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ABSTRACT

Inorganic pyrophosphatase (EC 3.6.1.1) has been purified to electrophoretic homogeneity from the soluble portion of the cytoplasm of rat Hepatoma 3924A and rat liver. It has a specific activity of 600 to 700 μmol inorganic orthophosphate liberated per min per mg protein at 25°, a value in the same range as the highly purified enzymes from yeast and *Escherichia coli*. By all criteria applied, the hepatoma inorganic pyrophosphatase is identical with the liver enzyme. It is a dimer with subunits with molecular weights of approximately 30,000 to 33,000 and has a pH optimum of 7.4, a K_m for pyrophosphate of 5 μM, and a K_a for Mg^{2+} of 0.3 mM with a pyrophosphate concentration of 0.2 mM. It is not inhibited by high Mg^{2+} concentrations up to 20 mM. Other metal ions such as Zn^{2+} and Ca^{2+} do not activate. Mn^{2+} activates to less than 10% that of Mg^{2+} at 0.6 mM and has no effect at 1 mM or higher. In the presence of optimal (4 mM) Mg^{2+} concentration, Ca^{2+}, Mn^{2+}, Hg^{2+}, and F^- at 0.2 mM inhibited strongly, but Zn^{2+} at 1 mM was not inhibitory.

The enzyme had no phosphatase activity toward any of the purine or pyrimidine nucleoside mono-, di-, and triphosphates or toward *p*-nitrophenyl phosphate, β-glycerophosphate, glucose 6-phosphate, or glucose 1-phosphate. Bromo- or iodoacetate at high concentration had no inhibitory effect, but *p*-chloromercuribenzoate and *p*-chloromercuriphenylsulfonate inhibited strongly at low concentration.

The purified enzyme was very unstable but was protected markedly at or above the pH optimum of 7.4 by cysteine, dithiothreitol, and glutathione.

INTRODUCTION

PP_i is a product of a wide range of reactions, including the biosynthesis of DNA, RNA, protein, and polysaccharide; a host of biochemically active intermediates; and the hydrolysis of nucleoside triphosphates (11, 17, 30, 32, 33). It must therefore be produced in large quantities, particularly in actively proliferating tissue. As part of a continuing study of the enzymatic basis of neoplastic behavior, we are focusing specific attention on PP_iase (EC 3.6.1.1.).

As a major if not sole mechanism for conversion of PP_i to P_i this enzyme plays a dual role: replenishing the P_i pool for the multitudinous metabolic reactions in which P_i is involved; and carrying to completion the endergonic reactions in which PP_i is formed (17). Considering its strategic importance, its potential regulatory role in P_i turnover has been relatively neglected; until our recent report on its activity in a series of rat and mouse neoplasms (32), virtually nothing was known of its properties and activities in tumors.

In the present study, we describe the purification of PP_iase from a poorly differentiated, rapidly growing Morris 3924A rat hepatoma, report data on some of its properties and kinetics, and compare it with a similarly purified PP_iase from rat liver.

MATERIALS AND METHODS

Normal male Sprague-Dawley rats were obtained from the Holtzmann Co., Madison, Wis., or Carworth Farms, New City, N. Y.; and ACI strain rats used for transplantation of Hepatoma 3924A were obtained from Laboratory Supply Corporation, Indianapolis, Ind. Hepatoma 3924A was originally obtained from Harold P. Morris but is now propagated in our laboratory by bilateral s.c. or i.m. implantation. Its properties have been described by Morris (21). In our colony, Hepatoma 3924A grows to a diameter of 1 to 2 cm in about 10 to 14 days, at which time rats were sacrificed by decapitation and the livers and tumors were dissected as described previously (32). To accumulate sufficient tissue for purification, generally 80 g, the tissue was either frozen immediately and stored at −20° for later homogenization or homogenized immediately in 4 volumes of 0.25 M sucrose containing 2 mM $MgCl_2$ and centrifuged 1 hr at 115,000 × *g*, after which the supernatant solution was stored at −20°. Under either condition, enzyme activity was nearly completely retained for at least 2 months. All subsequent steps were conducted as far as possible at 2 to 4°.

Enzyme Purification. Approximately 400 ml of liver or tumor supernatant solution equivalent to 80 g fresh tissue were adjusted to pH 6.2 in 25 mM histidine and brought to 50% saturation by addition of 135 g ammonium sulfate. After 20 min, the mixture was centrifuged for 25 min at 10,000 rpm, and the supernatant was brought to 70% saturation by addition of 65 g of solid ammonium sulfate. After the mixture was allowed to stand 1 hr, centrifugation was repeated, and the precipitate, containing approximately 74% of the total PP_iase activity, was transferred to a collodion bag (Schleicher and Schull Grade UH 100/25) and dialyzed against 350 ml 10 mM potassium phosphate, pH 7.2 with 2 changes, over a 16-hr period.

