ISOLATION, PURIFICATION, AND PARTIAL CHARACTERIZATION OF SEVERAL PLANT PHOSPHOMONOESTERASES

Thesis for the Degree of M. S.
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ISOLATION, PURIFICATION, AND PARTIAL CHARACTERIZATION OF SEVERAL PLANT PHOSPHOMONOESTERASES

By

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To Dalton and Marie

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INTRODUCTION

Phosphatases are enzymes which act on a variety of phosphate esters liberating inorganic phosphate. They are important in glucose metabolism, in bone formation, and a variety of disease conditions. Serum phosphatase levels in relation to cancer have been studied, in addition to the serum phosphatase assays routinely carried out to determine extent of liver or heart damage. Precise and definitive studies on the phosphatases are rare and this type of work on this class of enzymes is only beginning to develop.

The substrates of this group of enzyme are all esters of orthophosphate. Among the substrates which have been used for assay of phosphatase activity are sodium α-glycerophosphate and sodium β-glycerophosphate, p-nitrophenyl phosphate, o-carboxyphenyl phosphate, phenyl phosphate, and α-napthyl phosphate.

One of the greatest problems in work on the phosphatases has been that of attempting to classify the various types of phosphatase activity that have been found. One attempt at classification has been based upon specificity for substrate. The phosphatases have been classified as 1) general, that is acting on several substrates or 2) specific, acting on one compound or series of compounds only. However, since the specificities of many of the enzymes vary with the cofactor used in the assay of the enzyme activity, this classification is not too precise.

Another classification attempt has been based upon the pH optimum, but this can be carried out effectively only on highly purified enzyme preparations. There are in the literature many contradictory results arising from the use of preparations of a wide variety of purities. The majority of phosphatases of animal origin have pH optima in the alkaline range, while most of the phosphatases from plant sources have their optimum on the acid side.

Michaelis constants and competitive and noncompetitive inhibitors have also been used as a means of classification. Roche (1) has classified phosphomonoesterases into four different classes: Group I or alkaline phosphatases (found mostly in animals): inactivated after 30 minutes in an alkaline solution, pH optimum 9.2 to 9.6, activated by magnesium and inhibited by calcium, sulfhydryl compounds, cysteine, and fluoride ions. Group II or acid phosphatases (mainly occurring in plants): pH optimum 5.2 to 5.6, inhibitors are the most distinguishing characteristic of this group. Oxalate ions, fluoride, and molybdate ions inhibit while amino acids, magnesium and other divalent cations have no effect. Group III (a rather poorly characterized group, found generally in animals, unstable in neutral solution): pH optima 3.4 to 4.2, inhibited by magnesium ions. Group IV (yeast phosphatase): pH optimum

5.2 to 6.2, activated by magnesium and manganese ions (unlike phosphatases of Group II). The Group IV phosphatases are not well characterized.

Besides catalyzing the hydrolysis of the esters of phosphoric acid, alkaline phosphatases have been known to catalyze the synthesis of inorganic pyrophosphate from orthophosphate as described by Roche (2).

The purpose of this present work has been to isolate, purify, and somewhat characterize some phosphatases from convenient plant sources. A number of distinct, different acid phosphatases have been isolated from sunflower seedlings and wheat bran and have been partially characterized with respect to metal ion requirements, pH specificities, and temperature characteristics. The ability of these partially purified fractions from sunflowers and wheat bran to liberate inorganic phosphate from inositol polyphosphates has also been studied.

HISTORICAL

Much work has been done in this field by various workers who have approached this problem from many different angles. Some have considered the action of phosphatases with respect to substrate, such as α or β glycerophosphate, p-nitrophenyl phosphate, and some of the phosphorylated carbohydrates; some have considered inhibitors and activators as well as pH specificities and temperature requirements, while others have only attempted to purify partially this class of enzyme from a variety of sources, both plant and animal. Some of the work done in recent years is summarized in Table I. The references mentioned will be found in the Bibliography.

