PURIFICATION AND PROPERTIES OF AN ACID PHOSPHOMONOESTERASE FROM *NEUROSPORA CRASSA*

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SUMMARY

An acid phosphomonoesterase was purified 1400-fold from mycelium of *Neurospora crassa* with a 40 % recovery. The enzyme had a pH maximum of 5.6 with β-glycerol phosphate or glucosamine 6-phosphate as substrates, and cation or cofactor requirements could not be demonstrated. The substrate specificity of the enzyme was studied, using 46 compounds, α-glycerol phosphate being hydrolyzed most rapidly. When a free amino group and a phosphomonoester group were attached to adjacent carbon atoms, the compound either would not serve as a substrate or was slowly hydrolyzed. Deoxyguanosine 5'-phosphate and thymidine 5'-phosphate were not hydrolyzed although all other nucleoside monophosphates tested were able to serve as substrates. Fluoride and (+)tartrate were competitive inhibitors for the hydrolysis of β-glycerol phosphate, while concentrations of thyroxine that completely inhibited the hydrolysis of acetyl phosphate did not affect the hydrolysis of β-glycerol phosphate or *p*-nitrophenyl phosphate. No evidence was obtained for the presence of more than one phosphomonoesterase in the purified enzyme preparation. These properties distinguish this enzyme from previously described microbial phosphomonoesterases. The possibility of this acid phosphomonoesterase participating in metabolic control systems in *N. crassa* was discussed.

INTRODUCTION

Although acid phosphomonoesterases have been recognized in cells from animal, plant and microbial sources, relatively little is known about the properties, particularly the substrate specificity, of the enzymes derived from microbial sources. During the course of a study on hexosamine metabolism in the filamentous fungus *Neurospora crassa*, an acid phosphomonoesterase was discovered and the enzyme was partially purified[1]. There have been other observations of the presence of an acid phosphatase in crude extracts of *N. crassa*[2,3], but the enzyme was not purified by these investigators. The present report is concerned with the preparation of a 1400-fold purified *N. crassa* acid phosphomonoesterase and a description of its properties, which clearly differentiate it from other microbial phosphomonoesterases. Furthermore, a possible regulatory role for this enzyme in cellular metabolic processes is discussed.

Abbreviation: CTAB, cetyltrimethylammonium bromide.

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EXPERIMENTAL

Materials

The commercial compounds used were of the highest purity obtainable: purified sodium fructose 1,6-diphosphate (Sigma Chemical Co.); β-glycerol phosphoric acid, disodium salt with maximum α content of 0.1 %, and N,N-diethylaminoethyl cellulose "DEAE-cellulose" (Eastman Kodak Co.); crystalline thyroxine (Squibb). Glucosamine 6-phosphate was synthesized by the method of DISTLER *et al.*[4] and potassium phosphoramidate was prepared by the procedure of STOKES[5]. We are greatly indebted to the following investigators for these materials: glucosamine 1-phosphate and acetylglucosamine 1-phosphate, Dr. S. ROSEMAN; histidinol phosphate, Dr. B. AMES; 2,3-diphosphoglyceric acid, Dr. S. GRISOLIA; glucose 2- and 3-phosphates, Sir A. TODD; polyxenylphosphate, Dr. J. E. HUMMEL; bis-(p-nitrophenyl)-phosphate, Dr. I. A. BERNSTEIN; lithium acetylphosphate, Dr. P. SRERE; O-phosphohomoserine, Dr. M. FLAVIN; a number of nucleotides, Dr. R. POTTER; and β-napthylphosphate, Dr. R. L. HUNTER.

Analytical methods

Inorganic phosphate was determined colorimetrically by either the method of FISKE AND SUBBAROW[6] or the method of PEEL AND LOUGHMAN[7]. The latter method, modified to maintain the pH at 4.0 by a fourfold increase in the amount of acetate buffer, was used to assay Pi in the presence of acid-labile substrates. With p-nitrophenylphosphate as substrate, the p-nitrophenol liberated was measured[8]. Protein was routinely determined by measurement of the absorbancy of solution at 215 and 225 mμ by the method of WADDEL[9]. This method agreed within ± 5 % with the results obtained from micro-Kjeldahl analysis of representative fractions. Protein was also estimated in chromatographic fractions by the less sensitive method of WARBURG AND CHRISTIAN[10].

Enzyme assay

Enzymic activity was routinely measured in a system with a final volume of 0.4 ml containing 8 μmoles of sodium β-glycerolphosphate (pH 5.6) 20 μmoles of acetate buffer (pH 5.6) and enzyme. The incubation was carried out for 20 min at 30° and the reaction was stopped by addition of 0.1 ml of 10 % trichloroacetic acid. Any precipitate formed was removed by centrifugation and the supernatant fluid was used for the determination of Pi. The routine control was a complete assay tube to which the trichloroacetic acid had been added at zero time. When acid-labile substrates were used in the assay, the control tube contained substrate, buffer and boiled enzyme. At the end of the 20-min incubation period, the trichloroacetic acid was added and the Pi was assayed in the usual manner. The proportionality of Pi released with increasing enzyme concentration or time is shown in Fig. 1. A unit of enzyme activity was defined as the quantity of enzyme that would catalyze the liberation of 1 μmole of phosphorus/min under the conditions specified. Specific activity is defined as the enzyme units/mg of protein.