Gel Filtration. Approximately 15 ml (one-half volume) of the concentrated dialysate were applied to a 2.4- x 110-cm column of Ultrogel AcA44, previously equilibrated with the dialysis buffer. Protein was eluted with the same buffer at a flow rate of 30 ml per hr, and 3.2-ml fractions collected. As shown in Chart 1, the enzyme eluted in a single sharp peak, corresponding to a molecular weight 61,000. The recovery was 46% with a purification factor of 5.3. Elution was repeated with the second 15-ml fraction, and the 2 peak eluate fractions were combined.

Chromatography on ω-Aminohexyl Sepharose. The combined gel filtration eluates were concentrated to 3 ml by collodion bag dialysis and applied to a 1- x 12-cm column of ω-aminohexyl Sepharose 4B, previously equilibrated with a 10 mM potassium phosphate solution, pH 7.2, containing 10% glycerol. The column was washed with the same buffer and, after 40 ml of the enzyme were collected, the column was washed with 30 ml of the same buffer containing 50 mM KCl and then eluted with a linear concentration gradient of potassium pyrophosphate, pH 7.2, ranging from 0 to 25 mM in the same phosphate-KCl buffer. A total of 200 ml eluate was collected in 2.2-ml fractions. PP_iase

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eluted in a single, symmetrical band between 5 and 10 mM PP_i (Chart 2). Overall yield was 38% with specific activity of 68 units/mg and a purification factor of 115.

Hydroxylapatite Chromatography. The eluate was concentrated as above to 3 ml against a pH 6.8 buffer solution containing 10 mM potassium phosphate, 2 mM $MgCl_2$, and 10% glycerol and was applied to a 1.6- x 4.5-cm column of hydroxylapatite equilibrated previously with the same buffer. The enzyme was eluted with 200 ml of a linear gradient of potassium phosphate solution, pH 6.8, ranging from 0.01 to 0.40 M, and 1.8-ml fractions collected. The enzyme as shown in Chart 3 eluted between 0.08 and 0.12 M P_i. Sixteen % of the original enzyme was recovered with a specific activity of 630 units/mg protein, representing a 1070-fold purification (Table 1). The pattern of purification of the enzyme from normal rat liver was nearly identical. As shown in Table 1, a typical purification gave a yield of liver enzyme with a specific activity of 701 units/g and a purification factor of 531.

Assay. PP_iase was assayed as described previously (32) in 0.5 ml of 0.04 M histidine buffer, pH 7.2, containing 0.2 mM PP_i, 4 mM $MgCl_2$, and sufficient enzyme to give an absorbance change over the 10-min incubation period at 25° in the range of 0.2 to 0.5. The reaction was terminated by addition of 50 µl 0.6 M $KClO_4$, followed by 0.45 ml of molybdate-malachite green color reagent. After standing for 10 min at 0° for color development, samples were read in a 1-cm cuvet at 660 nm with a Gilford spectrophotometer against a water blank, and no-substrate blanks with absorbances of 0.03 to 0.04 were subtracted.

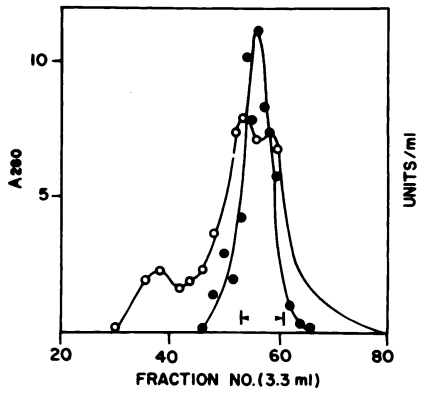

Chart 1. Gel filtration of Hepatoma 3924A PP_iase on Ultrogel AcA44. ○, absorbance at 280; ●, enzyme activity, units/ml. Fractions between the *vertical lines* were pooled for the next step.

