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Distribution and Properties of Alkaline Pyrophosphatases of Rat Liver

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A study of the distribution of alkaline inorganic pyrophosphatase [alkaline PP_iase, EC 3.6.1.1] in the subcellular fractions of rat liver indicated that in addition to the PP_iase present in the cytosol, there were two isoenzymes in mitochondria (Mt. I and Mt. II) which were electrophoretically different from the cytosol enzyme. PP_iase activities found in purified nuclei and lysosomes can be accounted for by contaminating mitochondria.

All three PP_iases which were partially purified had pH optima at 8 and estimated molecular weight of 68,000. They were activated by Mg^{2+}, and markedly inhibited by Zn^{2+}, Mn^{2+}, Hg^{2+}, Cd^{2+}, Ca^{2+} and F^-; they were rather unstable to air oxidation and stabilized in the presence of cysteine.

The three isoenzymes were different with regard to the effect of Mg^{2+}, substrate specificity and inactivation by urea. Mg^{2+} which was essential for the activity of all three enzymes was inhibitory when present in excess. This inhibition was more marked with the cytosol and Mt. II enzymes and less so with Mt. I. The cytosol PP_iase was rather specific for PP_i hydrolysis, while mitochondrial enzymes also hydrolyzed nucleoside polyphosphates at considerable rates. The urea concentrations required for 50% inactivation were 1.2 M for the cytosol enzyme and 2 M for mitochondrial PP_iases.

Inorganic pyrophosphate (PP_i) is not only a by-product in several biosynthetic reactions at the expense of nucleoside triphosphates, but also it apparently has a function in certain biological systems, *e.g.*, in bone metabolism preventing formation and dissolution of hydroxyapatite (*1*) or in photosynthetic reactions of certain microorganism donating energy (*2*). From these aspects, enzymes which hydrolyze PP_i might be expected to play an important roles in controlling metabolism in cells. Since there are several PP_i hydrolyzing enzymes with different properties, it is necessary to reinvestigate their subcellular distributions and substrate specificities for an understanding of the physiological roles of pyrophosphatases and PP_i. The present paper describes the subcellular distribution of alkaline inorganic PP_iase [pyrophosphate phosphohydrolase, EC 3.6.1.1] in rat liver and the presence of two isoenzymes in mitochondria which are different from the cytosol PP_iase.

MATERIALS AND METHODS

Enzyme assays

Alkaline PP_iase—Hydrolysis of PP_i was assayed by the colorimetric method of Fiske and SubbaRow (*3*). The assay mixture (2 ml) contained 50 mM Tris-HCl (pH 7.5), 1.1 mM Na pyrophosphate, 2.5 mM $MgCl_2$ and the enzyme. The reaction carried out at 37°C for 10 min was started by adding the enzyme and terminated by the addition of 1 ml of 10% trichloroacetic acid. Under these conditions, the rate of hydrolysis of PP_i was linear with time till up to 0.7 μmole of the substrate (30%) was hydrolyzed. One unit of enzyme was defined as that amount necessary to liberate 1 μmole of orthophosphate per min under the above conditions. Specific activity was the unit of enzyme per mg of protein.

Glutamate Dehydrogenase [*EC 1.4.1.2*]—The enzyme activity was assayed by measuring the glutamate formation from the reactants by the method of Beaufay *et al.* (*4*). One unit of enzyme catalyzed the oxidation of 1 μmole of NADH per min.

Alkaline Phosphatase [*EC 3.1.3.1*]—The assay mix-

ture (2 ml) contained 50 mM Tris-HCl (pH 7.5), 3.7 mM *p*-nitrophenyl phosphate, 2.5 mM $MgCl_2$ and the enzyme (*5*). The reaction was carried out at 37°C for 10 min and terminated by the addition of 1 ml of 0.1 N NaOH. After centrifugation to clarify the solution, the optical density of the supernatant solution was measured at 400 mμ.

Acid Phosphatase [*EC 3.1.3.2*]—In most cases, the assay conditions were the same as those for alkaline phosphatase except for the use of acetate buffer (0.1 M, pH 4.5). In the case of the subcellular fractionations, however, the assay mixture (2 ml) contained 50 mM acetate buffer (pH 5.0), 1.6 mM β-glycerophosphate and the enzyme. After incubated for 10 min at 37°C, the reaction was terminated by the addition of 10% trichloroacetic acid (1 ml) and P_i liberated in the supernate was measured. One unit of enzyme catalyzed the liberation of 1 μmole of P_i per min under the assay conditions used.

Subcellular Fractionation of Rat Liver—Adult male rats (Wistar or Sprague-Dawley) were sacrificed by decapitaion and bled. Livers were immediately removed, rinsed, homogenized in a Potter-Elvehjem homogenizer with ice cold 0.25 M sucrose (9 ml/g tissue), and fractionated into five fractions (nuclei, mitochondria, lysosomes, microsomes and soluble fraction (cytosol)) according to the method of de DUVE *et al.* (*6*).