Growth of Neurospora

Neurospora crassa 5297a (wild type) was maintained on potato–dextrose agar (Difco). A loopful of conidia was inoculated into a flask containing 100 ml of FRIES'

minimal medium[11] and incubated at least 48 h at 30° on a rotary shaker. The flask contents were blended aseptically for 30 sec in a Waring blendor and inoculated into a carboy containing 15 l of the same medium; growth was allowed to proceed at 30°

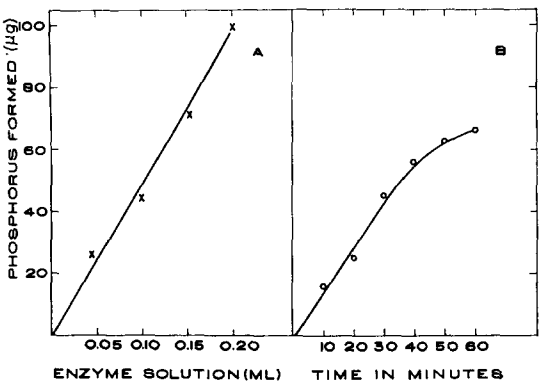

Fig. 1. Reaction rates as a function of enzyme concentration, A, and reaction time, B. In A, the reaction mixtures contained the designated volumes of diluted enzyme, purified through step 6, 8 μmoles of sodium β-glycerolphosphate (pH 5.6) and 20 μmoles acetate buffer (pH 5.6) in a final volume of 0.4 ml. The reaction time was 20 min for the results shown in A and 0.05 ml of enzyme solution was used for the results in B.

for 3 days with vigorous aeration. The mycelium was collected on several layers of cheese-cloth, washed twice with distilled water, squeezed dry, and stored at —20°. The acid phosphomonoesterase activity in the mycelia remained unchanged under these conditions over a period of several years. The wet-weight yield of mycelium from a carboy varied between 300 and 350 g. No phosphomonoesterase activity was observed in the culture medium at any time during this growth period.

Purification of enzyme

Unless otherwise specified, all operations were carried out in a cold room at 2–4° and deionized, glass-distilled water was routinely used.

Step 1. Preparation of crude extract: Frozen mycelia, washed (the beads were first washed in concentrated sulfuric acid and then rinsed with distilled water) glass beads (Superbrite, type 130, Minnesota Mining and Manufacturing Co.), and cold water in a ratio of 1:2:4 (w/w/v) were minced for 5 min in a Waring blendor. Cell debris and glass beads were subsequently removed by centrifugation for 10 min at 12 000 × *g*, yielding a milky supernatant fluid. Generally 60 g frozen mycelium were treated in this manner at one time. This process was repeated separately until the 3360 g of mycelium were extracted and the extracts were combined for further purification.

Step 2. Protamine sulfate treatment: Cold 2 % protamine sulfate (Eli Lilly and Co.), 1.4 l, was slowly added with stirring to 14 l of crude extract and allowed to stand in the cold for 10 min. The precipitate which formed was removed by centrifugation and the resultant supernatant solution, which was clear with a yellowish hue, contained all of the acid phosphatase activity.

Step 3. Ethanol fractionation: Cold absolute ethanol (7025 ml) at —20° was slowly added to 14050 ml of the supernatant fluid from Step 2 maintained at 2° while

gently mixing and allowed to stand for 2 h. The precipitate that formed was removed by centrifugation and discarded. An additional 21 075 ml of cold absolute ethanol was added to the supernatant fluid, bringing the ethanol concentration to 66 % (v/v), and the mixture was again allowed to stand for 2 h without further mixing. A large portion of clear supernatant was removed by siphoning and discarded, and the remaining precipitate was collected by centrifugation for 15 min at 10 400 × *g*. The precipitate was washed twice with 75 % ethanol and the ethanol washes were discarded. Then 250 ml of water was added to the precipitate and the enzyme was extracted into the water. The supernatant solution remaining after centrifugation was saved and this extraction process with water was repeated on the precipitate an additional three times. The aqueous extracts were combined and dialyzed overnight against 10 l of 0.02 *M* acetate buffer (pH 5.6) using dialysis tubing that was previously soaked for 30 min in 0.05 *M* EDTA and then rinsed in water.

Step 4. Selective precipitation with CTAB: The dialyzed enzyme from Step 3 was adjusted to pH 6.0 with cold 0.1 *N* NaOH, and an equal volume of a cold 1 % suspension of CTAB* was added slowly with gentle stirring, and allowed to stand for 1 h. The enzyme, which was completely precipitated by CTAB, was removed by centrifugation for 15 min at 6000 × *g*, leaving more than 90 % of the anthrone-reacting carbohydrate (as glucose) in the supernatant fluid. The enzyme was subsequently released from the insoluble CTAB–enzyme complex by stirring after addition of 80 ml of acetate buffer (0.1 *M*, pH 4.0) to the precipitate. The insoluble precipitate that was removed by centrifugation for 10 min at 30 000 × *g* was discarded, and the supernatant fluid was dialyzed overnight against 2 l of 0.02 *M* acetate buffer (pH 4.0). There usually was a trace of CTAB remaining in the dialyzed enzyme, at this point, that was subsequently completely removed by Step 6.

Step 5. Removal of inactive proteins by saturation with ammonium sulfate: The dialyzed enzyme from step 4 (120 ml) which contained approx. 8 mg of protein/ml, was adjusted to pH 6.0 with 0.1 *N* NaOH solution, and 84 g of finely powdered ammonium sulfate were slowly added while gently stirring until final saturation with ammonium sulfate was reached. After standing for 1 h in an ice-bath, the precipitate was removed by centrifugation for 20 min at 15 000 × *g*. The supernatant fluid, which contained most of the enzyme activity, was dialyzed against three changes of 0.02 *M* acetate buffer (pH 5.6), 10 l each, over a period of 18 h. The final volume after dialysis was about 480 ml.