In addition to these studies on the characteristics, requirements, and sources of phosphatases, other papers have been presented which deal with the mechanism of action of this class of enzymes. Harary (36) has suggested that phosphatases may also act as transferases as in the following set of reactions:

$$(1)\ \text{ATP} + 3\ \text{PGA} \xrightarrow{\text{phosphoglycerokinase}} \text{ADP} + \text{glyceric acid-1,3-diphosphate}$$

$$(2)\ \text{glyceric acid-1,3-diphosphate} \xrightarrow[\text{phosphatase}]{\text{acyl}} 3\ \text{PGA} + \text{Pi}$$

$$(1)+(2)\ \text{ATP} \xrightarrow[\text{acyl phosphatase}]{\text{phosphoglycerokinase}} \text{ADP} + \text{Pi}$$

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Table I

Summary of Some Previous Work on Phosphatases

(The following abbreviations will be used: p-nitrophenyl phosphate, pNPP; α or β-glycerophosphate, α- or β-GP; glucose-6-phosphate, G-6-P; adenosine triphosphate, ATP; adenosine monophosphate, AMP; ribonucleic acid, RNA; Ethylenediamine tetraacetate, EDTA; diethylaminoethyl cellutose, DEAE; inorganic phosphate, Pi; triphosphopyridine nucleotide, TPN.)

Source	Purity	$pH_{opt.}$	Substrates	Inhibitors	Activators	Other Data	Ref.
Belladonna Leaves	H_2O ext	4.0 - 5.0	not given	Fluoride molybdate	Mg^{++}, Zn^{++}, Mn^{++}, Ni^{++}, Co^{++}, Mg^{++}	no phytase activity	(3)
Ragweed Pollen	Crude	5.6	α-GP β-GP pNPP	Cu^{++}, arsenate cyanide fluoride	none found	Km GPA 3.0 x 10^{-3}m; Ea 7400 cal. Km 104 7.5 x 10^{-4}m; Ea 7600 cal.	(4)
Leaves	Crude	5.2	GP		$Mg^{++}Ni^{++}Co^{++}$	Similar to II	(5)
Leaves	Crude	4.0	GP	Fluoride molybdate	Mg, Zn, Mn, Ni, Co	Similar to alkaline phosphatase I	(5)
Wheat germ	Crude	acid	L-α GP gl-6-P	Hg^{++}	Mg^{++}		(6)
Wheat leaf	Crude	5.7	16 of them tested	28 of them tested	Mg^{++}	narrow substrate specificities	(7)

Pea plants	Crude	alkaline	fructose-1,6-diphosphate	Iodoacetamide & heavy metals	Mg^{++} cysteine	TPN specific phosphatases	(8)
Pea plants	Crude	5.0	various	Fluoride molybdate	none listed	non-specific not affected by Mg^{++}	(9)
Pea plant	55-75% ammonium sulfate fractionation	6.3	gl-6-P	Pi ICH_2COO^- Fluoride Molybdate	Mg^{++}		(10)
Potato	1300 fold purification	4.6 5.2 6.8	β-GP	can't find	can't find	acetone & Ethanol, or DEAE fractionation	(11)
Sunflower	Crude	not given	.1% CaGP RNA gl-1-P	not given	not given	non-specific	(12)
Cl. propionicum	partial	broad range	nucleotides	EDTA Glutathione	Fe^{++}	heat sensitive	(13)
Kidney Liver Bone		alkaline & acid. alkaline & acid. alkaline.		Fluoride, oxalate. Fluoride.			(14)

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Kidney Liver Bone		alkaline & acid. alkaline & acid. alkaline.		Fluoride, oxalate. Fluoride.			(14)

