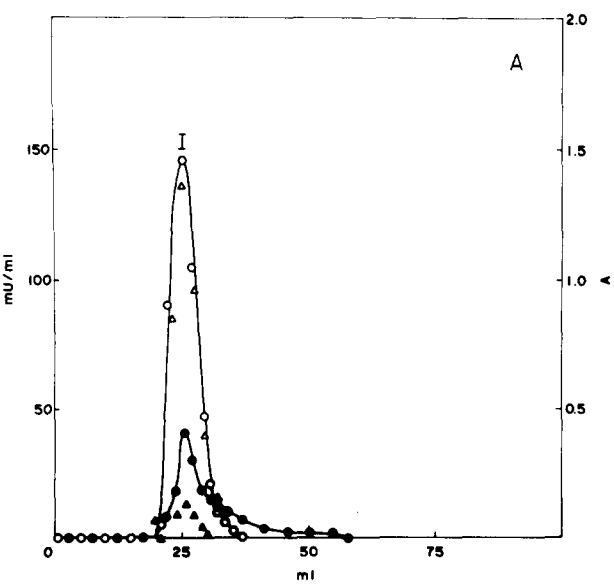

Fig. 3A. See opposite page for legend.

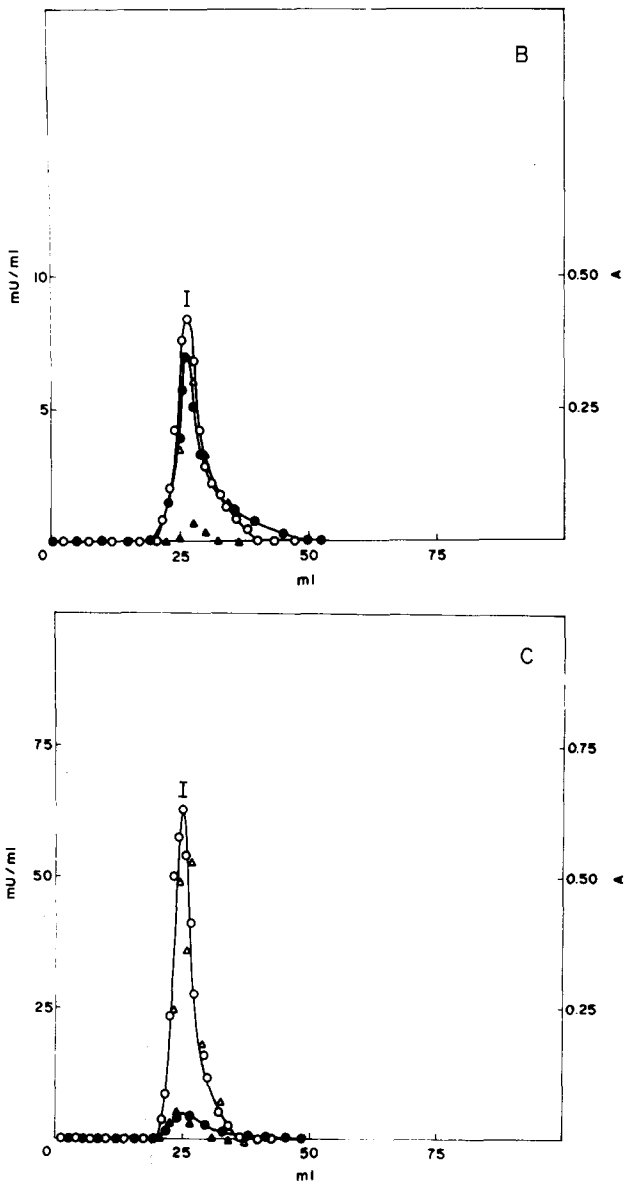

Fig. 3. Elution profiles of extracts of subcellular fractions. ○———○, acid phosphatase activity with *p*-nitrophenylphosphate as substrate; △———△, activity with $2 \cdot 10^{-6}$ M *p*-chloromercuribenzoate in the assay; ▲———▲, activity with $5 \cdot 10^{-3}$ M tartrate in the assay; ●———●, absorbance at 280 nm. For the assay conditions see Experimental Procedure. A, 2870 munits of a liver 10 000 × *g* pellet extract; B, 350 munits of a liver 105 000 × *g* pellet extract; C, 1020 munits of a kidney 10 000 × *g* pellet extract. I, high-molecular-weight acid phosphatase.

mercuribenzoate. Microsomes and mitochondria-lysosomes from brain, liver and kidney show only one class of acid phosphatase which was shown to be high-molecular-weight acid phosphatase by its elution volume and inhibition pattern.

