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Subcellular Localization and Some Properties of Intermediate-Molecular-Weight Acid Phosphatase from Rat Liver

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The distribution of acid phosphatases of intermediate molecular weight was determined in various rat tissues. The distribution study of intermediate-molecular-weight acid phosphatase (designated P-II) in the subcellular fractions of rat liver and kidney indicated that P-II was localized in the mitochondrial fractions. The P-II partially purified from liver showed a pI value of 7.0 on isoelectric focusing, and the apparent molecular weight was estimated to be 40000 by Sephadex G-100 gel filtration or 44000 by sodium dodecyl sulfate–polyacrylamide disc gel electrophoresis. The enzyme catalyzed the hydrolysis of a wide variety of natural phosphomonoesters, except for phosphoproteins, phosphoserine, *o*-phosphocholine and thiamine monophosphate. The enzyme showed a high activity on pyridoxal phosphate, β-glycerophosphate, flavin mononucleotide and adenosine 2′-monophosphate. It was markedly inhibited by Hg^{2+} and Ag^{+}, but not significantly by sulfhydryl blocking agents or by L-(+)-tartrate.

Keywords——acid phosphatase; mitochondria; rat liver; rat kidney; subcellular localization

Acid phosphatases [EC 3.1.3.2] have been identified in a wide variety of tissues, and are probably ubiquitous to all cells.[1] At least two types of acid phosphomonoesterases, of different molecular size, exist: high-molecular-weight (HMW) acid phosphatases with molecular weights of more than 200000 and 90000—120000 are associated with the microsomes and lysosomes, respectively,[2] and low-molecular-weight (LMW) acid phosphatases with molecular weights of 8000—18000 are localized in the cytosol.[2d,e,h,3]

In addition to the above two types of acid phosphatases, the existence of intermediate-molecular-weight (IMW) acid phosphatases with molecular weights of approximately 40000 in some mammalian tissues has also been reported.[3b,4] Recently, we purified IMW acid phosphatase from bovine kidney cortex and showed that the IMW enzyme can be clearly distinguished from the HMW and LMW acid phosphatases with respect to its enzymatic properties (substrate specificity and inhibitor sensitivity).[5] However, the properties of IMW acid phosphatase from tissues other than bovine kidney, the distribution in other mammalian tissues, and the subcellular localization remain to be established.

The present paper deals with the distribution of IMW acid phosphatase in various tissues of rat, the localization of the IMW enzyme in cells, and some properties of the partially purified IMW enzyme from rat liver.

Experimental

Materials——*p*-Nitrophenyl phosphate, flavin mononucleotide (FMN) and thiamine pyrophosphate were obtained from Wako Pure Chemical Industries; α-glycerophosphate, glucose-1-phosphate, phosphoethanolamine, ribose-5-phosphate, pyrophosphate (PPi) and *p*-chloromercuribenzoic acid (PCMB) were from Nakarai Chemicals; nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate ($NADP^+$) and adenosine 5′-diphosphate (ADP) were from Oriental Yeast Co.; adenosine 5′-triphosphate (ATP) and adenosine 5′-monophasphate (5′-AMP) were from Kojin Co.; thiamine monophosphate was from P-L Biochemicals; casein

(Hammarsten) was from Merck Co.; antipain, chymostatin, leupeptin and pepstatin were from the Protein Research Foundation; standard proteins, phenylmethylsulfonyl fluoride (PMSF), 6-ethylmercaptopurine and other substrates were from Sigma Chemical Co., except for acid phosphatase from sweet potato, which was prepared according to the method of Uehara *et al.*[6] Sephadex G-75 and 100, DEAE-Sephadex A-50, exchanger Mono P HR 5/20 column and polybuffer 96 were from Pharmacia Fine Chemicals; ampholyte was from LKB; hydroxylapatite was from BDH Chemicals. All other reagents used were of the highest purity available.