Mouse liver (normal & regenerating)	cell-free homogenate	5.5 9.2	phenyl phosphate	none given	none given	normal both acid & alkaline -more 9.2 in regenerating -no diff 5.5	(15)
Rat bone & intestine	Crude	alkaline	βGP	N-methyl amino acids & glycine	none listed	Km bone=.0019 m Km int=.0029 m in glycine Km .039 m	(16)
Rabbit liver	Crude	alkaline & acid	βGP	NaTCA NaTCA F^- oxalate	none listed	apparently 3 diff. enz.	(17)
Ling cod muscle (a no. of them)	DEAE fractions	acid	pNPP	cysteine EDTA Fluoride tartrate	varies in enzyme	5 enzymes separated	(18)
Ling cod muscle	Partial	6.5	yeast RNA	Zu^{++}, Cu^{++} Iodoacetate HCHO NaF	none listed	RNA-ases separated by electrophoresis	(19)
Ling cod muscle	DEAE fraction	6.4	nucleotides	Iodoacetate	none listed	Doesn't require a divalent metal cation for activation	(20)
Dog faeces	acetone powder	9.6	pNPP	none listed	Mg^{++} ($<$.0003 m)	A classic sep'n. & purification	(21)
Dog intestine	partial purification	8.8	Na β GP	Fluoride	alanine+ Mn^{++}, Fe^{++}, Zn^{++}, Ca^{++}, Mg^{++}	amino acid-incubation necessary for all metals to reactivate dialyzed preparation	(22)

Calf duodenum	very pure	alkaline	β GP phenyl phosphate	not given	not given	crystalline form obtained; hexosamine + hexose present as well as 17 amino acids	(23)
Bovine milk	Crude	alkaline	phenyl phosphate O-carboxy-phenyl phosphate	not given	not given		(24)
Calf intestine	purified	alkaline	phenyl phosphate βGP	Cyanide cysteine	Mg^{++} amino acids	F^- has no effect; 20% polysaccharide present	(25)
Calf tissues	Crude	alkaline	Na phytate	none listed	none listed	no phytase activity in intestine, muscle or kidney; liver & blood do have phytase activity	(26)
Ox kidney	Crude	broad	various	Cyanide	Mg^{++}, Zn^{++}		(27)
Rat int. bone kidney	Crude	alkaline	Na β GP	amino acids (alanine cysteine etc.)	none listed	inhibited by amino acids; reversed by dialysis	(28)
Human bone	Crude	8.4 9.4	sugar-phosphates glycerol-phosphates	Pi	none listed	adrenaline, pituitrin or thyroid has no effect	(29)

Brain	purified	6.0	acyl-phosphates	Pi, Adrenaline, dinitrophenol	Inosinic acid	Molecular weight of 13,000; Homogeneous solution in ultracentrifuge	(30)
Brain	Na picrate precipitate	6.0	carbamyl-phosphate acyl-phosphate	Zn^{++}, Pi	none listed	$Km=6.4 \times 10^{-3}$; inactivated by AMP, ATP Mg, Mn, Pb EDTA have no effect	(31)
Placenta	paper electrophoresis or butanol extract	alkaline	pNPP	none listed	none listed	(1) purified by electrophoresis & (2) ion exchange	(32)
Red cells	Purified	5.5	phenyl phosphate, GP, yeast adenylic acid	none listed	Mg^{++}(.01 Molar)		(33)
Leucocytes	crude	alkaline	not given	EDTA	Zn^{++}, Mg^{++}	Zn^{++} protects & restores; Mg^{++} protects & does not restore	(34)
Prostate	purified	5.3 - 5.6	α-L-GP	Fluoride	none listed	Km = $2.3-2.6 \times 10^{-3}$ m	(35)

There has been some discussion with regard to the possible relationship of this system to glycolysis. It has been pointed out that the acyl phosphatase will not hydrolyze ATP, ADP, or AMP, nor will it transfer a phosphate group to glucose or creatine. The mechanism of inhibition by oxalate has also been studied. There are three possible complexes which may be formed in the presence of enzyme, oxalate and inorganic phosphate; 1) An ES-complex, 2) An E-S-inorganic phosphate complex, and 3) An enzyme-oxalate complex. This mechanism does not appear to be supported by all of the available data (37).

The mechanism of cleavage of the phosphate esters has also been studied (38). Studies with several different substrates has shown the cleavage to occur between the oxygen-phosphate bond rather than between the carbon-oxygen bond. This has been determined using oxygen-18 water and has been confirmed by the fact that no inversion of configuration takes place on phosphatase action (39). A number of phosphatases of plant origin have been shown (40) to be able to transfer the phosphate group from phenyl phosphate or p-nitro phenyl phosphate to various alcohols; sugars and inositol did not act as acceptors in this system.