Purification of Nuclei—Purification of nuclei was performed principally based on the method of CHAUVEAU *et al.* (*7*) and WIDNELL and TATA (*8*). Nuclei and debris fractions obtained from 100 g of rat liver according to the method of de DUVE *et al.* (*6*) were suspended in 0.25 M sucrose (75 ml/25 g of original tissue), layered on 0.32 M sucrose (100 ml/25 g tissue) and centrifuged at 800×*g* for 15 min. The combined precipitates (crude nuclei) were suspended in 2.4 M sucrose-1 mM $MgCl_2$ (300 ml) and centrifuged at 65,000× *g* for 1 hr. The precipitate (Nuclei I) was resuspended in 2.4 M sucrose (300 ml) and centrifuged under the above conditions. The precipitate (Nuclei II) was rewashed in the same way to yield a colorless precipitate (Nuclei III). Each fraction was suspended in 0.25 M sucrose (10 mg protein/1 ml) and stored at −20°C.

Purification of Lysosomes—Purification of lysosomes was performed by the method of SAWANT *et al.* (*9*). Light mitochondria fraction (F I) was further purified to yield F II and F III. For the purpose of assay of these fractions, they were suspended in 0.7 M sucrose. As an indication of purity, β-glycerophosphatase activity was determined and it was found that specific activity of it in F III was four times higher than that in F I.

Polyacrylamide Gel Electrophoresis—Disk electrophoresis in polyacrylamide gel was performed by the methods of ORNSTEIN (*10*) and DAVIS (*11*) using pH 7.5 gel (*12*) and 0.4—0.8 unit of enzyme. The PP_iase active zone was stained yellow by the method of TONO and KORNBERG (*13*). The gel was incubated for 20—60 min at 30°C in a solution identical with that used for the enzyme assay, rinsed with water, and immediately immersed in SUGINO's triethylamine-molybdate reagent (*14*). Electrophoretic mobility was expressed as the ratio to that of bromophenol blue tracking dye.

Protein Determination—Protein was estimated by FOLIN and CIOCALTEU's method (*15*) with bovine serum albumin as a reference standard or by measuring the absorbance at 280 mμ (*16*).

RESULTS

Subcellular Distribution of Alkaline PP_iase—As shown in Table I, a predominant amount of alkaline PP_iase activity was present in the cytosol, in good agreement with findings of NORDLIE and LARDY (rat liver) (*17*), PYNES and YOUNATHAN (pigeon pancreas) (*18*), and SOODSMA and NORDLIE (rat heart) (*19*). The specific activity of mitochondrial PP_iase was higher than those of the other particulate fractions, but was only about 1/6 of that of the cytosol PP_iase.

Glutamate dehydrogenase activity and activity ratios of alkaline PP_iase to glutamate dehydrogenase of subcellular fractions are also given in Table I. The ratios of enzyme activities in four particulate fractions were in

TABLE I

Subcellular distribution of alkaline PP_iase in rat liver

	PP_iase	Glutamate dehydrogenase	Ratio
	Specific activity		
Homogenate	0.852	0.312	2.74
Nuclei	0.080	0.264	0.30
Mitochondria	0.118	0.422	0.28
Lysosomes	0.066	0.288	0.23
Microsomes	0.056	0.252	0.22
Cytosol	0.624	0.002	312.00

Specific activity: Units/mg protein.
One unit: 1 μmole of P_i liberated or 1 μmole of NADH oxidized per min.
Ratio: PP_iase/Glutamate dehydrogenase.

TABLE II

Alkaline pyrophosphatase and glutamate dehydrogenase in purified nuclei and lysosomes

Fractions	β-Glycero-phosphatase	PPiase	Glutamate dehydrogenase	PPiase / Glutamate dehydrogenase
	Specific activity			
Mitochondria		0.100	0.315	0.32
Crude nuclei		0.137	0.145	0.94
Nuclei I		0.021	0.061	0.34
II		0.014	0.043	0.33
III		0.009	0.029	0.31
Lysosomes F I	0.010	0.060	0.161	0.37
F II	0.022	0.060	0.152	0.39
F III	0.041	0.043	0.116	0.37

the range of 0.22—0.30 and very similar to that of mitochondria. Similar comparisons with nuclei and lysosomes at various stage of purification are summarized in Table II. While the specific activity of β-glycerophosphatase of lysosomes increased, those of alkaline PPiase and glutamate dehydrogenase decreased with the advanced steps of purification. Furthermore, PPiase/glutamate dehydrogenase ratios were not much altered with purification and approximately the same as that of mitochondria. The ratio of two enzymes decreased from 0.94 (crude nuclei) to 0.31 (Nuclei III), which was very similar to that of mitochondria (0.32).

Since glutamate dehydrogenase is known to be localized in mitochondria (*4*, *20*), the constant ratios of alkaline PPiase and glutamate dehydrogenase activities in each fraction (Table I) and in purified nuclei and lysosomes (Table II) suggest that alkaline PPiase activities found in lysosomes and nuclei were due to the contaminating mitochondria.

Polyacrylamide Gel Electrophoresis

(i) *Extraction of PPiase from the Particulate Fractions*—Mitochondria were washed three times with 0.25 M sucrose, suspended in the same medium (about 0.5 ml/g of original tissue), frozen (centrifuge tubes placed in a dry ice-alcohol bath) and thawed three times. The samples were then centrifuged at 13,000×*g* for 20 min at 0°C. The sediment was resuspended in 0.1 M Tris-HCl (pH 7.5), freeze-thawed three more times and again centrifuged. Fifty to seventy per cent of the original mitochondrial alkaline PPiase activity was recovered in the combined extracts. PPiase from lysosomes and nuclei was obtained by the procedure used for mitochondria.