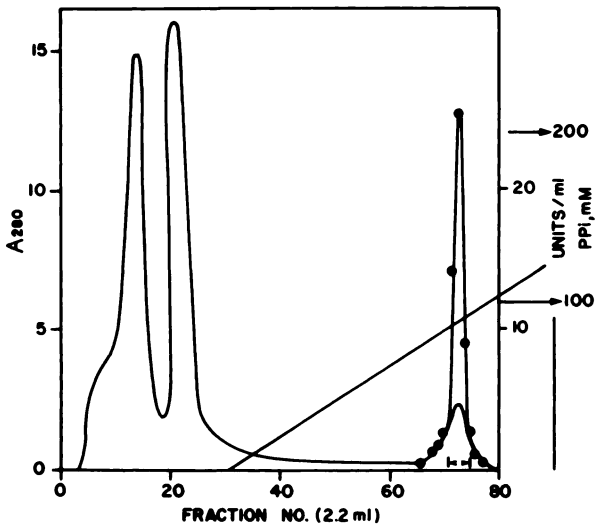

Chart 2. Chromatography on ω-aminohexyl Sepharose 4B. *Curved line*, absorption at 280 nm; ●, enzyme in units/ml; *straight line*, PP, concentration. *Vertical lines* enclose the enzyme fraction used for the next step.

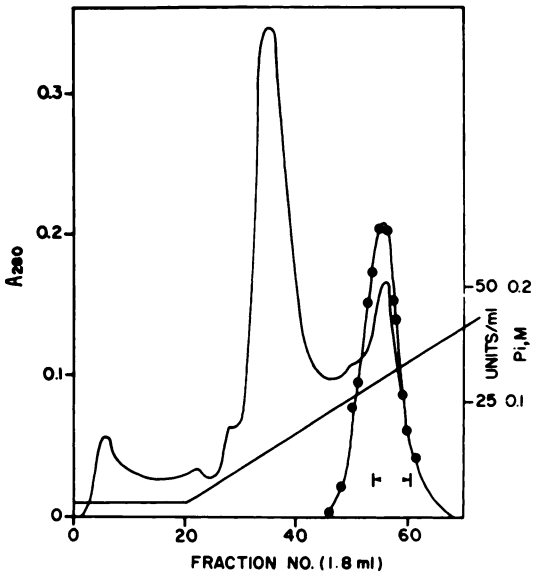

Chart 3. Chromatography on hydroxylapatite. *Solid line*, absorbance at 280 nm; ●, PP_iase activity. The material enclosed by the *vertical lines* had a specific activity of 630 units/mg protein.

Table 1

Purification of rat liver and Hepatoma 3924A PP_iase

Starting material in each instance was a 105,000 × *g* supernatant solution obtained from a 1:5 (w/v) homogenate prepared from 80 g fresh tissue in 0.25 M sucrose. A unit is equivalent to 1 µmol P_i liberated per min at 25°.

	Total protein (mg)	Total units	Specific activity (units/mg protein)	Recovery (%)	Purification (-fold)
Normal liver					
Supernatant	6602	8724	1.32	100	1
Ammonium sulfate (0.5–0.7 saturated)	2099	6575	3.13	75	2.4
Ultrogel	480	5190	10.8	60	8.2
ω-Aminohexyl Sepharose	14.7	3674	250	42	189
Hydroxyapatite	3.8	2648	701	30	531
Hepatoma 3924A					
Supernatant	3984	2335	0.59	100	1
Ammonium sulfate (0.5–0.7 saturated)	944	1728	1.83	74	3.1
Ultrogel	350	1084	3.10	46	5.3
ω-Aminohexyl Sepharose	13.0	885	68.1	38	115
Hydroxyapatite	1.02	643	631	28	1070

Activities are given in units, µmol P_i formed per min, and specific activity is given in units per mg protein. Protein concentrations were determined according to Waddell (35) by difference in absorption at 215 and 225 nm, using bovine serum albumin as the standard. Molecular weight was estimated according to the method of Andrews (1) by gel filtration through Sephadex G-100 in a 1- x 110-cm column eluting with 10 mM potassium phosphate, pH 7.0, containing 2 mM $MgCl_2$ and 0.06 M KCl. Marker proteins were bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin, and RNase.

RESULTS

The PP_iase, as purified from Morris Hepatoma 3924A, had a specific activity of approximately 600 to 700 units/mg protein and gave a single sharp band on polyacrylamide gel electrophoresis carried out according to the method of Davis (7), with

10 μg enzyme and 2 mM $MgCl_2$ in the gel and stock buffers. The molecular weight of the native enzyme determined by gel filtration on Sephadex G-100 according to the report of Andrews (1) was 60,300, and that for the liver enzyme was 61,000. Electrophoresis carried out in 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate according to the report of Weber (38) yielded a subunit molecular weight of 33,000, in accord with other data (8, 13, 26).

pH Optimum. As shown in Chart 4, the pH optimum of the Hepatoma 3924A enzyme was 7.4. The activity at pH 5.8 was only 5% of the optimum but rose steeply to reach 70% of the optimum at pH 7.0. The decline was more gradual on the alkaline side, falling to about 40% of the optimum at pH 9.5. Essentially the same pattern was found for the purified rat liver enzyme.