Step 6. Fractionation on a DEAE-cellulose column: The dialyzed enzyme from Step 5 was concentrated to about 10 ml by lyophilization and dialyzed for 3 h against 0.02 *M* acetate buffer (pH 5.6). DEAE-cellulose that had previously been washed with 1 % NaOH in 10 % NaCl solution, and equilibrated with 0.02 *M* acetate buffer (pH 5.6) was used to pack a column 18.7 × 160 mm. The dialyzed enzyme was placed on the column and the column was washed with 100 ml of 0.02 *M* acetate buffer (pH 5.6); enzyme elution was accomplished with a gradient of increasing acetate buffer of the same pH. The concentration of acetate buffer in the top reservoir was 0.3 *M* (pH 5.6), while the mixing bottle contained 120 ml 0.02 *M* acetate buffer of the same pH. The rate of elution was about 30 ml/h and 9-ml samples of the eluate were collected with a fraction collector. Each fraction was assayed for the presence of protein and

* 1 % CTAB was not completely soluble at this temperature; the turbid suspension was used.

acid phosphatase activity with β-glycerolphosphate as substrate. In some experiments, the fractions were also assayed using glucosamine 6-phosphate as a substrate. Three major protein peaks were generally observed with all the phosphatase activity for

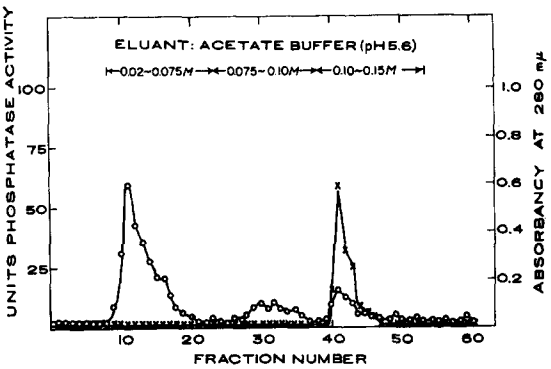

Fig. 2. Chromatography of *Neurospora* acid phosphomonoesterase, purified through step 5, on a DEAE-cellulose column. After adsorption of the enzyme on the column, it was washed with 100 ml of 0.02 *M* acetate buffer (pH 5.6). The column was then eluted with a gradient of increasing molarity of pH 5.6 acetate buffer, as described in the text. The molarity of the buffer was calculated and is shown at the top of the figure. The phosphomonoesterase peak was eluted when the buffer was about 0.1 *M*. In the figure, O—O, indicates the curve for protein; ×—×, indicates the curve for acid phosphomonoesterase with β-glycerolphosphate as the substrate.

either substrate residing in the last peak (Fig. 2). The appropriate tubes (40–45) were combined and lyophilized. The lyophilized powder was dissolved in a small volume of water, dialyzed in an EDTA-washed dialysis tube against 0.01 *M* acetate buffer (pH 5.6), and stored at —20°. Such preparations could be stored at least 6 months without any significant loss of enzymic activity. The results of a typical preparation, in which the enzyme was purified 1400-fold with a recovery of 40% of the enzyme activity, are summarized in Table I.

TABLE I

PURIFICATION OF ACID PHOSPHATASE FROM *N. crassa*

Fractionation step	*Total volume (ml)*	*Total units*	*Total protein (mg)*	*Specific activity (Units/mg protein)*	*Yield (%)*
1. Crude extract*	14 000	5110	128 800	0.039	100
2. Protamine supernate	14 050	5090	84 000	0.060	99
3. Ethanol precipitate; H_2O extract; dialyze	1 800	3442	2 270	1.512	67
4. CTAB precipitate; pH 4.0 extract; dialyze	120	2714	954	2.835	53
5. 100% $(NH_4)_2SO_4$ precipitate; dialyze	480	2160	303	7.125	42
6. DEAE-cellulose chromatography; lyophilize; dialyze	—	2075	28	74.100	40

* In this purification procedure, 3360 g of frozen mycelia were processed.

RESULTS

Properties of acid phosphomonoesterase

The properties of the enzyme were determined with enzyme purified through step 6, unless otherwise noted.

Influence of pH

The results of experiments on the effect of variation in pH on enzyme activity are presented in Fig. 3. The maximum activity with either β-glycerolphosphate or glucosamine 6-phosphate as substrate was observed at pH 5.4–5.6. The substrates themselves are buffers and at the high concentrations used they must be adjusted to the desired pH prior to the addition of the enzyme. When enzyme purified through step 5 was stored at pH 6.1 and 5.1 for 5 h at 0°, 10 and 3 %, respectively, of the enzyme activity was lost. In the same manner, 28, 50 and 82 % of the activity was lost after storage at 0° for 5 h at pH 4.2, 9.0 and 11.4, respectively.

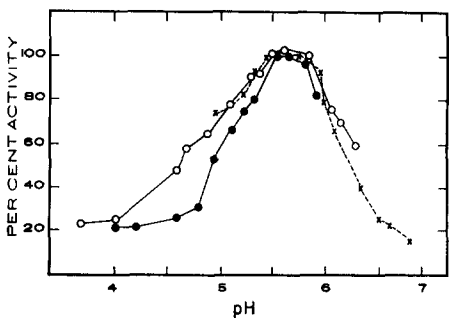

Fig. 3. The effect of the variation of pH on the liberation of phosphorus. The enzyme activities are indicated as the percentage of the activity at pH 5.6 using either β-glycerolphosphate or glucosamine 6-phosphate as substrate in an acetate buffer. The standard assay described in the text was performed except for the variation in type and/or pH of the buffer. The pH shown is the actual pH at the completion of the experiment as measured with a glass electrode on a duplicate sample. O—O, β-glycerolphosphate in acetate buffer; ●—●, glucosamine 6-phosphate in acetate buffer; ×—×, β-glycerolphosphate in acetate–barbital buffer.

Substrate specificity

Forty-six compounds were tested as substrates for the enzyme under the conditions of the standard assay at pH 5.6, except for the concentration of substrate (Table II). The specificity of the enzyme for the phosphomonoester bond was fairly broad, but the phosphodiester, inorganic or organic pyrophosphate, or phosphoprotein bonds were not cleaved. α-Glycerolphosphate was hydrolyzed most rapidly while the same concentrations of β-glycerolphosphate, riboflavin 5-phosphate and glucosamine 6-phosphate were cleaved at a slightly slower rate. The phosphomonoesters of both primary or secondary alcohols readily served as substrates.

Influence of substrate concentration

The effect of substrate concentration on the velocity of hydrolysis of eight substrates was determined under conditions of constant pH. The LINEWEAVER-BURK[12] plots of $1/V$ *versus* $1/S$ for the substrates β-glycerolphosphate, *p*-nitrophenylphosphate

TABLE II

SUBSTRATE SPECIFICITY OF *Neurospora* ACID PHOSPHATASE

The enzyme assay was performed as described in the text except that the amount of substrate is indicated in the table. All substrates were adjusted to pH 5.6.