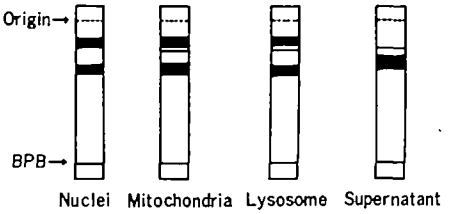

FIG. 1. Electrophoretic patterns of alkaline PPiases of rat liver.

(ii) *Gel Electrophoresis*—As shown in Fig. 1, alkaline PPiase of the cytosol moved towards the anode essentially as a single band, but the enzyme extracted from mitochondria formed two distinct zones. The faster moving PPiase moved just ahead of the cytosol PPiase, and the mobility of the slower one was about a half of that of the faster moving one.

In the case of PPiases from freeze-thawed lysosomes and nuclei, again two alkaline PPiases with the same relative mobilites as those of mitochondrial enzymes were detected. From these results, it was concluded that three different alkaline PPiases were present in the rat liver cells, one in the cytosol and two in

the mitochondria, and that no detectable amount of alkaline PP_iase was present in microsomes.

Effect of Cysteine on PP_iase—The crude mitochondrial and cytosol PP_iases were preincubated in Tris-HCl buffer (pH 7.5, 0.1 M) at 37°C and the residual enzyme activities were measured as appropriate time intervals. PP_iase activities decreased to about 50% of the initial values in 60 min. In a nitrogen atmosphere, the decrease was less marked than in air, indicating the instability of the enzyme to air oxidation (Fig. 2).

Under similar conditions at 20°C, however, the enzyme activities were almost completely retained in the presence of cysteine (0.01 M). Furthermore, when the enzyme preparation partially inactivated by standing for 2—3 hr at room temperature was incubated with 0.01 M cysteine for 30 min at 0°C, it was reactivated to the original level at zero time (Fig. 3).

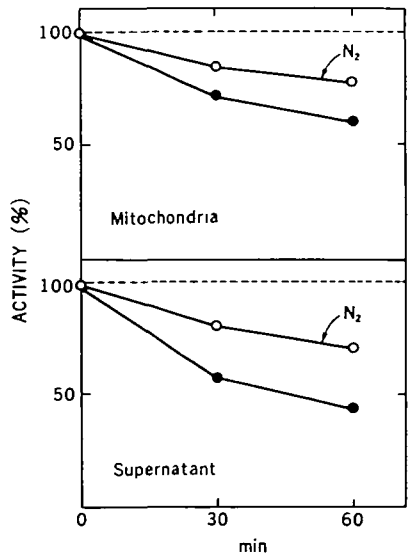

FIG. 2. Stability of alkaline PP_iases at 37°C. The cytosol- or crude mitochondrial PP_iase of rat liver was incubated in Tris-HCl (pH 7.5, 0.1 M) at 37°C in a pair of Thunberg tubes. One tube was evacuated and filled with nitrogen several times and the other tube was left filled with air. Activities are expressed as percentage of initial activities.

—●— In air, —○— In nitrogen.

Effect of pH on Alkaline PP_iase—Cytosol and crude mitochondrial PP_iases were incubated at various pHs with and without cysteine (final concentration, 1 mM), and then assayed at pH 7.5. In the absence of cysteine, pH optimum for the enzyme stability was 7.0—7.5, and in the presence of cysteine, it was shifted to 6.5 (Fig. 4). This shift was probably due to the combination of two factors, the stability of cysteine at acidic pHs and that of enzyme at pH 7—7.5 in the absence of cysteine.

Since cysteine seemed to protect the enzyme from the air oxidation and to renature the partially oxidized enzyme, this amino acid was added to the buffer used for the enzyme purification.

Purification of PP_iase

(i) *Cytosol Enzyme*—Cytosol of rat liver was fractionated with ammonium sulfate (40—

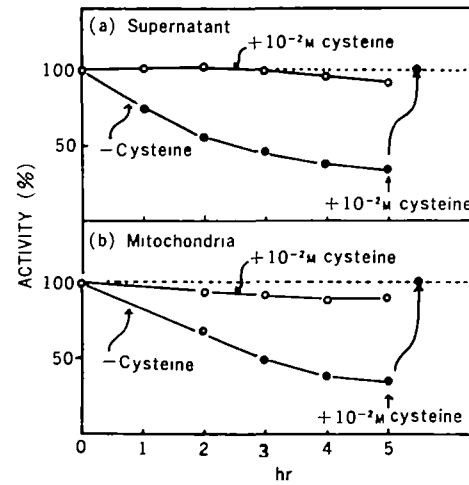

FIG. 3. Effect of cysteine on PP_iase, protection of activity and renaturation of inactivated enzyme.

(a) Cytosol alkaline PP_iase was incubated at 20°C in Tris-HCl (pH 7.5, 0.1 M) in the presence of 0.01 M cysteine or in its absence. 0.5 ml aliquots (0.028 unit of enzyme) were withdrawn and assayed as described in the text. After 5 hr incubation, cysteine was added to a concentration of 0.01 M to the incubation mixture containing no cysteine, incubated at 0°C for 30 min and assayed.

(b) The same experiments as above were carried out using crude mitochondrial enzyme. Enzyme concentration was 0.028 unit per ml.