Substrate Concentration. With a large excess of Mg^{2+} and PP_i concentrations ranging from 2.5 to 25 μM, the reciprocal plot, $1/V$ *versus* $1/S$, was linear, indicating a K_m of approximately 5 μM for both liver and hepatoma enzymes (Chart 5).

Mg^{2+} Concentration. These enzymes have an absolute requirement for Mg^{2+}. With a saturating concentration of PP_i at 0.2 mM, the concentration of Mg^{2+} for half-maximal activity was about 0.3 mM and was nearly optimal at 1 mM (Chart 6). Higher concentrations of Mg^{2+}, up to 20 mM under the same conditions, were not inhibitory.

Other Metal Ions. Chart 6 reveals that at a concentration of 1 mM, at which Mg^{2+} activates nearly optimally, other metal ions were ineffective. Mn^{2+} at 0.6 mM was weakly active, exhibiting less than 10% of that of Mg^{2+} at the same concentration, but higher concentrations were much less active. Activation by Ca^{2+} and Zn^{2+} was hardly detectable at concentrations ranging from 0.1 to 4 mM.

Effects of Metal Ions on PP_i, ATP, and *p*-Nitrophenyl Phosphate as Substrates for Hepatoma 3924A PP_iase. The experiments in Table 2 were conducted to assess the substrate

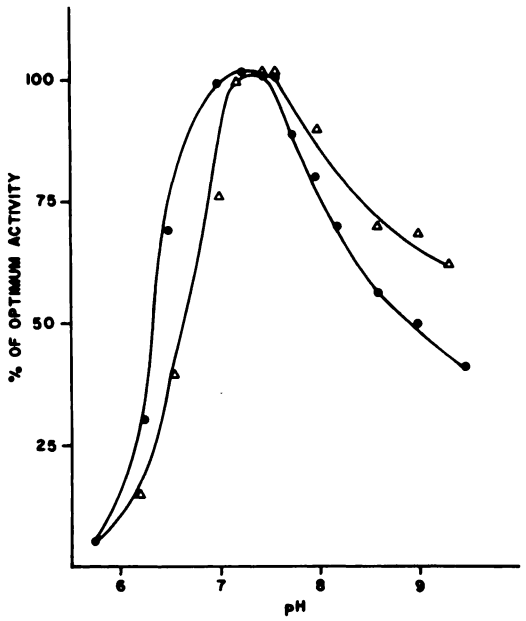

Chart 4. Effect of pH on enzyme activity. The assay medium contained 0.2 mM pyrophosphate, 40 mM histidine, and 4 mM $MgCl_2$ adjusted to the designated pH, and the reading began by addition of an appropriate dilution of the enzyme. △, Hepatoma 3924A; ●, liver.

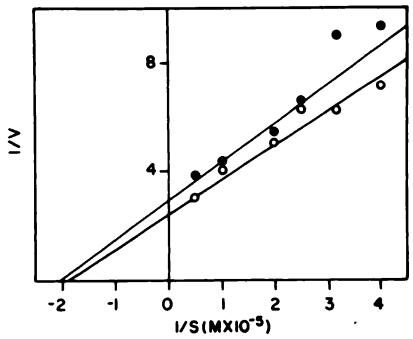

Chart 5. Plot of $1/V$ *versus* $1/S$ for Hepatoma 3924A and rat liver. Using 4 mM $MgCl_2$ in our regular assay system, the PP_i concentration was varied from 2 to 22.5 μM. ●, Hepatoma 3924A; ○, rat liver.