Preparation of Crude Extracts from Various Rat Tissues——Male Wistar rats weighing 200—250 g, which had been fasted for 20 h, were used in all experiments. Each tissue was perfused with cold 0.9% NaCl, then immediately removed, chilled and chopped. Each tissue was homogenized with a Waring blender for 90 s in 3 volumes of ice-cold 3 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose. The homogenate was extracted for 15 min at 4 °C by the addition of Triton X-100 to a final concentration of 1.0% (v/v). The supernatant was obtained by centrifugation at 105000 × *g* (60 min), and applied to a Sephadex G-100 column (2.0 × 108 cm) equilibrated with 50 mM Tris–acetate buffer (pH 7.0) containing 0.1 M NaCl.

Fractionation of Rat Liver——The perfused liver was homogenized in 9 volumes of 3 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose with a Teflon-glass homogenizer at 4 °C. The Teflon pestle was driven at 1000—1100 rpm. The homogenization time for each up and down stroke was about 5 s. Subcellular fractions were prepared by differential centrifugation according to the procedure of de Duve *et al.*[7] The extraction of P-II from the subcellular fractions was performed by resuspension in the above buffer containing 1% Triton X-100. The resulting suspension was centrifuged at 105000 × *g* (60 min) and the supernatant was subjected to Sephadex G-100 gel filtration as described in the above section.

Purification of P-II——The liver (300 g) perfused as described above was homogenized with a Teflon-glass homogenizer in 9 volumes of 3 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose. The homogenate was centrifuged at 5000 × *g* (10 min) to obtain the precipitate. After being washed with the sucrose solution, the precipitate was resuspended in 10 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl (buffer A). The suspension was combined with the same volume of 10% Triton X-100 containing 0.1 mM PMSF and 20 μg/ml each of antipain, chymostatin, leupeptin and pepstatin in buffer A. The suspension was stirred for 1 h at 4 °C and then centrifuged at 105000 × *g* (60 min). The supernatant obtained was loaded onto a Sephadex G-75 column (5.6 × 115 cm) equilibrated and eluted with buffer A. Three peaks, corresponding to P-I containing P-I′, P-II and P-III (see Fig. 1) were obtained, and the pooled P-II activity peak was applied to a Sephadex G-100 column equilibrated and eluted with buffer A. The eluted P-II fraction was dialyzed against 10 mM phosphate buffer (pH 7.0), and then applied to a DEAE-Sephadex A-50 column (2.0 × 45 cm) equilibrated with the same buffer. After extensive washing of the column with the same buffer, the enzyme was eluted with a linear 0—0.5 M NaCl gradient at pH 7.0. The active fractions were pooled, concentrated by ultrafiltration with an Amicon PM-10 membrane, and dialyzed against 10 mM phosphate buffer (pH 7.0) containing 10 mM NaCl. The enzyme solution was then applied to a hydroxylapatite column (2.0 × 23 cm) equilibrated with the dialyzing buffer and eluted with buffer A. The active fractions were pooled and concentrated by ultrafiltration. The enzyme solution was subjected to isoelectric focusing[8] in 0.8% ampholyte with a gradient of sucrose (0—48%) in the pH range of 3.5—10 using a 110 ml column. Electrophoresis was performed at 800 V for 48 h at 4 °C. The P-II fraction exhibited a single activity peak with a pI value of 7.0. The active fractions were pooled, concentrated, and then loaded onto a Sephadex G-100 column (1.5 × 68 cm) equilibrated and eluted with buffer A. The active fractions were pooled and concentrated. The enzyme preparation thus obtained was used as P-II for further experiments.

Enzyme Assays——Acid phosphatase activity was routinely determined at 37 °C using 2.5 mM *p*-nitrophenyl phosphate in 0.1 M acetate buffer (pH 5.0), in a final volume of 1.0 ml, unless otherwise noted. The reaction was stopped by the addition of 3.0 ml of 0.25 M NaOH, and the absorbance was measured at 410 nm. One unit of the enzyme activity was defined as an increase in the absorbance of 1.0 per minute under the above conditions. The enzyme activity for a number of other phosphorylated compounds was determined under the above conditions by estimation of orthophosphate (Pi) liberation. The liberated Pi was determined by the method of Chen *et al.*[9] or Delsal and Manhouri.[10] Various marker enzymes including 5′-nucleotidase,[11] alkaline phosphatase,[12] glutamate dehydrogenase,[13] NADPH-cytochrome c reductase,[14] acid phosphatase,[14] and lactate dehydrogenase[15] were assayed as described.