80% saturation). The precipitate from centrifugation at 10,000×*g* for 15 min was dissolved in a small volume of distilled water and chromatographed on a Sephadex G-100 column (3×32 cm), which had been pre-equilibrated with elution fluid (0.1 M Tris-HCl, pH 6; 0.005 M cysteine-HCl). Alkaline PPiase was eluted in tubes No. 13—30 (5 ml each aliquot), and acid phosphatase appeared in two peaks (Fig. 5a), the faster moving peak overlapping with alkaline PPiase. Fractions No. 18—25 were combined and applied to a DEAE-Sephadex A-50 column (3×36 cm), which had been pre-equilibrated with 0.05 M acetate buffer (pH 6.8) containing 1 mM PP_i and 5 mM cysteine-HCl. Elution was carried out by a NaCl

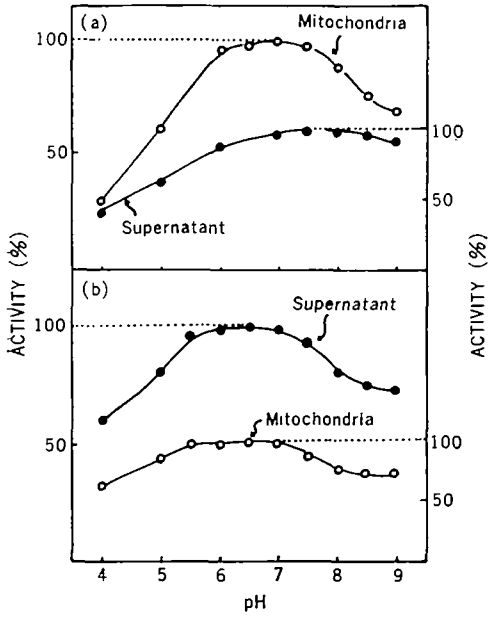

FIG. 4. Effect of pH on alkaline PPiase stability.

(a) Crude mitochondrial and cytosol PPiases were incubated at 25°C for 20 min in various 0.1 M buffers (acetate, pH 4—6.5 and Tris-HCl, pH 7.0—9). The enzyme activity (0.02—0.03 unit per ml) was then measured at pH 7.5 as described in the text. The activities were represented as the percentage of the maximum activity.

(b) Similar experiments in the presence of 1 mM cysteine at 22°C for 45 min.

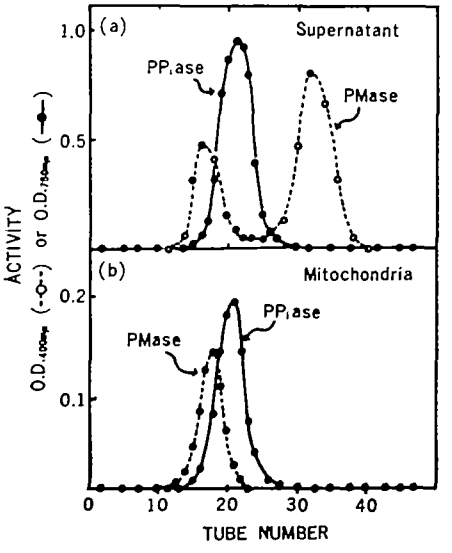

FIG. 5. Column chromatography of alkaline PPiase on Sephadex G-100.

(a) The cytosol alkaline PPiase (40—80% saturation with ammonium sulfate) was applied to Sephadex G-100 column (3×32 cm) pre-equilibrated with 0.1 M Tris-HCl 5 mM cysteine. The protein applied was about 1 g in 50 ml. The PPiase and acid phosphomonoesterase activities (acid PMase) were measured as described in the text. Eech tube contained 5 ml of effluent.

(b) Mitochondrial extract obtained by freez-thawing was treated as in (a). Alkaline phosphomonoesterase (alkaline PMase) was assayed by the method described in the text.

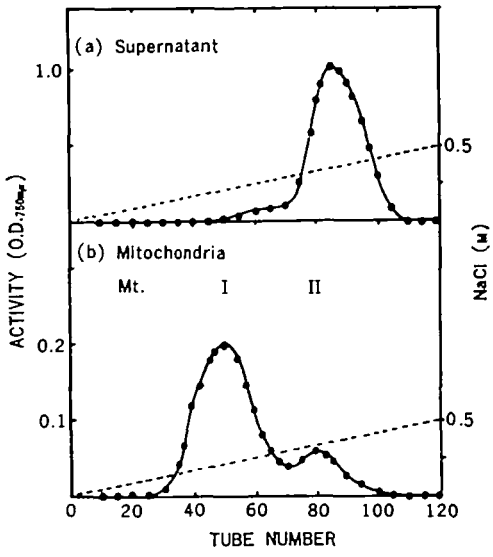

FIG. 6. Column chromatography of alkaline PP_iase on DEAE Sephadex A-50.

(a) Cytosol alkaline PP_iase fraction from Sephadex G-100 was applied on a DEAE Sephadex A-50 column (3×36 cm) pre-equilibrated with 0.05 M Tris-acetate—5 M cysteine-HCl—1 mM PP_i (pH 6.8) and eluted with linear gradient of NaCl. Protein applied was 1,080 mg.

(b) Mitochondrial alkaline PP_iase fraction from Sephadex G-100 was treated as in (a). Protein applied was about 314 mg.