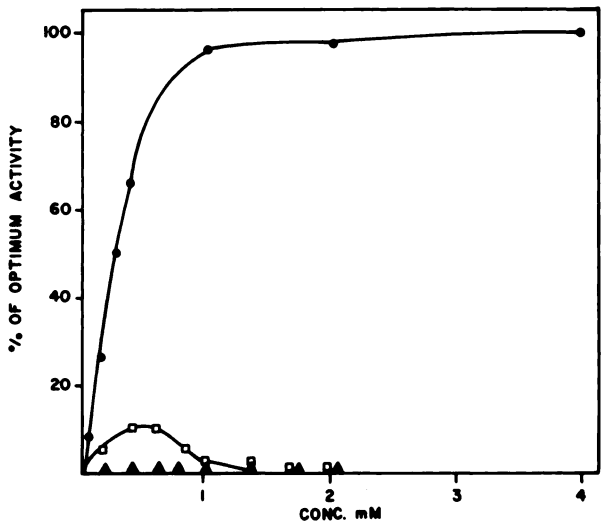

Chart 6. Effects of Mg^{2+}, Mn^{2+}, and Zn^{2+} on Hepatoma 3924A PP_iase activity. In our regular assay system, the metal ion concentration was varied from 0.1 to 4 mM. Activity is expressed as percentage of optimum activity with 4 mM Mg^{2+}. ●, $MgCl_2$; □, $MnCl_2$; ▲, $ZnCl_2$.

specificity of the purified enzyme and the effects thereon of various metal ions. Under our optimal assay conditions, the enzyme is virtually inactive toward ATP as substrate. It is also inactive toward *p*-nitrophenyl phosphate at the optimal pH of 10.4 for alkaline phosphatase. With 4 mM Mg^{2+} present, Ca^{2+} and Mn^{2+} at 0.2 and 0.4 mM inhibited the PP_iase to 14 to 30% of control activity. In contrast, Zn^{2+} was not inhibitory at 1 mM and inhibited 60% at 5 mM. With Mg^{2+} absent, neither Ca^{2+}, nor Mn^{2+}, nor Zn^{2+} at 4 mM activated PP_iase or alkaline phosphatase activity, and ATPase activity of PP_iase was questionable at the borderline of measurement. The enzyme is obviously highly specific for PP_i as substrate and for Mg^{2+} as activator.

Substrate Specificity. Further data on substrate specificity shown in Table 3 reveal that the enzyme has no appreciable organic phosphatase activity under the assay conditions used. None of the mono-, di-, or triphosphates of adenosine, guanosine, uridine, or inosine exhibited any significant activity when assayed at 0.2 mM, nor was there any significant alkaline phosphatase activity when assayed at pH 10.4 with 1 mM *p*-nitrophenyl phosphate or β-glycerophosphate. Other inactive substrates were glucose 6-phosphate and glucose 1-phosphate at 1 mM and inorganic tripolyphosphate and trimetaphosphate at 0.2 mM. Possible pyrophosphate analogs, meth-

ylene diphosphonate and aminomethylphosphonate, did not inhibit, and the carbon analogs oxalate, malonate, and glycolate exhibited minor if any inhibition at 0.5 or 5.0 mM.

Effect of Temperature on Reaction Rate of Hepatoma and Liver PP_iases. Pynes and Younathan (26) observed a biphasic curve for log *v versus* $1/T$ with an inflection at 29° with a partially purified rat erythrocyte PP_iase. Using our regular 10-min assay, we also obtained a similar biphasic curve with an inflection at 28°. However, at the higher temperature, our rates deviated from linearity with time, a result we attributed to instability of the enzyme. By lowering the incubation time to 5 min and taking care to minimize exposure of the enzyme to higher temperatures, the log *v versus* $1/T$ plots were linear, as shown in Chart 7, and gave essentially identical activation energies for the tumor and liver enzymes of 9,600 and 10,200 calories/mol, respectively.

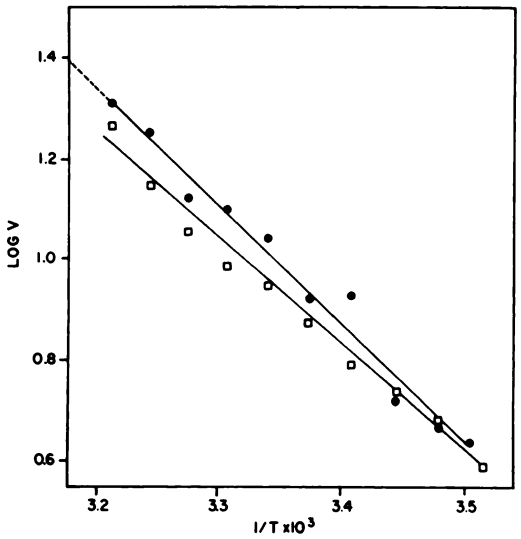

Chart 7. Plot of log *v versus* $1/T$ over the range, 40° to 13°. Reaction was initiated by addition of enzyme to regular assay medium and terminated after 5 min incubation at the designated temperature. ●, rat liver; □, Hepatoma 3924A.