Determination of Protein, Deoxyribonucleic Acid (DNA) and Ribonucleic Acid (RNA)——Protein was determined by the method of Lowry *et al.*[16] using bovine serum albumin as a standard or by measuring the absorbance at 280 nm. DNA and RNA were determined according to Rip *et al.*[17]

Molecular Weight Determination——Molecular weight data was obtained by gel permeation chromatography[18] using a Sephadex G-100 column (2.0 × 67 cm) equilibrated with buffer A and a series of proteins of known molecular weights as standards, and also by sodium dodecyl sulfate (SDS)–polyacrylamide disc gel electrophoresis[19] in 10% acrylamide gels.

Results and Discussion

Distribution of P-II in Various Rat Tissues

The crude extracts from various rat tissues were subjected to gel filtration in order to detect acid phosphatases which may be separated on the basis of differences in molecular size. Figure 1 shows the elution patterns of acid phosphatases obtained when crude extracts of liver were applied to a Sephadex G-100 column. The peaks of acid phosphatase activity illustrated in Fig. 1 were designated P-I′, P-I, P-II and P-III in order of elution. None of the enzyme fractions showed hydrolytic activity toward *p*-nitrophenyl phosphate as a substrate in the alkaline pH range. This indicates the absence of alkaline phosphatase in the enzyme fractions. The isolated acid phosphatase fractions exhibited the same chromatographic pattern in subsequent runs of gel filtration under various conditions of ionic strength and detergent. This finding suggests that the multiple forms are distinct enzymes and the appearance of P-II is not due to the aggregation of LMW components or to the dissociation of HMW components. In the figure, P-I (I′: $M_r > 200000$,[20] I: $M_r = 100000$[2b]) and P-III ($M_r = 15000$[20]) are isozymes corresponding to the HMW and LMW acid phosphatases, respectively. Thus, P-II corresponds to IMW acid phosphatase. The extractability of P-II from liver was examined with various media such as 3 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose (medium A),

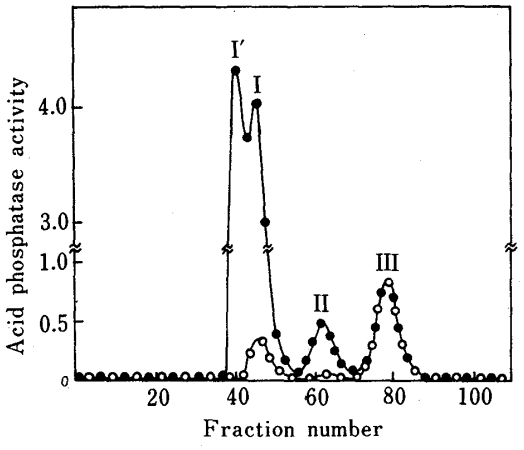

Fig. 1. Gel Filtration of the Crude Extracts from Rat Liver on a Sephadex G-100 Column

Five milliliters of the crude extract prepared with medium containing 0.25 M sucrose (—○—) or 1% Triton X-100 (—●—) was applied to a Sephadex G-100 column. Experimental conditions for the preparation of the crude extracts and gel filtration were as described under Experimental and in the text. The fractions, 3 ml each, were collected and analyzed for acid phosphatase activity.

TABLE I. Composition of Acid Phosphatases Separated by Gel Filtration of Various Rat Tissue Extracts

Tissue	Acid phosphatase			Total activity[a] (units/g wet weight)
	P-I	P-II	P-III	
	% total			
Liver	85	6	9	155
Kidney	63	22	16	164
Brain	42	14	44	56.0
Spleen	63	24	13	121
Heart	44	7	49	17.9
Lung	64	15	21	56.0
Stomach	61	10	29	21.1
Small intestine	49	30	21	62.2
Skeletal muscle	56	—	44	11.2

Experimental conditions for the preparation of the tissue extracts and the gel filtration were as described under Experimental. P-I (containing P-I′), P-II and P-III refer to acid phosphatase activity peaks eluted at positions corresponding to those shown in Fig. 1. The percentage compositions were calculated by measurement of the total acid phosphatase activity of each of the pooled activity peaks. *a*) The values are expressed as the activity extracted per gram wet weight.