TABLE III

Purification of cytosol alkaline PP_iase of rat liver

Fraction	Volume (ml)	Protein (mg)	Total units	Recovery (%)	Specific activity	Purification
Homogenate	625	15,500	23,200	100	1.5	1
$(NH_4)_2SO_4$ precipitate 40—80% saturation	195	4,100	7,200	31	1.8	1.2
Sephadex G-100	270	1,080	7,100	30	6.6	4.4
DEAE Sephadex A-50	250	176	4,400	19	25.0	16.7
Sephadex G-100	85	79	3,600	15	45.0	30.0

TABLE IV

Purification of mitochondrial alkaline PP_iase of rat liver

Fraction	Volume (ml)	Protein (mg)	Total units	Recovery (%)	Specific activity	Purification
Mitochondria	100	3,430	760	100	0.22	1.0
Freez-thawing						
Supernatant	125	1,290	425	56	0.33	1.5
Precipitate	45	1,040	131	17	0.12	
Sephadex G-100	110	314	220	29	0.71	3.2
DEAE Sephadex A-50						
Fraction I	180	90	191	25	2.12	9.6
Fraction II	360	47	75	10	1.60	7.3
Sephadex G-100						
Fraction I	80	24	138	18	5.8	26.4
Fraction II	60	7.6	44	6	5.8	26.4

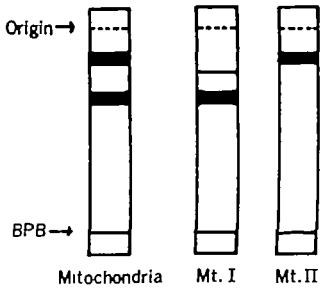

FIG. 7. Electrophoresis of mitochondrial PPi-ases on polyacrylamide gel.

The experimental conditions were described in the text. Crude mitochondria extract, partially purified enzymes, Mt. I. and Mt. II containing 0.4—0.8 unit of PPiase activity were applied to the gels. The lowest line indicates the position of bromophenol blue (BPB) used as marker.

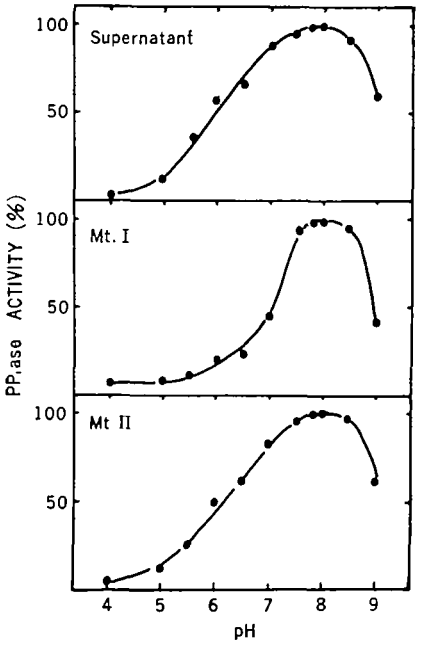

FIG. 8. pH Optimum of alkaline PPiase.

The experimental conditions are described in the text except for the use of buffers of various pH's. The enzyme activities are expressed as the percentage of the maximal activities at pH 8.

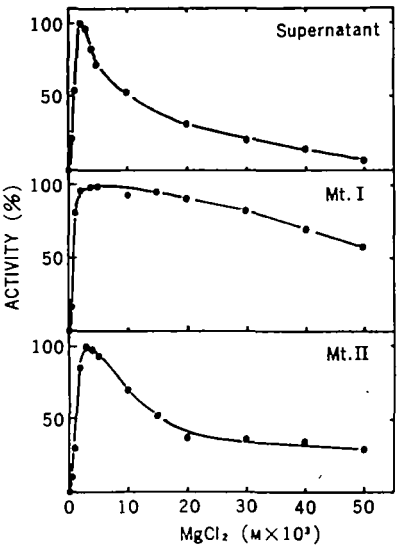

FIG. 9. Effect of Mg^{2+} concentration on alkaline PPiase.

The enzyme assays were carried out at pH 8 as described in the text except for the use of various concentrations of Mg^{2+}. Enzyme activities are expressed as percentage of the maximum activities.

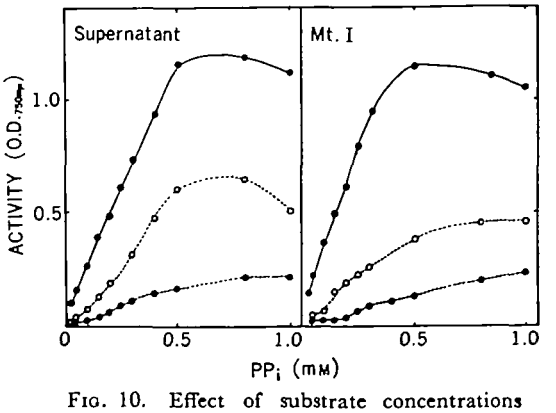

FIG. 10. Effect of substrate concentrations on PPiase activities at different Mg^{2+} concentrations.

Enzymes used were 2.4 units of cytosol enzyme and 1.7 units of Mt. I. Incubation times were 10 and 15 min for cytosol and Mt. I enzyme, respectively.

—●— $PP_i/Mg^{2+}=0.1$,
···○··· $PP_i/Mg^{2+}=1.0$,
···●··· $PP_i/Mg^{2+}=2.0$.

gradient using the same buffer (Fig. 6a). The combined alkaline PPiase fractions (No. 70—100) were concentrated to a small volume in a visking tubing covered with Carbowax 6,000 at 0°C. The concentrated enzyme was chromatographed on a Sephadex G-100 column

(3×32 cm) to eliminate the remaining alkaline phosphatase and Carbowax. The PP_iase preparation at this stage was almost free from acid and alkaline phosphatases and had 30 fold higher specific activity than the original liver homogenate. The purification procedures are summarized in Table III.