Table 2

Activity of Hepatoma 3924A PP_iase toward PP_i, ATP, and p-nitrophenyl phosphate and effects thereon of metal ions

PP_i and ATP hydrolysis were measured with 0.2 mM substrate and 4 mM metal ion under our optimal conditions for PP_i assay in 50 mM histidine buffer, pH 7.2. Alkaline phosphatase activity was measured with 0.2 mM *p*-nitrophenyl phosphate and 4 mM metal ion in a 50 mM glycine-NaOH buffer at pH 10.4. Values are in percentage of PP_iase activity in the presence of 4 mM Mg^{2+}.

	Added ion		% of PP_iase activity with Mg^{2+} alone		
Mg^{2+} (mM)	Ion	Concentration (mM)	PP_i	ATP	*p*-Nitrophenyl phosphate
4			100	1.4	0.7
4	Ca^{2+}	0.2	30		
4	Ca^{2+}	0.4	20		
4	Mn^{2+}	0.2	29		
4	Mn^{2+}	0.4	14		
4	Zn^{2+}	1.0	100		
4	Zn^{2+}	5.0	40		
0	Ca^{2+}	4.0	0.4	0.3	0.0
0	Mn^{2+}	4.0	0.0	1.7	0.0
0	Zn^{2+}	4.0	0.2	0.5	0.0

Table 3

Substrate specificity and inhibitory effects on activity of purified Hepatoma 3924A PP_iase

Substrates were added to our regular assay system with 4 mM Mg^{2+} and were tested for inhibitory effects by adding to the regular assay system containing 0.2 mM PP_i. Values are given in percentage of control values. Concentrations were 0.2 mM except where indicated in parentheses.

	Activity (% of PP_iase activity toward 0.2 mM PP_i)	
Substrate	As substrate	As inhibitor
PP_i	100	100
AMP, ADP, ATP	0.3 ± 0.3[a]	100 ± 1
GMP, GDP, GTP	0.0 ± 0.1	101 ± 1
UMP, UDP, UTP	0.3 ± 0.2	104 ± 2
IMP, IDP, ITP	0.4 ± 0.2	
p-Nitrophenyl phosphate (1.0 mM)	0.2	104
Glucose 1-phosphate (1.0 mM)	0.2	
Glucose 6-phosphate (1.0 mM)	0.1	
β-Glycerophosphate (1.0 mM)	0.1	
Tripolyphosphate	0.2	
Trimetaphosphate	0.5	
Methylenediphosphonate (0.5 mM)		120
Methylenediphosphonate (5.0 mM)		71
Aminomethyl phosphonate (0.5 mM)		94
Aminomethyl phosphonate (5.0 mM)		94
Oxalate (0.5 mM)		105
Oxalate (5.0 mM)		71
Malonate (0.5 mM)		100
Malonate (5.0 mM)		76
Glycolate (0.5 mM)		130
Glycolate (5.0 mM)		99
Urea (2 M)		80

[a] Mean ± S.E.

Further data on inhibitory effects of various ions and SH[3] reagents are summarized in Table 4 in terms of concentrations giving 50% inhibition of PP_iase activity under our optimal assay conditions. Ca^{2+}, Mn^{2+}, and F^- inhibited 50% at 65 to 70 μM whereas Zn^{2+} was very much less inhibitory. The effect of Hg^{2+} ions was time dependent. When added immediately before assay, 50% inhibition occurred at 0.4 mM; but when added 10 min prior to assay, inhibition was essentially complete and was partially protected by prior incubation with PP_i. (Effects of Hg^{2+} are complex and are under continued study.) The haloacetates, even with prior 5-min incubation, did not inhibit appreciably; however, both *p*-chloromercuribenzoate and *p*-chloromercuriphenylsulfonate inhibited strongly at very low concentrations.

Stability of Liver and Hepatoma PP_iase. Although the enzyme was reasonably stable in whole, concentrated liver or tumor cytosol (32), the purified enzyme was very unstable; at 25° and at pH 7.5, activity was lost very rapidly. With 4 mM Mg^{2+} present in our usual assay system, 64% of initial activity was lost in 1 hr and about 90% in 6 hr. Activity under the same conditions was protected by 0.5 mM GSH or DTT but the loss was still about 40% in 1 hr. Loss of activity also occurred at 0°, to about 40% in 6 hr, and there was no protection by the same SH compounds. To examine the protective effect of GSH and DTT in more detail, the purified enzyme was subjected to a range of pH from 5.2 to 10.0 for 10 min at 25° and then brought to pH 7.2 and assayed. The data collected in Chart 8 reveal that the stability is highly pH dependent. Activity was most stable at pH 7.5, with 20% loss in 10 min. At pH 6.0, however, 80% was lost, and at pH 9.0 85% was lost during 10 min at 25°. In the presence of 2 mM DTT, complete protection occurred at pH 7.5, and activity was optimal at pH 9.0. Protection by DTT was marked even at pH 10, but there was little or

[3] The abbreviations used are: SH, sulfhydryl; GSH, glutathione; DTT, dithiothreitol.