Substrate	*Substrate added (μmoles)*	*Relative rate of hydrolysis**	*Michaelis constant (M)*
α-Glycerolphosphate	8	100	$5.8 \cdot 10^{-3}$
β-Glycerolphosphate	8	94	$4.6 \cdot 10^{-3}$
3-Phosphoglyceric acid	1	21	
2,3-Diphosphoglyceric acid	1	20	
Acetyl phosphate**	3.2	56	
Phosphoenol pyruvate***	2.4	10	
α-Glucose 1-phosphate	8	23	
Glucose 2-phosphate	2	11	
Glucose 3-phosphate	2	13	
Glucose 6-phosphate	8	45	$6.2 \cdot 10^{-3}$
Glucosamine 6-phosphate	8	89	$3.8 \cdot 10^{-3}$
6-Phosphogluconate	8	14	
Fructose 6-phosphate	5	66	
Fructose 1,6-diphosphate	5	27	$4.6 \cdot 10^{-2}$
α-Glucosamine 1-phosphate	8	0	
N-Acetylglucosamine 1-phosphate	8	14	
Ribose 5-phosphate	5	28	
Adenosine 5'-phosphate	5	30	$1.5 \cdot 10^{-3}$
Adenosine 3'-phosphate	5	35	
Adenosine 2'- and 3'-phosphate	2.8	30	
Cytidine 2'- and 3'-phosphate	2.8	24	
Uridine 2'- and 3'-phosphate	2.8	16	
Guanosine 3'-phosphate	2.8	26	
Adenosine triphosphate	5	0	
Deoxyadenosine 5'-phosphate	2.8	23	$4.1 \cdot 10^{-4}$
Deoxycytosine 5'-phosphate	2.8	21	
Deoxyguanosine 5'-phosphate	2.8	0	
Thymidine 5'-phosphate	2.8	0	
Carbamyl phosphate§	2	7	
Potassium phosphoramidate§	3	28	
o-Phospho L-homoserine	4.9	5	
o-Phospho DL-serine	10	0	
o-Phospho DL-threonine	10	0	
Phosphocholine	10	0	
Ethanolamine phosphate	10	0	
L-Histidinol phosphate	5	7	
p-Nitrophenyl phosphate§§	4	51	$2.5 \cdot 10^{-3}$
Bis-(*p*-nitrophenyl) phosphate§§	1	0	
Potassium pyrophosphate	8	0	
Phosphovitin	8	0	
Riboflavin 5-phosphate	8	96	
Thiamine pyrophosphate	8	0	
Diphosphopyridine nucleotide§§§	2	0	
Reduced diphosphopyridine nucleotide	2	0	
Triphosphopyridine nucleotide	1	3	
β-Naphthyl phosphate	2	0	

* The amount of phosphate liberated from α-glycerolphosphate, 0.71 μmole, was considered to be 100 and the other values are indicated as percentages of this value.

** Acetyl phosphate was determined by the method of LIPMANN AND TUTTLE[52].

*** Phosphoenolpyruvate was added as the cyclohexylamine salt.

§ Evolution of ammonia was measured in a Conway diffusion vessel[53].

§§ *p*-Nitrophenol was determined by the method of BESSEY *et al.*[54].

§§§ Diphosphopyridine nucleotidase activity was also checked by the method of COLOWICK *et al.*[8].

and adenosine 5′-phosphate are presented in Fig. 4; the K_m values for these and five other substrates are listed in Table II.

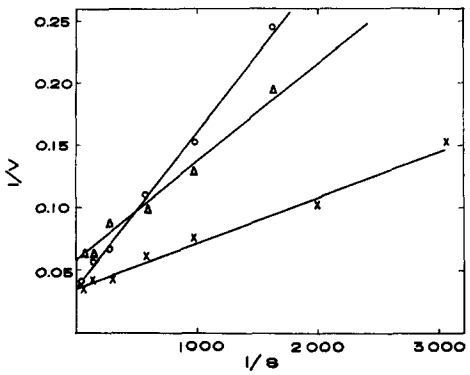

Fig. 4. Effect of substrate concentration, (S), on the rate of hydrolysis, V, by *Neurospora* phosphomonoesterase. The results are plotted by the method of LINEWEAVER AND BURK[12]. O—O, β-glycerolphosphate; △—△, *p*-nitrophenylphosphate; ×—×, adenosine 5′-phosphate.

Influence of ions

When the divalent cations Mn^{2+}, Zn^{2+}, Co^{2+}, Mg^{2+}, Fe^{2+}, Be^{2+} or Cu^{2+} were added to the purified enzyme in concentrations as high as 0.025 M, no change in the rate of liberation of Pi from β-glycerolphosphate was observed. Further evidence that metallic ions were not involved in the activity of the *N. crassa* acid phosphomonoesterase was obtained in experiments with the metal-binding agents EDTA, diethyldithiocarbamate and potassium cyanide. Concentrations of these agents as high as $5 \cdot 10^{-2} M$ had no effect on the enzymic activity. However, the enzyme was completely inhibited by $6 \cdot 10^{-6} M$ molybdate ion. Cu^{2+} has been reported[2] to stimulate, and molybdate to competitively inhibit, the hydrolysis of *p*-nitrophenylphosphate by the acid phosphatase in crude extracts of *N. crassa*. As mentioned previously, Cu^{2+} had no effect on the hydrolysis of β-glycerolphosphate by the purified enzyme used in the present studies.