0.3 M NaCl in medium A (medium B), 2 mM ethylenediaminetetraacetic acid in medium A (medium C), 25% v/v 1-butanol (medium D), 1% Lubrol in medium A (medium E), 1% deoxycholate in medium A (medium F), and 1% Triton X-100 in medium A (medium G), under the conditions described in Experimental, except for the medium. As shown in Fig. 1, a significant quantity of P-II was extracted with medium G, but not with medium A. The most effective extraction was observed with medium G, containing Triton X-100; in media A—F, the quantities of P-II extracted were approximately 4, 7, 6, 5, 28, and 29%, respectively, of that in medium G. These results indicate that P-II may be bound to the particulate fraction(s) of the cells.

Table I lists the compositions of acid phosphatase isozymes from various rat tissues: P-II was found in various tissues, including liver, kidney, brain, spleen, heart, lung, stomach and small intestine, but not in skeletal muscle. The activity of P-II per g wet weight of tissue was highest in the kidney among the tissues tested.

Subcellular Localization of P-II

Figure 2 shows the subcellular distributions of marker enzymes and P-II from liver. The data on these marker enzymes indicated that separation of the subcellular fractions was reasonably effective. The distribution pattern of P-II, which was obtained by gel filtration on Sephadex G-100 from each of the subcellular fractions, was similar to that of glutamate dehydrogenase, a mitochondrial marker enzyme. These results indicate that P-II is localized in the mitochondria. To examine whether P-II of tissues other than liver was also localized in the mitochondria, the subcellular distribution of kidney P-II was also determined. The kidney P-II was also detected mainly in the mitochondrial fraction of the kidney cells (not shown in the figure).