(ii) *Mitochondrial PP_iase*—Crude mitochondrial alkaline PP_iase was chromatographed on a Sephadex G-100 column (Fig. 5b) as described for the cytosol PP_iase. Fractions No. 18—28 were combined and re-chromatographed on a DEAE-Sephadex A-50 column. Alkaline PP_iase activities were found in two peaks (Fractions No. 30—70 and 71—100) (Fig. 6b). The faster moving (Mt. I) and slower moving (Mt. II) peaks were pooled separately and concentrated to a small volume by means of Carbowax 6,000 and then each chromatographed on a Sephadex G-100 column. Both mitochondrial PP_iases (Mt. I and Mt. II) appeared to be essentially homogeneous as judged from the activity on polyacrylamide gel electrophoresis (Fig. 7). The enzyme preparations were purified about 26 fold over the original mitochondria and relatively free from alkaline phosphatase. Specific activities of these enzymes, however, were 1/7 of that of the cytosol PP_iase. The purification procedures are summarized in Table IV.

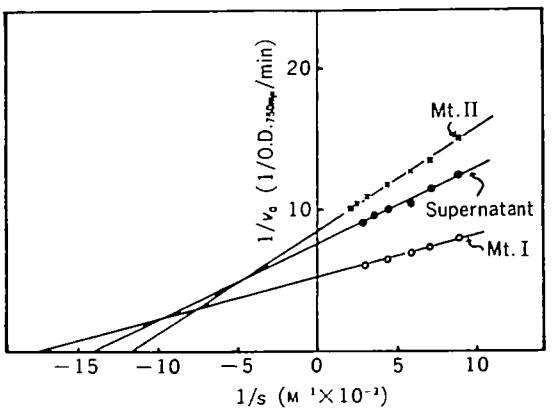

FIG. 11. Double reciprocal plots of initial velocity of alkaline PP_iases against PP_i concentrations.

Mg^{2+} was added to keep the ratio of $(Mg^{2+})/(PP_i)=4.5$ for all experiments. Units of enzyme used were; 5.2 (cytosol), 2.5 (Mt. I) and 0.72 (Mt. II). Incubation time was; 3 min (cytosol), 10 min (Mt. I) and 25 min (Mt. II). K_m values obtained from the above experiments were 7×10^{-5} M (cytosol), 5.6×10^{-5} M (Mt. I) and 8.6×10^{-5} M (Mt. II).

—●— Cytosol,
—○— Mt. I,
—×— Mt. II.

Properties of Partially Purified PP_iases

(i) *pH Optimum*—All three alkaline PP_iases had the same optimum at pH 8 (Fig. 8). Cytosol and Mt. II enzymes had somewhat broader pH optima than Mt. I.

(ii) *Effect of Mg^{2+} Concentrations*—At the fixed PP_i concentration (1.1 mM), three alkaline PP_iases from rat liver showed maximum activities with Mg^{2+}/PP_i ratio=4—5. Further

TABLE V

Relative rates of hydrolysis of PP_i and other substrates by rat liver alkaline PP_iases

Substrates	PP_iases		
	Cytosol	Mt. I	Mt. II
PP_i	100	100	100
ATP	7.2	35	17
GTP	29	65	35
UTP	13	89	33
CTP	14	98	27
ITP	11	31	80
ADP	22	96	37
GDP	9.6	57	68
UDP	3.2	13	59
CDP	5.2	47	20
IDP	6.0	71	86
AMP	1.7	4.3	8.5
GMP	1.0	4.3	7.8
UMP	1.1	4.3	8.5
CMP	1.2	4.0	8.5
IMP	1.0	3.6	8.0
β-Glycerophosphate	0.9	3.6	7.5
p-Nitrophenylphosphate	1.1	4.5	7.5

Final substrate concentration: 1 mM.

The enzyme activities are calculated from P_i liberated from various substrates under assay conditions. Relative activities are expressed as the percentage of those for PP_i.

increase of Mg^{2+} concentration was inhibitory. The inhibition by excess Mg^{2+} was more marked with the cytosol and Mt. II enzymes and less so with Mt. I PPiase (Fig. 9).

(iii) *Effect of Substrate Concentration*—As HORN *et al.* reported for the cytosol PPiase of mouse liver (*21*), partially purified cytosol- and Mt. I enzymes showed allosteric properties (*22*). As shown in Fig. 10, the substrate saturation curves took S-shaped forms when $(PP_I)/(Mg^{2+})=1$ and 2, and they were converted to a Michaelis type hyperbola when Mg^{2+} concentration was increased to make $(PP_I)/(Mg^{2+})=0.1$.

(iv) *Michaelis Constants*—With $(Mg^{2+})/(PP_I)$ ratio fixed at 4, Michaelis constants were calculated from LINEWEAVER-BURK's plot (*23*). K_m's (PP_I) were 7.0×, 5.6× and 8.6×10^{-5} M for the cytosol-, Mt. I- and Mt. II PPiases, respectively (Fig. 11).