Effect of inhibitors

A number of compounds were tested as inhibitors of the acid phosphomonoesterase (Table III). (+)Tartrate, but not (—) or *meso*-tartrate, was found to be an inhibitor of the enzyme. The acid phosphatase of crude *N. crassa* extracts had previously been reported to be inhibited by (+) but not (—)tartrate[3]. (+)Tartrate was a competitive inhibitor of the hydrolysis of β-glycerolphosphate by the enzyme as shown by the intersection on the $1/V$ axis of a plot of $1/V$ against $1/S$ both with and without the inhibitor present (Fig. 5). The *Neurospora* phosphatase was much less sensitive to (+)tartrate inhibition of β-glycerolphosphate hydrolysis than was the prostatic enzyme. Even though both of these enzymes have similar K_m values with β-glycerolphosphate as the substrate, the prostate enzyme has K_i values near $10^{-5} M$ (see refs. 13 and 14) while the K_i with the *Neurospora* enzyme was $2.3 \cdot 10^{-2} M$. The *Neurospora* phosphatase was quite sensitive to fluoride, which was also found to be a competitive inhibitor, with a K_i of $2.4 \cdot 10^{-4} M$ (Fig. 5).

TABLE III

EFFECT OF VARIOUS INHIBITORS ON *Neurospora* ACID PHOSPHOMONOESTERASE

Assay medium contained 20 μmoles of (pH 5.6) acetate buffer, 8 μmoles of sodium β-glycerolphosphate, diluted enzyme solution and inhibitor. The enzyme, buffer and neutralized inhibitor were pre-incubated together at 30° for 5 min before addition of the substrate to initiate the reaction. The incubation was at 30° for 20 min.

Inhibitor	*Concentration (M)*	*% Inhibition*
p-Chloromercuriphenyl sulfonate	$2 \cdot 10^{-3}$	33
p-Chloromercuriphenyl sulfonate plus reduced glutathione	$2 \cdot 10^{-3}$ $2 \cdot 10^{-3}$	25*
NaF	$7.5 \cdot 10^{-4}$	94
(+)Tartrate	$1.2 \cdot 10^{-2}$	100
(—)Tartrate	$5.0 \cdot 10^{-2}$	0
meso-Tartrate	$5.0 \cdot 10^{-2}$	0
Thyroxine**	$1.0 \cdot 10^{-5}$	0
Thyroxine***	$1.0 \cdot 10^{-5}$	100
Polyxenylphosphate	$1.25 \cdot 10^{-4}$	77

* Reduced glutathione was pre-incubated with the enzyme and buffer for 5 min at 30° before addition of the inhibitor. After addition of the inhibitor, incubation was continued for 5 min at 30° before addition of the substrate to initiate the assay.

** β-Glycerolphosphate or *p*-nitrophenylphosphate (8 μmoles) used as substrate.

*** Acetyl phosphate (1 μmole) used as substrate in place of β-glycerolphosphate.

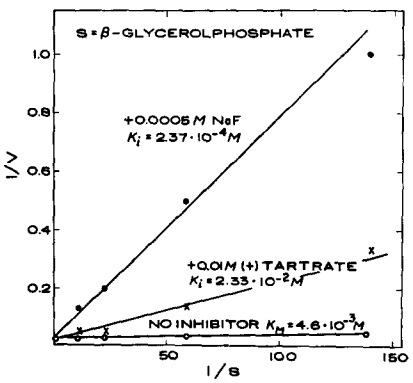

Fig. 5. Competitive inhibition by fluoride and (+)tartrate on the hydrolysis of β-glycerolphosphate by *Neurospora* phosphomonoesterase. The assays were performed as described in the footnote to Table III. Results are plotted by the method of LINEWEAVER AND BURK[12].

The anionic polymer, polyxenylphosphate[15], was a strong inhibitor of the *Neurospora* acid phosphomonoesterase. At pH 5.6, the hydrolysis of *p*-nitrophenylphosphate was completely inhibited at a concentration of $1.6 \cdot 10^{-4} M$. Furthermore, it was observed that the enzyme liberated Pi from the polyxenylphosphate preparation itself and as a consequence, controls for this enzymic cleavage had to be included. Although the monomer, 4,4'-xenyldiphosphoric acid has been reported to be hydrolyzed by prostatic acid phosphatase at about the same rate as β-glycerolphosphate the monomer was not an inhibitor for that enzyme[15].

Experiments with the sulfhydryl inhibitor *p*-chloromercuriphenyl sulfonate at $2 \cdot 10^{-3} M$ resulted in only a 33 % inhibition of the enzyme activity. Prior incubation

of the enzyme with the same concentration of reduced glutathione or cysteine, only reduced the inhibition to 25 %. These results are somewhat similar to those obtained with purified yeast acid phosphomonoesterase[16] and leave in doubt whether sulfhydryl groups are required for the activity of the *Neurospora* acid phosphomonoesterase.

The hormone thyroxine, at a concentration of 10^{-5} *M*, was found to have no effect on the hydrolysis of either *p*-nitrophenylphosphate or β-glycerolphosphate although this same concentration of thyroxine completely inhibited the hydrolysis of acetyl phosphate (Table III). Thyroxine has been reported to be a potent inhibitor of the cleavage of acetyl phosphate by the mammalian acyl phosphatase[17]. NIGAM *et al.*[13] have reported differences in the inhibition by various carboxylic acids on the hydrolysis of phenylphosphate, *p*-nitrophenylphosphate and β-glycerolphosphate by the prostatic acid phosphatase. That this differential inhibition is not common for all carboxylic acid inhibitors, even with the same prostate enzyme, is indicated by the results of KILSHEIMER AND AXELROD[14]. These investigators reported that (+)-tartrate was a competitive inhibitor for five substrates, including acetyl phosphate, and the K_i values were the same for all of the substrates. In the present work, several carboxylic acids were tested as inhibitors of the hydrolysis of β-glycerolphosphate and *p*-nitrophenylphosphate by the *Neurospora* enzyme (Table IV). The results with (+)tartrate, oxalate and glucuronate were similar to those obtained with the prostate enzyme[13] in that the first two acids inhibited the hydrolysis of both substrates whereas glucuronate inhibited the hydrolysis of β-glycerolphosphate but not *p*-nitrophenyl-phosphate. On the other hand, the results with saccharate and pyruvate were different in that these acids did not inhibit the hydrolysis of either substrate by the *Neurospora* enzyme.

Evidence for a single phosphomonoesterase

A number of experiments were performed to determine if the observed properties of the purified phosphomonoesterase preparation might actually be the result of the summation of the properties of a number of closely related phosphomonoesterases.