Studies on some of the alkaline phosphatases (41) have shown that the optimum pH varies with the concentration of substrate; the higher the concentration, the higher the pH. By dilution of the substrate, the pH optimum can be reduced almost to neutrality, and it is felt that this is in some way related to the action of the metal ion which is bound to the enzyme.

Chemical composition studies have also been carried out on some of the more highly purified phosphatases. Binkley (42) has reported glucose to be an active constituent of the alkaline phosphatase of hog kidney. This enzyme was also reported to contain a pyrimidine moiety as part of its structure. Portmann (43) has reported that during purification of the alkaline phosphatases of rat, swine, and bovine intestine, the increase in specific activity of the enzyme was paralleled by an increase in hexoseamine content. When the proteins of human semen were separated by starch electrophoresis (44) 11 fractions were found, of which one consistently represented the acid phosphatase activity. This band was found between the F-alpha$_2$ and albumin fractions. Highly purified preparations of alkaline phosphatase have been prepared from dog intestine and from ox kidney and attempts have been made to crystallize these (45). What was at one time believed to be crystals of enzyme was later shown to be crystals of magnesium phosphate bound to the enzyme; further attempts at purification of alkaline phosphatases have been claimed (46) when the crude enzyme is incubated with trypsin for several hours at 37° C.

Electrophoretic studies of intestinal alkaline and acid phosphatases have also been carried out by Harris and Mehl (47). They achieved some degree of purification by repeated separations on starch-gel electrophoresis. As a result of electro-dialysis studies on intestine, kidney, and liver alkaline phosphatases (48) it is claimed that normal alkaline phosphatases contain three components: (1) the protein apoenzyme, (2) an organic dialyzable coenzyme, (3) magnesium. It is claimed that the differences between the enzymes from various sources largely represent differences in binding between the apoenzyme and coenzyme involved as well as slight differences in the apoenzyme portions from different sources.

A number of reviews are available having reference to the phosphatases: Altman and Dounce (49) have included many phosphatases in their review of non-oxidative and non-proteolytic enzymes. Akamatsu (50) has given special emphasis to inhibition constants, Michaelis-Menten values, and activation energies. The chemical structure of the phosphatase molecule has also been reviewed (51). Other reviews are those of Axelrod (52) and Burnham (53).

RESULTS AND DISCUSSION

In the work done in this paper, p-nitrophenyl phosphate has been used as the substrate for determining the activity of the enzyme fractions. Since this was the compound used in the majority of routine screening procedures, phosphatases which are not capable of catalyzing the hydrolysis of this compound would not be apparent. The reactions involved are:

$$\text{p-nitrophenyl phosphate} \xrightarrow{\text{enzyme}} \text{p-nitrophenol} + H_3PO_4$$

$$\text{p-nitrophenol} \xrightarrow{OH^-} \text{p-nitrophenoxide}^- + H_2O$$

It must also be noted that magnesium ion was used also in the routine screening procedures. Again some of the enzymes may not have been detected due to inhibition of these enzymes by magnesium. However, when one of the sunflower separations was re-assayed using zinc as the metal ion, no activity in any of the fractions was detected.

Ammonium Sulfate Fractionations

As a result of the ammonium sulfate fractionations, there was little separation of activity found. The activity seemed to concentrate in the 0-33% fraction as concluded from Table II.

Table II

Ammonium Sulfate Fractionation of Sunflower Extracts

Fraction	Optical Density	Enzyme Units	Ratio $\frac{(\text{Acid})}{(\text{Alkaline})}$
0-33%			
pH 5.0	.44	330	3.06
pH 9.6	.145	109	
33-66%			
pH 5.0	.29	70	19.4
pH 9.6	.015	3.6	
66-100%			
pH 5.0	.03	0	0
pH 9.6	.00	0	

Note: A unit of activity is defined as that amount of enzyme which causes an increase in optical density of 0.100 per hour at 35° C.

It may be noted that most of the activity was found below 80% saturation which influenced further separation attempts.

Since the 0-33% fraction seemed to contain the most activity, it was refractionated into 0-10, 10-20, and 20-30% fractions, and found to contain the distribution of units described in Table III.