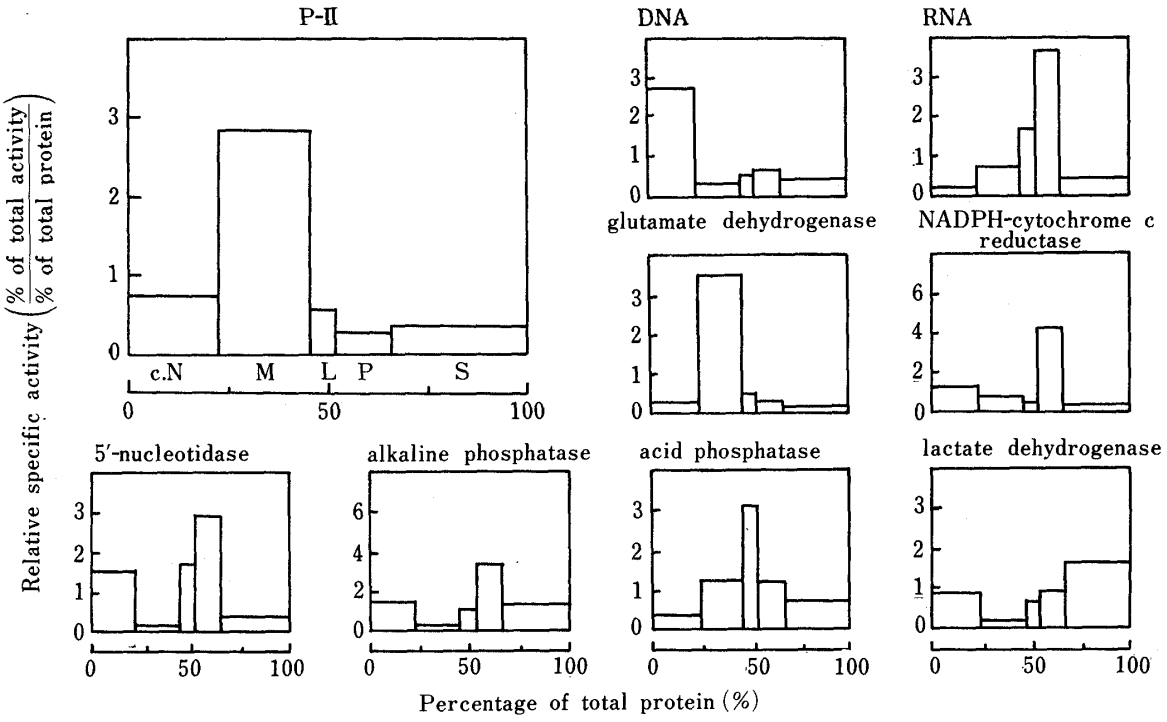

Fig. 2. Subcellular Distribution Patterns of Acid Phosphatase, P-II, and Marker Enzymes in Rat Liver Fractions

The ordinate represents the relative specific activity of the enzyme in the fractions. The abscissa represents in terms of their relative protein content, in the order in which they are isolated, *i.e.* from left to right: crude nuclear (c.N), mitochondrial (M), lysosomal (L), microsomal (P), and soluble (S) fractions. Experimental conditions for the separation of subcellular fractions and assays for enzymes, protein, DNA, and RNA were as described under Experimental.

TABLE II. Purification of P-II from Rat Liver

Step	Total protein[a] (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Crude extract[b]	71638	26342	0.37	1	100
Sephadex G-75	1452	1408	0.97	2.6	5.35
Sephadex G-100	699.4	884	1.26	3.4	3.36
DEAE-Sephadex A-50	69.7	261	3.74	10	0.99
Hydroxylapatite	8.6	188	21.8	59	0.71
Isoelectric focusing	1.9	45.6	24.5	66	0.17
Sephadex G-100	0.9	40.8	45.3	123	0.15

a) Protein concentration was determined from the absorbance at 280 nm, assuming that the extinction coefficient, $E_{1\%}^{1cm}$, at 280 nm was 10.0. *b*) Starting from 300 g of rat liver.

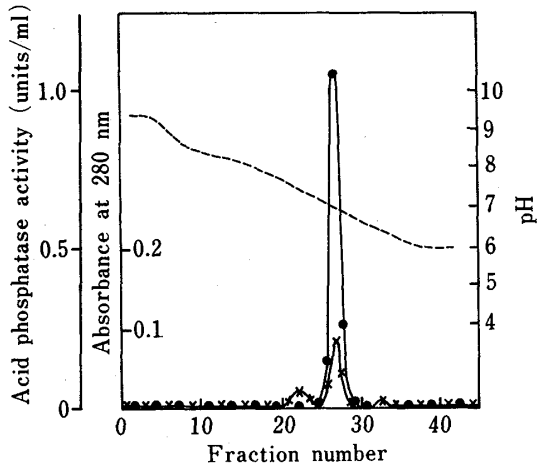

Fig. 3. Chromatofocusing Profile of P-II Fraction Obtained from the Final Purification Step

P-II fraction obtained from the final purification step was loaded onto a polybuffer exchanger Mono P HR/20 column (0.5 × 20 cm) equilibrated with 75 mM Tris–acetate buffer (pH 9.3), and eluted with 1 : 10 diluted polybuffer 96 (pH 5.8). The fractions, 1 ml each, were collected and analyzed for absorbance at 280 nm (—×—), acid phosphatase activity (—●—) and pH (----).

Purification and Purity of P-II

The results of a typical purification are summarized in Table II. P-II was purified about 120-fold with a 0.15% recovery from the crude extract of rat liver. On the basis of the fact that P-II comprised about 6% of total acid phosphatase activity in the crude extract from rat liver (Table I), the recovery of P-II through the purification is estimated to be about 3%. The recovery of P-II was low, possibly because of a tendency to be adsorbed nonspecifically on the resins (including DEAE-Sephadex) or because of instability.