It has previously been mentioned that the individual fractions obtained during chromatography on DEAE-cellulose were assayed separately using either β-glycerol-phosphate or glucosamine 6-phosphate as substrates. No evidence was obtained for a separation of these two phosphomonoesterase activities. In a more direct test, the

TABLE IV

INHIBITION BY CARBOXYLIC ACIDS OF ACID PHOSPHOMONOESTERASE ACTIVITY ON TWO SUBSTRATES

The assays were performed in the same manner described in the Table III using 8 μmoles of either β-glycerolphosphate or *p*-nitrophenylphosphate as the substrate.

Inhibitor (0.0125 M)	*% Inhibition*	
	β-Glycerolphosphate	*p-Nitrophenylphosphate*
(+)Tartrate	100	96
Oxalate	100	72
D-Glucuronate	50	0
L-Malate	11	0
L-Glutamate	32	27
Pyruvate	0	0
D-Saccharate	0	0

TABLE V

SIMULTANEOUS HYDROLYSIS OF TWO SUBSTRATES BY ACID PHOSPHATASE

All substrates were 0.005 *M*. Where 2 substrates were used together, each substrate was 0.005 *M*. The assays were performed under the conditions described in the EXPERIMENTAL section.

Substrate	*Phosphate formed, observed (μmoles)*	*Phosphate formed, calculated* (μmoles)*
β-Glycerolphosphate	0.324	—
Glucosamine 6-phosphate	0.298	—
Glucose 6-phosphate	0.128	—
β-glycerolphosphate + glucosamine 6-phosphate	0.390	0.378
β-glycerolphosphate + glucose 6-phosphate	0.313	0.320
Glucosamine 6-phosphate + glucose 6-phosphate	0.266	0.276

* As calculated from the equation of BRIGGS AND HALDANE[18] using the Michaelis constants listed in Table II.

purified enzyme was tested against combinations of two substrates together. If separate enzymes were responsible for the enzymic activity against either β-glycerolphosphate, glucosamine 6-phosphate, two rapidly hydrolyzed substrates, or glucose-6-phosphate, a less rapidly hydrolyzed substrate, then when various combinations of two substrates were tested, one would expect to see some additive effects. The results, presented in Table V, indicated that the amount of Pi formed corresponded closely with the amount expected from a single enzyme acting simultaneously on two substrates. The formula of BRIGGS AND HALDANE[18] was used to make the calculations.

In another attempt to detect the existence of more than one phosphomonoesterase, the purified enzyme in 0.05 *M* acetate buffer (pH 5.6) was heated at 30°, 40°, 50°, 60°, or 70° for 15 min and cooled. Then aliquots of the heated enzyme were

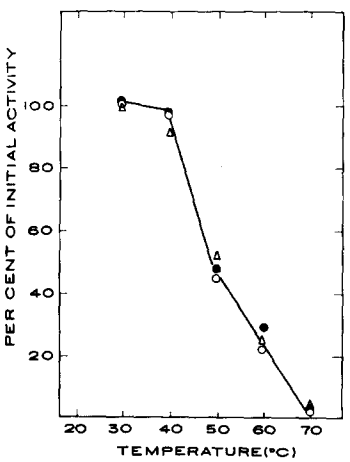

Fig. 6. Relative rates of hydrolysis of three substrates during the course of heat-inactivation of the *Neurospora* phosphomonoesterase. Samples of purified enzyme, in 0.05 *M* acetate buffer (pH 5.6) were heated for 15 min at the indicated temperatures and cooled in ice. Then aliquots were assayed for residual phosphomonoesterase activity with three different substrates by the standard assay procedure. O—O, α-glycerolphosphate; ●—●, β-glycerolphosphate; △—△, glucose 6-phosphate. All 3 values at 30° and 70° were 100% and 0%, respectively.

assayed for activity using α-glycerolphosphate, β-glycerolphosphate and glucose 6-phosphate as substrates. The results of a typical experiment are shown in Fig. 6. Although slight differences in the amount of inactivation were occasionally observed at one temperature, there never was any consistent indication of a loss of activity against one substrate at a rate different than the others over the whole course of an experiment.

Finally, the relative rate of hydrolysis of a number of substrates remained fairly constant throughout the course of enzyme purification. A comparison of the results of the relative rate of hydrolysis, as a percentage of the rate of β-glycerolphosphate cleavage, using the crude extract and enzyme purified through step 6 are shown in Table VI. The relative rates of hydrolysis of α- and β-glycerolphosphate, glucosamine 6-phosphate and glucose 6-phosphate were similar with the crude and purified enzyme. Only the activity with fructose 6-phosphate dropped significantly from the crude to the most purified enzyme preparation. The pyrophosphatase activities present in the crude extract were completely absent in the purified enzyme.

TABLE VI

RELATIVE RATES OF HYDROLYSIS OF SOME SUBSTRATES WITH THE CRUDE AND PURIFIED PHOSPHATASE PREPARATIONS

The standard conditions of assay described in the text were used with 8 μmoles of each substrate. The activities are expressed as percentages of that obtained with β-glycerolphosphate as the substrate.

Substrate	*Enzyme Fraction*	
	Step 1	*Step 6*
α-Glycerolphosphate	105	107
β-Glycerolphosphate	100	100
Fructose 6-phosphate	91	70
Glucosamine 6-phosphate	87	94
Glucose 6-phosphate	46	46
Thiamine pyrophosphate	39	0
Potassium pyrophosphate	58	0

In the absence of any evidence for the presence of more than one phosphomonoesterase, it was concluded that a single enzyme was responsible for all of the phosphomonoesterase properties observed.

Physical properties of the enzyme

The ultraviolet absorption spectrum of the purified enzyme showed a sharp maximum at 277 mμ, with no peak or shoulder near 260 mμ to indicate a significant contamination of the enzyme by nucleotides. Starch gel electrophoresis of the enzyme[19], employed at a number of pH values from 4.0 to 5.6, revealed the presence of two protein components in each instance. The major band, containing about 80–90 % of the protein, contained all of the phosphomonoesterase activity. Recovery of enzyme from the gel by a variety of procedures was uniformly poor, and thus starch gel electrophoresis could not be used as a preparative procedure. From the mobility of the enzyme in the gel, the isoelectric point of the enzyme was estimated to be in the range of pH from 4.5–5.0.