Table III

Refractionation of 0 to 30% Ammonium Sulfate Precipitate

Fraction	280mµ	260 mµ	Protein Concentration	Total Units Acid	Alkaline
0-10%	1.25	1.48	8.37 mg./ml.	2520	540
10-20%	.377	.400	280 mg./ml.	6300	3150
20-30%	.540	.562	4.1 mg./ml.	4350	330

As a result of these data, a second series of fractionation attempts was carried out on a new batch of seedling extracts. In Table IV dialyzed and undialyzed fractions are compared for activity. Preparation of the extracts is described in the Experimental section.

Table IV

A Comparison of the Activity of Dialyzed and Undialyzed Ammonium Sulfate Fractions of Sunflower Seedling Extracts

Fraction	280mµ	260mµ	Protein Concentration	% Nucleic Acid	Total Acid	Units Alkaline
0-10%	.385	.475	2.35 mg./ml.	6.25	2272	288
0-10% D	.137	.168	0.837 mg./ml.	6.12	1760	0
10-20%	.357	.445	21.5 mg./ml.	6.50	5440	4640
10-20% D	.610	.790	31.5 mg./ml.	7.60	4800	3840
20-30%	.325	.405	19.5 mg./ml.	6.50	4480	2336
20-30% D	2.03	2.39	13.5 mg./ml.	5.37	4480	2336
30-100%	1.43	1.65	9.72 mg./ml.	5.15	4000	2944
30-100% D	.620	.630	4.78 mg./ml.	3.65	4160	768

There was, however, still no satisfactory separation of acid activity from alkaline activity.

DEAE-Cellulose Column Separations

A. Sunflower Column I

As Figure I shows, the sunflower seedlings, after being eluted from a column of DEAE-cellulose with an increasing concentration of tris buffer, pH 7.5, (see Experimental section), were separated into several active fractions, having as their peaks tubes 37, 45, and 53. As a result of this work, a more extensive separation was carried out as described in the Experimental section.

When different metal ions, other than magnesium, re used in the assays of the peak tubes, differing results