Table 4

Inhibitors of purified PP_iase activity of Hepatoma 3924A

Inhibitors were added to the regular assay system containing 0.2 mM PP_i and 4 mM Mg^{2+} in concentrations spanning the range of inhibitory effects. The values given are the concentrations giving 50% inhibition. Results were identical for the tumor and liver enzymes.

Inhibitor	Concentration for 50% inhibition (mM)
Ca^{2+}	0.06
Mn^{2+}	0.07
Zn^{2+}	3.8
F^-	0.07
Hg^{2+} (immediate)	0.4
Hg^{2+} (prior 10-min incubation)	0.00002
Hg^{2+} (prior 10-min incubation with 0.2 mM PP_i)	0.0001
Iodoacetamide (prior 5-min incubation)	80
Iodoacetate (prior 5-min incubation)	15
Bromoacetate (prior 5-min incubation)	15
p-Chloromercuribenzoate (prior 10-min incubation)	0.0005
p-Chloromercuriphenyl sulfonate (prior 5-min incubation)	0.0001

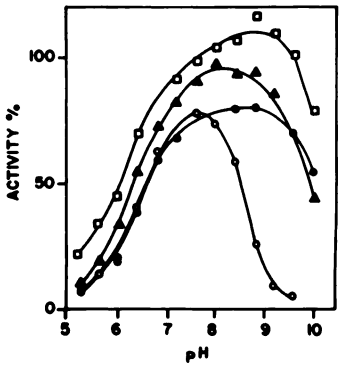

Chart 8. Protective effects of reducing agents on thermal inactivation of Hepatoma 3924A PP_iase. Where indicated, the reducing agent was added at 2 mM to the assay medium containing 0.1 M histidine adjusted to the designated pH and 5 mM $MgCl_2$ at 25°. After 10 min, samples were removed and assayed by our regular procedure at pH 7.2. Activity is expressed as percentage of activity obtained at pH 7.2 without incubation. ○, no addition; □, DTT; ▲, cysteine; ●, GSH.

no protection by DTT on the acid side. Essentially the same pattern of protection was observed for L-cysteine. Although protection by GSH was not apparent at pH 7.5, it markedly enhanced the stability at higher pH. It is clear from these data that all 3 thiols protect the enzyme, and the effect is most marked at high pH.

DISCUSSION

The purified rat liver and Hepatoma 3924A have specific activities of 600 to 700 units/mg protein and appear to be reasonably homogeneous. This range of activity is within those of the highly purified and crystallized *Escherichia coli* (14) and yeast PP_iases (6, 15, 28, 35, 36), which are reported to range between 600 and 1300 units/mg. PP_iase from rabbit muscle was also purified to electrophoretic homogeneity by Morita and Yasui (20). Their preparation also had a specific activity of 680 units/mg protein, thus pointing to this level of activity as a common feature of PP_iases of diverse origin. Since the crude liver cytosol had a specific activity of 1.57 units/mg, we estimated that 1.57/600 = 0.26% of the liver cytosol protein is PP_iase. Similarly estimated, the 3924A tumor PP_iase constitutes 0.14% of the cytosol protein. Josse and Wong (14) reported that PP_iase is 0.2% of the *E. coli*-soluble protein, and from data of Kunitz (18) it is about 1% of the yeast-soluble protein. However, the enzyme from rabbit muscle estimated from the data of Morita and Yasui (20) is only 0.014% of the soluble protein.

PP_iase has also been partially purified from rat liver (13), human erythrocytes (10, 26, 34), pig scapula cartilage (8, 9), chick aorta (5), and pig brain (31) to specific activities ranging up to <100 units/mg protein. The enzyme was also purified partially from *Rhodospirillum rubrum* (16).