The finding that the phosphomonoesterase enzyme was soluble in water saturated with ammonium sulfate suggested that the enzyme might either have a relatively low molecular weight or be contaminated with large amounts of polysaccharide[20]. Since enzyme preparations after step 4 had a ratio of protein to carbohydrate (as glucose, by an anthrone procedure) of over 100, it appeared unlikely that the solubility of the enzyme in a saturated ammonium sulfate solution was due to the presence of large amounts of contaminating polysaccharide.

DISCUSSION

The properties of the *Neurospora* phosphatase indicate that it can best be classified as a relatively non-specific acid phosphomonoesterase most closely fitting ROCHE's classification of a Type-II phosphomonoesterase[21]. This is based on the pH optimum of 5.6, the absence of a cation or cofactor requirement and the ability to hydrolyze phosphomonoesters but not pyrophosphate esters. Although the *Neurospora* enzyme was purified 1400-fold, it was, at best, only about 80–90 % pure. The results of a number of experiments, however, made it appear unlikely that the purified enzyme preparation contained a number of related phosphomonoesterases rather than a single phosphomonoesterase.

The properties of the *Neurospora* acid phosphomonoesterase clearly differentiate it from the acid phosphomonoesterases derived from yeast[16], *Escherichia coli*[22], or other microbial sources[21,23]. It is closest in its properties to those of the human prostatic acid phosphomonoesterase[24]. For example, both enzymes have similar pH maxima, are inhibited competitively by fluoride and (+)tartrate, and are sensitive to the anionic inhibitor polyxenylphosphate[15]. Furthermore, they do not require cations or cofactors, have phosphotransferase activity[25–27] and similar Michaelis-Menten constants[24]. Although both enzymes act on a number of substrates in a similar manner, such as approximately equal activity with either α- or β-glycerolphosphate, the *Neurospora* enzyme utilizes a somewhat narrower range of phosphomonoesters than the prostate enzyme. Thus O-phosphothreonine, ethanolamine phosphate and choline phosphate, which are all substrates for the prostatic enzyme[24,28], are not hydrolyzed by the *Neurospora* enzyme.

It is likely that *Neurospora* contains other enzymes for the catabolism of those phosphomonoesters which are not active substrates for the acid phosphomonoesterase. Enzymes such as the specific O-phosphoserine phosphatase of yeast[29] and the threonine synthetase of *N. crassa*[30], which converts O-phosphohomoserine to threonine and Pi by a complex non-hydrolytic reaction[31], are known to exist. Also, L-histidinol phosphate, which is cleaved only very slowly by the *Neurospora* acid phosphomonoesterase, is rapidly hydrolyzed by either the specific *Neurospora* L-histidinol phosphate phosphatase[32] or the *Neurospora* alkaline phosphomonoesterase[32,33].

Some of the factors controlling the substrate specificity of the *Neurospora* acid phosphomonoesterase have been ascertained. It was observed that when a free amino group and a phosphomonoester group were attached to adjacent carbon atoms, the compound would either not serve as a substrate, as in the case of O-phosphothrenonine, O-phosphoethanolamine or O-phosphoserine, or would be hydrolyzed only very slowly, as with L-histidinol phosphate. The exact degree of separation of the amino and phosphomonoester groups necessary before the phosphomonoester would

be hydrolyzed was not determined, although intervention of a single methylene group, such as in O-phosphohomoserine, restored only very slight substrate activity. The fact that phosphocholine would not serve as a substrate for the *Neurospora* phosphomonoesterase also suggested that the amino group could be replaced by a quaternary ammonium group in inhibiting the cleavage of a nearby phosphomonoester bond. A compound such as glucosamine 6-phosphate, where 3 carbon atoms separate the carbon atoms bearing the amino and phosphomonoester groups, was found to be an excellent substrate for the enzyme. The fact that glucosamine 1-phosphate was inert as a substrate whereas N-acetylglucosamine 1-phosphate was hydrolyzed at a slow rate indicated that the inhibitory effect of the free amino group could be somewhat neutralized by N-acetylation.

The presence of a carboxyl group in the region of the free amino group also augmented the inhibiting effect of the amino group. For example, histidinol phosphate, which contains the structure $-CH_2-CHNH_2-CH_2OPO_3H_2$, was hydrolyzed at a relatively slow rate, while O-phosphoserine, with the structure $HOOC-CHNH_2-CH_2OPO_3H_2$, was completely inert. The presence of a $-NH_2$ group close to the bond that was to be hydrolyzed by the enzyme was not always completely inhibitory. So carbamyl phosphate, which has $-NH_2$ and phosphomonoester moieties attached to the same carbon atom, was cleaved at a slow rate while phosphoramidate, which is an amide of phosphoric acid, was hydrolyzed at a moderate rate.

An unexpected example of substrate specificity was observed among the ribonucleoside and deoxyribonucleoside phosphates. The *Neurospora* enzyme hydrolyzed all of the ribonucleoside 5'-, 3'- or mixtures of 2'- and 3'-phosphates tested. However, among the deoxynucleoside phosphates, thymidine 5'- and deoxyguanosine 5'-phosphate were inert although both deoxyadenosine- and deoxycytosine 5'-phosphate were hydrolyzed by the enzyme. This behavior should be contrasted with the nonspecific *E. coli* alkaline phosphomonoesterase, for example, which hydrolyzed all of the ribonucleoside and deoxyribonucleoside phosphates examined at similar rates[34,35]. The specificity of the *Neurospora* enzyme with the deoxyribonucleotides suggests the possibility that it may play some regulatory role in the biosynthesis of *Neurospora* deoxyribonucleic acid.

Obviously, factors other than the charge on the substrate molecule must play a role in the substrate specificity of the *Neurospora* enzyme. For example, fructose 6-phosphate is hydrolyzed more readily than is glucose 6-phosphate. A larger number of appropriate substrates will have to be examined, though, before any conclusions can be drawn with regard to the role of these factors on the specificity of the enzyme.