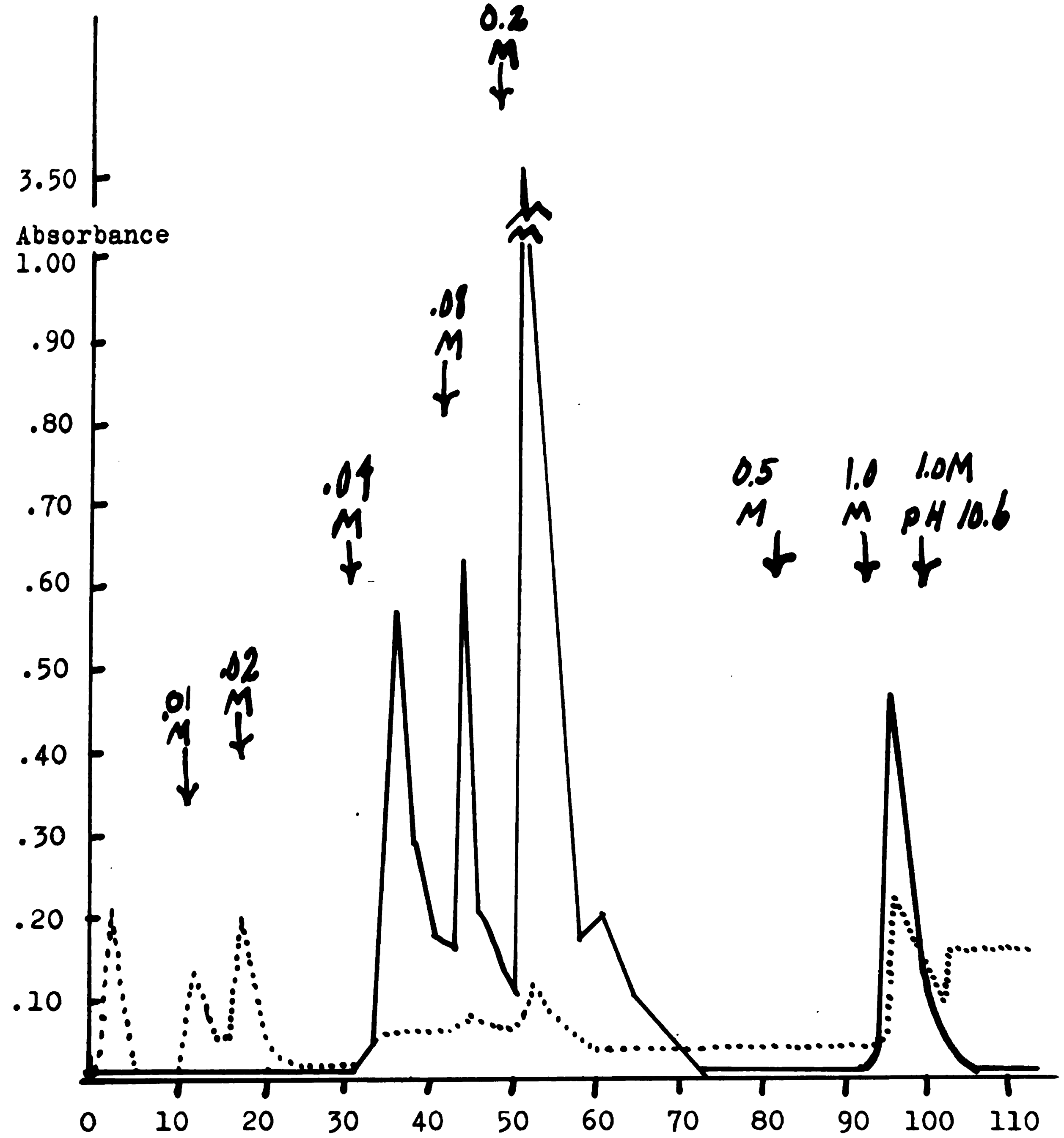

Figure I. Separation of Phosphatase Activity from Sunflower Seedlings on DEAE-Cellulose: Column I

Note: The dotted line represents absorbance at 280mμ.

were obtained depending upon the ion used. A typical result is expressed in Table V.

Table V

Metal Ion Studies on the Peak Tubes from Sunflower Column I

Tube No.	No Metal	Optical Density with Mg^{++}	Zn^{++}	Mn^{++}	$Mg^{++}+Zn^{++}$
37 pH 5.0	0.00	0.097	0.000	0.192	0.07
37 pH 9.6	0.00	0.012	0.000	0.110	0.005
45 pH 5.0	0.032	0.160	0.010	0.210	not studied
45 pH 9.6	0.010	0.065	0.005	0.172	not studied
53 pH 5.0	0.375	0.480	0.030	0.500	0.060
53 pH 9.6	0.00	0.085	0.000	0.160	0.005

At this point it was decided to repeat the procedure on a larger column in order to get larger amounts of the enzymes for more extensive characterization.

B. Sunflower Column II

The results of this second column are summarized in Figure II. Again the column was eluted with increasing concentrations of tris buffer, pH 7.5, and the tubes were assayed at pH 5.0 according to the procedure described in the Experimental section. Again several peaks were found which will be designated as peak numbers 305, 440, 480, and 625. Metal ion studies were carried out on these peak fractions. A typical result is expressed in Table VI.