Chromatofocusing of the P-II fraction obtained from the final purification step gave one major protein peak at around pH 6.9 and two minor protein peaks. Acid phosphatase activity was found only in the major protein peak (Fig. 3).

Properties of P-II

Molecular Weight——The molecular weight of P-II was estimated to be approximately 40000 by gel filtration (Fig. 4A). When P-II fraction purified by chromatofocusing was subjected to SDS-polyacrylamide disc gel electrophoresis, a single protein band at a position corresponding to a molecular weight of approximately 44000 was detected (Fig. 4B). These results indicate that P-II is a monomeric protein.

Substrate Specificity——The enzyme activity against various phosphate esters is shown in Table III. P-II efficiently catalyzed the hydrolysis of not only *p*-nitrophenyl phosphate but also pyridoxal phosphate, FMN, 2′- and 5′-AMP, α- and β-glycerophosphates, *o*-phosphotyrosine, and various sugar phosphate esters. P-II showed particularly high activity towards 2′-AMP, FMN, pyridoxal phosphate and β-glycerophosphate among the physiological substances tested. Phosvitin, casein, phosphoserine, *o*-phosphocholine, thiamine mon-

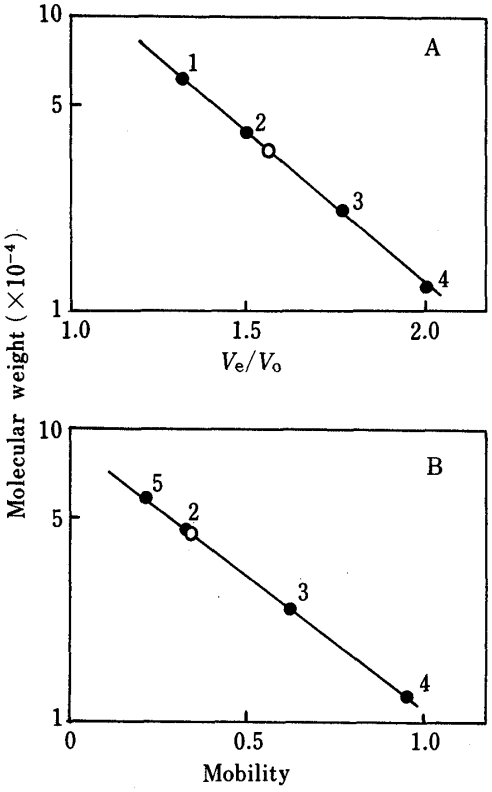

Fig. 4. Molecular Weight Determination by Gel Filtration (A) and by SDS–Polyacrylamide Disc Gel Electrophoresis (B)

Experimental conditions for the gel filtration and the electrophoresis were as described under Experimental. Blue dextran 2000 (average $M_r = 2 \times 10^6$) was used to measure the void volume (V_o) of the column, and the elution volume (V_e) was determined from the absorbance at 230 nm for standard proteins or by assay of acid phosphatase activity for the sample. (●): standard proteins [1, bovine serum albumin ($M_r = 68000$); 2, ovalbumin ($M_r = 45000$); 3, chymotrypsinogen A ($M_r = 25000$); 4, horse heart cytochrome c ($M_r = 12500$); 5, sweet potato acid phosphatase (subunit $M_r = 55000$)]. (○): sample (P-II).

TABLE III. Substrate Specificity of P-II

Substrate	Relative activity[a] (%)	Substrate	Relative activity[a] (%)
p-Nitrophenyl phosphate	100	*myo*-Inositol-2-phosphate	15
2′-AMP	95	NAD^+	0
3′-AMP	5	$NADP^+$	3
5′-AMP	45	Phosphoserine	0
α-Glycerophosphate	50	*o*-Phosphocholine	0
β-Glycerophosphate	84	*o*-Phosphotyrosine	48
Glucose-1-phosphate	13	Diphenyl phosphate	0
Glucose-6-phosphate	27	Bis(*p*-nitrophenyl)phosphate	
Fructose-6-phosphate	22	Phospho(enol)pyruvate	
Fructose-1,6-diphosphate	56	Phosphoethanolamine	
Pyridoxal-5′-phosphate	96	Thiamine monophosphate	
Pyridoxamine-5′-phosphate	5	Thiamine pyrophosphate	
FMN	114	PPi, ADP, ATP	
3-Phosphoglycerate	27	Casein, phosvitin	
Ribose-5-phosphate	4		

a) The rate with 2.5 mM substrate relative to that with 2.5 mM *p*-nitrophenyl phosphate in 0.1 M acetate buffer (pH 5.0).