In spite of innumerable investigations on the properties of alkaline and acid phosphatases, their role in cellular metabolism is almost completely unknown. Indeed, the suggestion has been made[36] that phosphatase activity may possibly be an artifact, the hydrolase activity representing the remains of another more important function, such as group transfer. Although this suggestion, based on the acetyl phosphatase activity of crystalline glyceraldehyde 3-phosphate dehydrogenase preparations[37] should be borne in mind, there is no evidence, as yet, that the hydrolase activities of phosphatases are artifacts.

Phosphomonoesters are important intermediates in a wide variety of biochemical reaction cycles. One can visualize how control of the amount of activity of the phosphomonoesterases that have a limited substrate specificity, the so-called specific phospho-

monoesterases, might have an important role in regulating specific cellular biochemical processes[38,39]. One might expect, though, that it would be less likely that those phosphatases capable of hydrolyzing a wide variety of phosphomonoesters would be capable of acting in a similar manner. There is some evidence, however, that this latter rationalization may not always be true since factors other than the *in vitro* substrate specificity of an enzyme are apparently operative *in vivo*.

The role of glucose 6-phosphatase in contributing to the regulation of glucose metabolism in liver and kidney is generally accepted[38]. Rat microsomal glucose 6-phosphatase, which is often thought of as a specific phosphomonoesterase, has already been shown to hydrolyze 18 phosphomonoesters and about half of these are cleaved at 20 % or more of the rate of glucose phosphate. Thus, the enzyme is certainly not specific *in vitro* although its function *in vivo* strongly suggests that it is functioning in a specific regulatory manner on glucose production and glycogen synthesis. With this example in mind, it is possible that an enzyme such as the *Neurospora* acid phosphomonoesterase, may also be capable of acting in a regulatory manner *in vivo* even though it can hydrolyze at least twenty phosphorylated compounds *in vitro* at 20 % or more of the rate of hydrolysis of α-glycerolphosphate. This could be accomplished, for example, by the selective hydrolysis of those few phosphomonoesters available to the enzyme at its particular location in the cell. Although relatively little is known about the localization of phosphatases in microbial cells, there are reports of its presence in cell membranes. Thus, more than 90 % of the total acid phosphomonoesterase of *Staphylococcus aureus* was found in its cytoplasmic membrane fraction[40] while about two-thirds of the total yeast acid phosphatase was located on the "cell surface[41]".

An instance where the level of non-specific acid phosphomonoesterase activity was apparently involved in the control of glucose metabolism in *Salmonella typhimurium* has also recently been reported[42]. From the examples of the results with the mammalian glucose 6-phosphatase and the acid phosphatase of *S. typhimurium*, it would appear that even phosphomonoesterases that are not limited in their *in vitro* substrate specificity may still play a role in metabolic control mechanisms.

The substrate specificity of acid phosphomonoesterases might be made more selective *in vivo* by the presence of an inhibitor in the cell that would act selectively in preventing the hydrolysis of one or a few key biochemical substrates while not affecting others. Several examples of *in vitro* differential inhibition were observed in the present study. Thus, the hydrolysis of acetyl phosphate was completely inhibited by 10^{-5} *M* thyroxine while this same concentration of thyroxine had no effect on the hydrolysis of either β-glycerolphosphate or *p*-nitrophenylphosphate (Table III). The specific acyl phosphatase of muscle, which has also been found in yeast[43], was reported to be inhibited by low concentrations of thyroxine[17]. It has already been suggested that acyl phosphatase plays a regulatory role in glycolysis[44] by catalyzing the decomposition of 1,3-diphosphoglyceric acid[45,46]. The marked differential inhibition of the *Neurospora* phosphomonoesterase by thyroxine serves as a good example of the type of inhibition that might be involved in metabolic control mechanisms through modification of the substrate specificity of the phosphomonoesterase *in vivo*.

The observation that both glucuronate and malate did not inhibit the hydrolysis of *p*-nitrophenylphosphate while inhibiting the hydrolysis of β-glycerolphosphate 50 and 11 %, respectively (Table IV), again can be used to support the hypothesis

that the *in vivo* substrate specificity of the phosphatase may be modified by other materials present in the cell. A number of organic acids were previously observed to differentially inhibit the hydrolysis of some substrates by the prostatic acid phosphomonoesterase[13].

Phosphomonoesterases might also play a regulatory role by controlling the level of Pi in the cell. Pi is both a product of phosphatase action and a competitive inhibitor of the enzyme and it has been suggested[47] that the level of Pi might be maintained through control of the activity of the phosphatases. The level of Pi might, in turn, also control biochemical processes sensitive to Pi, such as the Embden-Meyerhof and/or hexosemonophosphate shunt glycolytic pathways[48].

It has recently been shown in certain bacteria, that the presence of Pi repressed the formation of alkaline phosphomonoesterase[49,50] and it was suggested that a Pi-phosphatase control system might play a regulatory role in the phosphorus economy of the cell[49]. With the strain of *N. crassa* used in the present study, however, the presence of Pi did not repress the formation of either the acid or the alkaline phosphomonoesterases[51].

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SUCCINIC SEMIALDEHYDE DEHYDROGENASE: PURIFICATION AND PROPERTIES OF THE ENZYME FROM MONKEY BRAIN

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SUMMARY

DPN-dependent succinic semialdehyde dehydrogenase from rhesus monkey brain retains full activity when purified by fractionation in the absence of reducing agents. In this state, the enzyme is relatively insensitive to arsenite. Addition of mercaptoethanol markedly increases the arsenite sensitivity. Both DPN and mercaptoethanol are effective in protecting the enzyme from auto-oxidation. Additional experiments are presented in support of a role of DPN in stabilizing the tertiary structure of the enzyme in the vicinity of the aldehyde binding site.

Abbreviations: SSA, succinic semialdehyde; SSADH, succinic semialdehyde dehydrogenase; PCMB, *p*-chloromercuribenzoate.