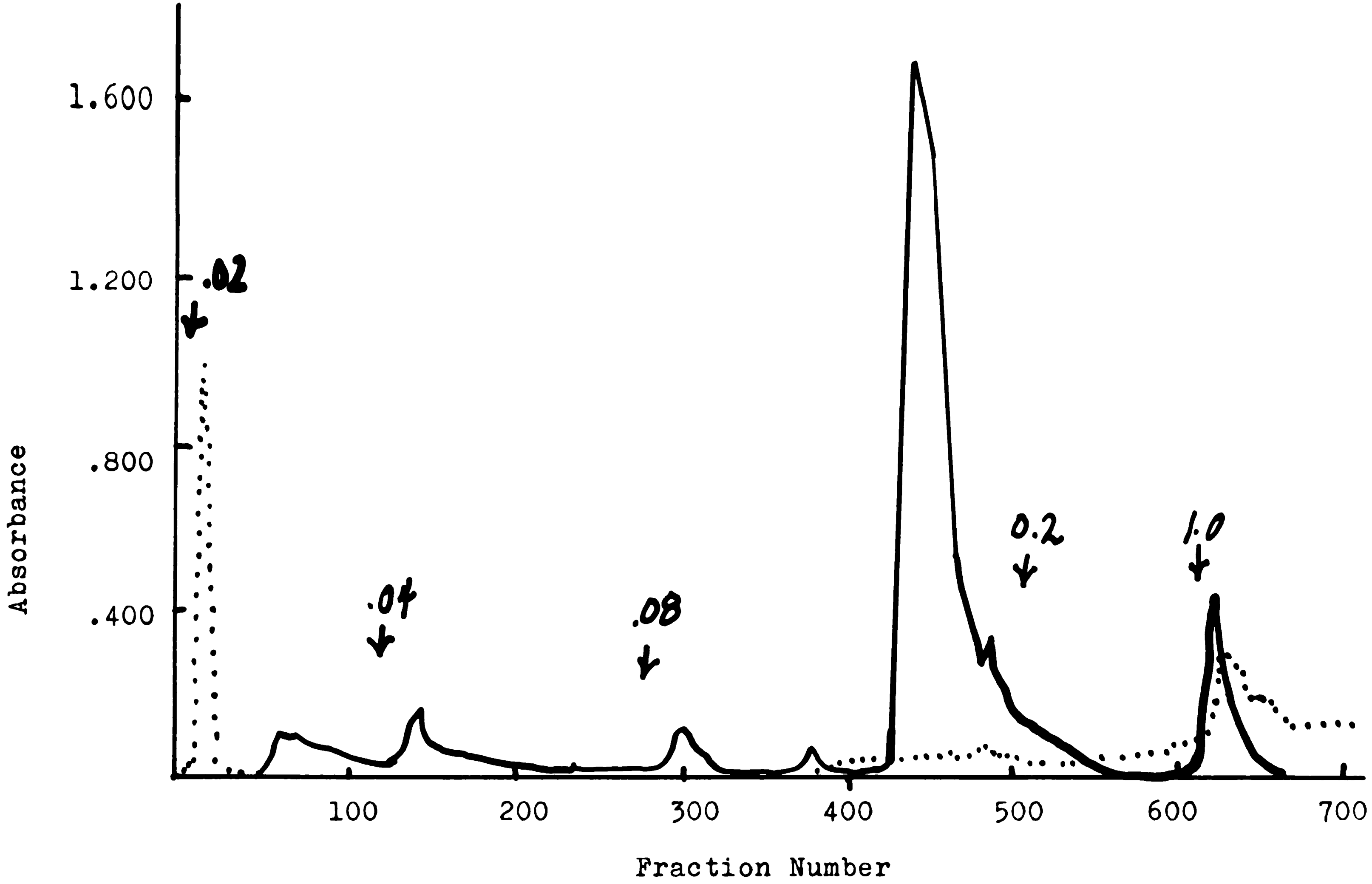

Figure II. Separation of Phosphatase Activity from Sunflower Seedlings on DEAE-Cellulose: Column II

Note: The dotted line represents absorbance at 280mµ.

Table VI

Metal Ion Studies of Peak Tubes from Sunflower Column II

	Optical Density with				
Peak No.	EDTA	Mg^{++}	Zn^{++}	Mn^{++}	$Mg^{++}+Zn^{++}$
305	0.025	0.250	0.115	0.175	0.050
440	0.86	0.84	0.31	0.85	0.32
480	0.02	0.24	0.00	0.19	0.00
625	0.38	0.44	0.175	0.435	0.155

From these results it is probable that tubes 440 and 625 are the same enzyme, while tubes 305 and 480 contain two other distinct enzymes. Thus further studies involving temperature characteristics and pH specificities were undertaken. The results of this work are summarized in Figures III and IV respectively. These provide additional support for the conclusion that tubes 440 and 625 represent the same enzyme while the other peaks represent different enzymes.

C. Wheat Bran Column I

Similar studies were carried out on another source of enzyme, this time one which is known to contain phytase activity. A commercial grade of wheat bran was extracted with tris buffer as described in the Experimental section. The activity of these fractions was ascertained and the results are expressed in Figure V. Metal ion studies were again carried out on the two peak tubes, 32 and 38. Again a typical example of the results is given in Table VII.