ophosphate, PPi, thiamine pyrophosphate, ATP, and NAD^+ were not hydrolyzed to any measurable extent.

P-II exhibited an optimum pH of around 5 for *p*-nitrophenyl phosphate hydrolysis. However, the optimum pH was shifted slightly towards the neutral range with the above physiological substances as substrates: near 5.5 with 2′-AMP and β-glycerophosphate and near 6 with pyridoxal phosphate and FMN.

TABLE IV. Effects of Various Substances on the Activity of P-II

Substance added	Concentration (M)	Relative activity (%)
None		100
L-(+)-Tartrate	1×10^{-3}	98
	1×10^{-2}	63
KF	1×10^{-5}	100
	1×10^{-4}	46
	1×10^{-3}	8
PCMB[a)]	1×10^{-7}	94
	1×10^{-6}	56
N-Ethylmaleimide[a)]	1×10^{-2}	101
Iodoacetate[a)]	1×10^{-2}	89
Adenine	1×10^{-3}	103
6-Ethylmercaptopurine	1×10^{-3}	101
KH_2PO_4	1×10^{-4}	99
	1×10^{-3}	73
Na_2HAsO_4	1×10^{-3}	83
$(NH_4)_6Mo_7O_{24}$	1×10^{-6}	102
	1×10^{-5}	68
$FeCl_3$	1×10^{-7}	104
	1×10^{-6}	66
$Cu(CH_3COO)_2$	1×10^{-5}	90
	1×10^{-3}	56
$Hg(CH_3COO)_2$	1×10^{-10}	104
	1×10^{-8}	49
	1×10^{-6}	8
$AgNO_3$	1×10^{-9}	103
	1×10^{-7}	65

The activity was determined by incubation of the enzyme at 37 °C in the presence of 2.5 mM *p*-nitrophenyl phosphate and the indicated additions in a total volume of 1.0 ml of 0.1 M acetate buffer (pH 5.0). The activity was expressed as percent of the no-addition run. *a*) After a 5 min preincubation of the enzyme at 37 °C in 0.1 M acetate buffer (pH 5.0), aliquots were withdrawn and the activity was assayed under the standard conditions.

These findings are similar to those obtained with bovine kidney IMW acid phosphatase.[5)]

Effects of Various Compounds——Table IV summarizes the effects of various substances on the acid phosphatase activity of P-II. L-(+)-Tartrate and sulfhydryl blocking agents are effective inhibitors of HMW and LMW acid phosphatases, respectively,[2b,e,g,3c,e,f)] while 6-ethylmercaptopurine is an activator of LMW acid phosphatase.[3d,5)] However, P-II was less sensitive to inhibition by the above inhibitors, and was not affected by purine compounds. Fluoride strongly inhibited HMW and IMW acid phosphatases, but not the LMW enzyme.[5)] In the case of P-II, effective inhibition by fluoride was found. These findings are consistent with the properties of IMW acid phosphatase.[5)]

Among the cations tested, Ag^+, Hg^{2+}, Fe^{3+} and Cu^{2+} were found to be effective inhibitors of P-II, though the inhibitory effects of the metal ions clearly differed from each other. Other metal ions, including Ca^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+} and Zn^{2+}, showed no significant effects at 1 mM on the activity of P-II (data not shown).

In conclusion, the results obtained in the present study indicate that the molecular and enzymatic properties of P-II from rat liver are similar to those of IMW acid phosphatase from bovine kidney cortex, recently reported.[5)] Furthermore, the subcellular fractionation study indicated that IMW acid phosphatase is localized in the mitochondria.

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