(v) *Substrate Specificity*—Rates of hydrolysis of PP_I and a number of other nucleoside poly- and monophosphates are summarized in Table V. Under the conditions employed, PP_I was hydrolyzed by the cytosol enzyme at much greater rates than any other compound tested. The rates of hydrolysis of mononucleotides were less than 2% of that of PP_I and very similar to those of β-glycerophosphate and *p*-nitrophenyl phosphate. Therefore, it is very likely that slower hydrolysis of mononucleotides was due to a little contamination by phosphatase. In contrast to this, mitochondrial PPiase hydrolyzed nucleoside polyphosphates at much greater rates than the cytosol enzyme.

(vi) *Inhibitors*—All three alkaline PPiases were markedly inhibited by Zn^{2+}, Mn^{2+}, Hg^{2+}, Cd^{2+}, Ca^{2+} and F^-. Cytosol PPiase was moderately inhibited by Ni^{2+}, Co^{2+} and Cu^{2+}, while mitochondrial enzymes were more strongly inhibited by these metal ions. MoO_4^- which was reported as an inhibitor for glucose 6-phosphatase (*24*) showed almost no effect. Alloxan, an inhibitor for human erythrocyte PPiase (*25*), was not inhibitory to rat liver PPiase (Table VI).

TABLE VI

Effect of divalent cations and other compounds on the PPiase activity

Compounds added	PPiase		
	Cytosol	Mt. I	Mt. II
	Relative specific activity		
None	100	100	100
Ca^{2+}	23	15	13
Cd^{2+}	5	7	6
Co^{2+}	74	12	30
Cu^{2+}	87	9	7
Hg^{2+}	20	8	11
Mn^{2+}	9	15	9
Ni^{2+}	57	28	18
Zn^{2+}	6	7	6
F^-	11	13	7
MoO_4^-	103	110	114
Alloxan	100	95	105

Experimental conditions were described in the text.

Enzymatic activities were measured in the presence of various inhibitors (final concentration = 1 mM).

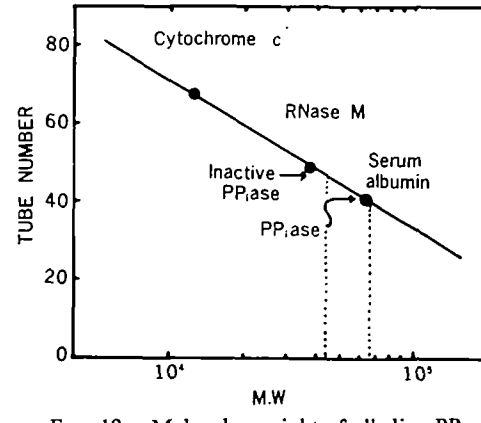

FIG. 12. Molecular weight of alkaline PPiase. Cytosol alkaline PPiase or marker protein (3 ml) was applied on a Sephadex G-100 column (2.8×50 cm) and eluted with Tris-HCl (0.1 M, pH 7.5), collected as 4 ml aliquots. The marker proteins used were cytochrome *c*, bovine serum albumin and ribonuclease from *Asp. saitoi* (*25*). Cytochrome *c* was determined by measuring optical density at 550 mμ, serum albumin by FOLIN and CIOCALTEU's colorimetric procedure (*15*), and ribonuclease activity was measured according to the procedure of IRIE (*27*).

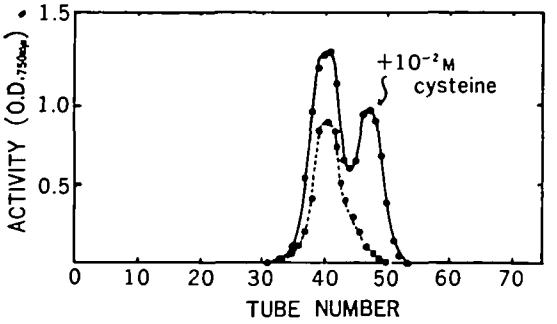

FIG. 13. Sephadex G-100 column chromatography of partially inactivated cytosol alkaline PP_iase.

Three ml of cytosol alkaline PP_iase partially inactivated by incubation at 37°C were applied on a Sephadex G-100 column (2.8×50 cm). The elution conditions were the same as in Fig. 10.
--○-- PP_iase activity was measured directly.
—●— PP_iase activity was measured after addition of 0.01 M cysteine.

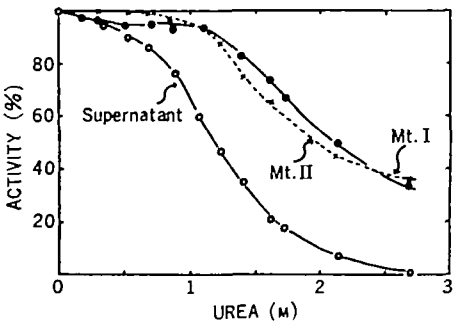

FIG. 14. Inactivation of alkaline PP_iase in urea.

Alkaline PP_iases were incubated at 20°C for 60 min in various concentrations of urea containing 0.04 M Tris-HCl, pH 7.5 and then the activities were measured in the buffer solution without urea as described in the text. Activities were expressed as percentage of the initial activities.