TABLE II

DISTRIBUTION OF ACID PHOSPHATASES TOTAL ACTIVITY IN RAT LIVER SUBCELLULAR FRACTIONS

Fractions were prepared and assayed as described under Experimental Procedure. Results are given as the average ± S.E. of 5 experiments.

	Total units	% of total activity	Specific activity units/mg ($\times 10^{-3}$)
Homogenate	77.4 ± 8.5	100	70 ± 10
10 000 × *g* precipitate	45.1 ± 3.1	58	55 ± 4
105 000 × *g* precipitate	17.3 ± 3.5	22	94 ± 5
Cell sap	16.1 ± 4.8	21	35 ± 3
Nuclei *	0.22	—	8.5

* Average of 3 experiments.

Discussion

Although acid phosphatases have been studied in detail [2—16] no systematic description of the low- to high-molecular-weight acid phosphatase ratios in different tissues has been presented.

As shown here these ratios depend critically on the homogenization procedure employed. Using a buffered, isotonic medium for preparing the homogenates, always only two classes of acid phosphatases were found in the extracts.

As judged by the elution volumes from Sephadex G-75 [3] one is of high-molecular-weight ($M_r \geqslant 100\,000$), eluting in the void volume of Sephadex G-75, and the other is eluted close to cytochrome *c* indicating a molecular weight of less than 30 000 in agreement with previous results [4,5,6,10,12].

High-molecular-weight acid phosphatase was always inhibited by tartrate and insensitive to *p*-chloromercuribenzoate and low-molecular-weight acid phosphatase was always inhibited by *p*-chloromercuribenzoate.

Although α-glycerophosphate is a very poor substrate for low-molecular-weight acid phosphatase, superimposable elution profiles and the same inhibition patterns as observed for *p*-nitrophenylphosphate were obtained in all experiments.

Our results cannot be compared to others [31] in which Triton has been used as solubilizing agent due to the interactions of proteins with the detergent [32] that can produce different forms of association of the native proteins, and also because detergents will solubilize acid phosphatase activity known to be present in cell membranes [33].

Dziembor et al. [34] demonstrated that prostate high-molecular-weight acid phosphatase can be separated in two isoenzymes which differ by their N-acetyl neuraminic acid content. Neuraminidase treatment of prostatic high-molecular-weight acid phosphatase results in the formation of molecular species with identical isoelectric point and the same chromatographic pattern in DEAE-cellulose. Rat liver high-molecular-weight acid phosphatase can be isolated in two forms having different isoelectric points but with the same molecular weight [2].

Needleman and Koenig [35] demonstrated that the isoelectric focusing profiles of the lysosomal acid phosphatases were markedly sensitive to the method of extraction and subsequent treatment of the extract. Rybarska and Ostrowsky [36] showed that prostate acid phosphatases can be separated in subunits after tyrosine and tryptophan residues were modified. Kubicz [37] showed that potato tuber high-molecular-weight acid phosphatase (molecular weight of 96 000) can be separated in two subunits of 46 000 daltons in sodium dodecyl sulfate polyacrylamide electrophoresis.

The numerous results in the literature [4,5,8,10,11,16] presenting more than two classes of acid phosphatases as judged by their molecular weight, must be regarded cautiously and the intermediate molecular weight forms ($M_r \sim$ 60 000) could be regarded as high-molecular-weight acid phosphatase degradation or dissociation products.

Our results show that high molecular weight acid phosphatase is associated with cell organelles, being predominant in lysosomes, and that no interconversion between high- and low-molecular-weight acid phosphatases is detected by the method used here. Low-molecular-weight acid phosphatase is a totally soluble enzyme, a different molecular species, and not a degradation product or a form derived by rupture of lysosomes or even a monomer of high molecular weight acid phosphatase [38].

This study clearly shows that acid phosphatase is present in eukaryotic cells, confined mainly to the mitochondria-lysosomal fraction and to the cell sap.

It is evident from the data present here that in rat tissues, and, probably, in most eukaryotes, only two major classes of acid phosphatases can be isolated and that these are different molecular species which come from lysosomes and from cytoplasm respectively.

Acknowledgments

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