The purified rat liver and hepatoma enzymes were identical by all criteria, and from our previous study (32), which demonstrated identical electrophoretic migration for the PP_iases of many normal and neoplastic rat tissues, we conclude that there is a single major cytosolic form in the rat accompanied by 2 minor forms.[4] In nearly all respects, the rat liver and hepatoma enzymes closely resembled the purified rabbit muscle enzyme. The molecular weights were similar at 60,000 to 67,000, and the K_m values for PP_i at 5 μM and the pH optima at 7.5 were identical. Both were labile and were protected somewhat by DTT and cysteine and inhibited by *p*-chloromercuribenzoate and F^-. A few differences were observed and remain to be resolved; for example, the muscle PP_iase was inhibited competitively by ATP and *p*-nitrophenyl phosphate, whereas the liver and hepatoma enzymes were unaffected.

In most respects, the less purified PP_iases from other tissues were also closely similar, with an absolute requirement for Mg^{2+}, a dimeric structure, lability protected partially by thiols, and inhibition by *p*-chloromercuribenzoate, F^-, and heavy metals. Although generally higher K_m values for PP_i were reported, up to as high as 140 μM, these variations are not entirely surprising in view of many differences in enzyme purity and assay methodology.

Some differences were apparent from the soluble PP_iase reported by Irie *et al.* (13).[5] They reported a 10-fold higher K_m for PP_i and appreciable activities toward nucleoside di- and triphosphates in contrast with the high substrate specificity we observed for the rat liver and tumor PP_iase.

Effect of Mg^{2+}. A significant feature of all of the animal PP_iases thus far studied is the absolute requirement for Mg^{2+}. As first discovered by Bloch-Frankenthal (2), Mg^{2+} is required in excess for optimal activity, it is generally agreed that free PP_i is a potent inhibitor, and Mg^{2+} both activates the enzyme and ligates with PP_i to yield the true substrate, $MgPP_i^{2-}$ (5, 12, 13, 15, 16, 26, 34, 37). The purified liver and tumor enzymes as shown in Chart 6 were inhibited by free PP_i and also showed high specificity for Mg^{2+} activation. With 0.2 mM PP_i, activity was half-maximal at 0.3 mM Mg^{2+}. At these concentrations, essentially all of the PP_i is in the form of $MgPP_i^{2-}$. This is established by reference to the complexity constants deter-

[4] Electrophoresis on agarose gels at pH 7.2 has revealed that rat normal and neoplastic tissues give identical PP_iase migration patterns, a major form migrating to the anode, a minor anodic form that migrates more slowly, and a minor cathodic form.

[5] Irie *et al.* (13) found 2 minor PP_iases in the mitochondria in addition to the major cytosol enzyme. Others have also reported enzyme activity in particulate fractions, notably in rat liver nuclei (15), mitochondria (23), and microsomes (24) [later identified with glucose-6-phosphatase (25) and *R. rubrum* membranes (27)]. In work to be published, we found that 97 to 98% of the total rat liver and Hepatoma 3924A PP_iases was in the cytosol and that no appreciable activity could be extracted from the particulate fractions other than what could be accounted for as entrained cytosolic enzyme. According to the report of Waked and Kerr (36), essentially all of the PP_iase in dog brain was found in the cytosol after systematic cell fractionation.

mined by Lambert and Watters (19), from which the concentrations of the 2 major forms, $MgPP_i^{2-}$ and $Mg_2PP_i^0$, can be calculated (4, 22, 29, 34) for various ratios of total Mg^{2+} and PP_i. At high Mg^{2+} to PP_i ratios of 20, as in our regular assay procedure, activity was not inhibited; nor even when the Mg^{2+} concentration reached 10 to 20 mM (15, 16, 32, 37). At these concentrations, the preponderant species is $Mg_2PP_i^0$. From the lack of inhibition at these high Mg^{2+} concentrations, it can be inferred that both $MgPP_i^{2-}$ and $Mg_2PP_i^0$ are active substrates. Kinetic studies on the yeast enzyme (3) also indicate that both forms are substrates; but studies of the *E. coli* enzyme (14), which differs in many respects from the yeast enzyme, indicate that $MgPP_i^{2-}$ is a sole substrate and that $Mg_2PP_i^0$ is either inactive or weakly inhibitory. The yeast and *E. coli* PP_iases differ not only from each other but differ in many respects from the animal PP_iases, notably in stability, metal requirements, and substrate specificity (3, 14).

—SH Groups. Preliminary data on the powerful inhibitory action of *p*-chloromercuribenzoate and Hg^{2+} and protection by —SH reagents and partial protection of Hg^{2+} inhibition by PP_i suggest the presence of —SH groups at the active site. However, neither iodoacetamide nor iodoacetate nor bromoacetate were inhibitory at low concentration. The nature of the active site as well as other properties of the enzyme require further study.

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