(vii) *Molecular Weights*—Molecular weights of rat liver alkaline PP_iases were estimated by Andrew's Sephadex G-100 filtration method (*26*) using cytochrome *c*, bovine serum albumin and ribonuclease from *Aspergillus saitoi* (*27*) as reference proteins. From the plot of elution patterns *vs* logarithm of molecular weights (Fig. 12), the molecular weight of cytosol PP_iase was estimated as approximately 68,000. Since the peaks of mitochondrial PP_iases were eluted in the same tube under the same conditions, the molecular weights of all three PP_iases should be close to each other.

The cytosol PP_iase partially inactivated by air oxidation was also filtered through the same Sephadex G-100 column under the same conditions, and the enzyme activity of each fraction was measured before and after incubation with 0.01 M cysteine at 0°C for 30 min. Only one peak was found before incubating with cysteine, and two after the treatment (Fig. 13). The peak before the cysteine treatment and the first peak after the treatment coincided with the native cytosol alkaline PP_iase. The second peak after the cysteine treatment, having the estimated molecular weight of 42,000, seemed to correspond to the air oxidized- and inactivated species of cytosol PP_iase which was renatured by reduction with cysteine. When the renatured slower moving fractions were applied to the same Sephadex G-100 column again, only one active peak came out in the tubes corresponding to the native PP_iase.

(viii) *Inactivation by Urea*—As shown in Fig. 14, the cytosol alkaline PP_iase was more sensitive to urea treatment than the mitochondrial enzymes. The urea concentrations required for 50% inactivation were 1.2 M for the cytosol enzyme and about 2 M for the mitochondrial PP_iases. The inactivation by urea was irreversible and the activities were not recovered by dilution with water.

DISCUSSION

Although the presence of alkaline PP_iase activities in the particulate fractions, in addition to that in the cytosol, of animal tissues has been reported (*17—19*), their enzymic properties have not been carefully compared. In our present investigation, three different alkaline PP_iases, one in the cytosol, two others in the mitochondrial fraction and none in other particulate fractions of rat liver, were detected first by polyacrylamide gel electrophoresis (Fig. 1) and further confirmed by partial purifications.

They were distinguished each other with respect to elution patterns from DEAE Sephadex A-50 column (Fig. 6), pH profile of

activity (Fig. 8), the effect of Mg^{2+} (Fig. 9), Michaelis constants (Fig. 11), substrate sepcificity (Table IV), and the sensitivity to urea (Fig. 14).

As to specificity of substrates other than PP_i, SCHLESINGER and COON (*28*) reported that yeast alkaline PP_iase catalyed phosphate liberation from nucleoside polyphosphates only in the presence of Zn^{2+} and Co^{2+}, and SOODSMA and NORDLIE (*19*) observed that rat heart cytosol alkaline PP_iase hydrolyzed phosphoanyhydrides other than PP_i or phosphate esters at much smaller rates than PP_i. Our preparation of cytosol PP_iase from rat liver hydrolyzed nucleoside polyphosphates at rates equal to about 3% of PP_i hydrolysis, but the mitochondrial enzyme (Mt. I and II) hydrolyzed these substrates at rates slightly greater than 30% of PP_i hydrolysis (Table IV). Since mitochondrial PP_iases had little activity with respect to hydrolysis of β-glycerophosphate or AMP, contamination by 5'-nucleotidase or nonspecific phosphomonoesterase does not seem a likely explanation for the greater rates of hydrolysis of nucleoside polyphosphates.

PP_iases from various sources had different requirements for activating metal ions. While yeast (*29*) and *E. coli* (*30*) required Mg^{2+}, less effectively Mn^{2+} or Co^{2+} for PP_i hydrolysis, and Zn^{2+}, Co^{2+} or Mn^{2+} for nucleoside polyphosphates hydrolysis (*29*, *30*), PP_iase from human erythrocytes (*25*) required Mg^{2+}, Co^{2+} or Mn^{2+} for PP_i hydrolysis. With rat liver PP_iase, Mg^{2+} was the only effective divalent cation for maximal enzyme activation, and other cations such as Zn^{2+} or Ca^{2+} were inhibitory. These observations agree well with that of KESSELRING and SIEBERT (*31*) for rat liver nuclear PP_iase. From our results with purified nuclei, however, it seems unlikely that rat liver nuclei have any alkaline PP_iase.

Molecular weights of rat liver alkaline PP_iases were estimated by the Sephadex G-100 filtration method to be about 68,000, which is very similar to that for crystalline PP_iase from yeast, 63,000 (*32*). The molecular weight of cytosol PP_iase inactivated by air oxidation decreased to about 48,000, which is slightly more than half that of the native protein. This may be interpreted to indicate that a drastic structural change occurred by air oxidation which was favorable for enzyme penetrating into or adhering to Sephadex particles, because the enzyme partially inactivated by air oxidation is reactivated by cysteine and the molecular weight decrease is not explained by the expected formation of S-S bond(s).

PYNES and YOUNATHAN (*25*) reported the renaturation of human erythrocyte alkaline PP_iase by 3 mM 2-mercaptoethanol. In our observation, cysteine-HCl was very effective for protecting the enzyme and for renaturing the inactivated enzyme. It was also observed that other SH compounds such as glutathione and 2-mercaptoethanol were also effective for this purpose. These observations together with the findings of enzyme protection in a nitrogen atmosphere and of molecular weight decrease after air oxidation indicate that some oxidizable group, probably SH-, plays very important roles for the enzymic activity and for the possible dissociation into subunit proteins. Irreversible inactivation by urea at a concentration as low as 1—2 M, also possibly indicates the presence of subunit structure in PP_iase.

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