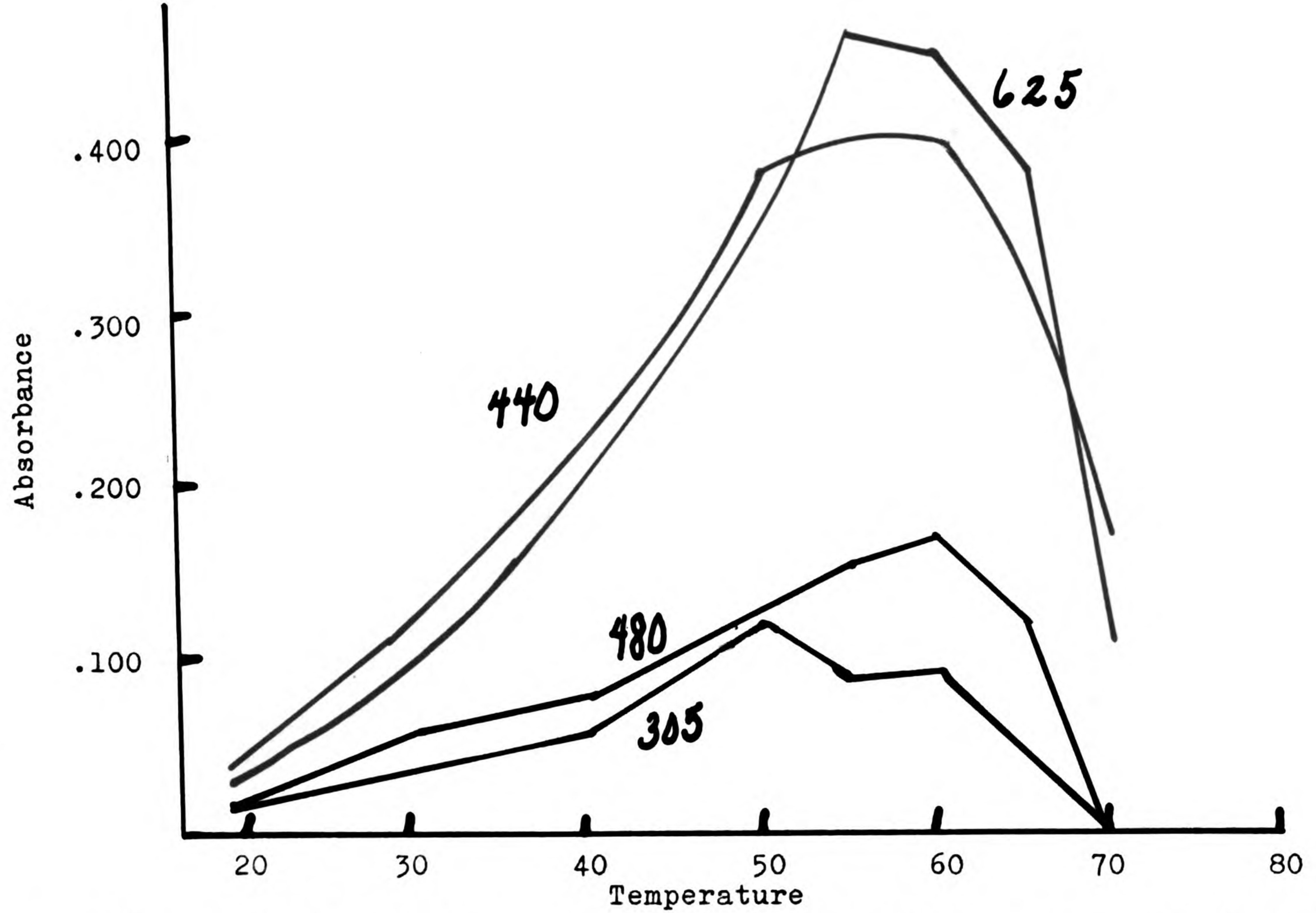

Figure III. Influence of Temperature on Activity of Phosphatases Obtained from Sunflower Column II

Note: The absorbance values for the 440 curve have been divided by two.

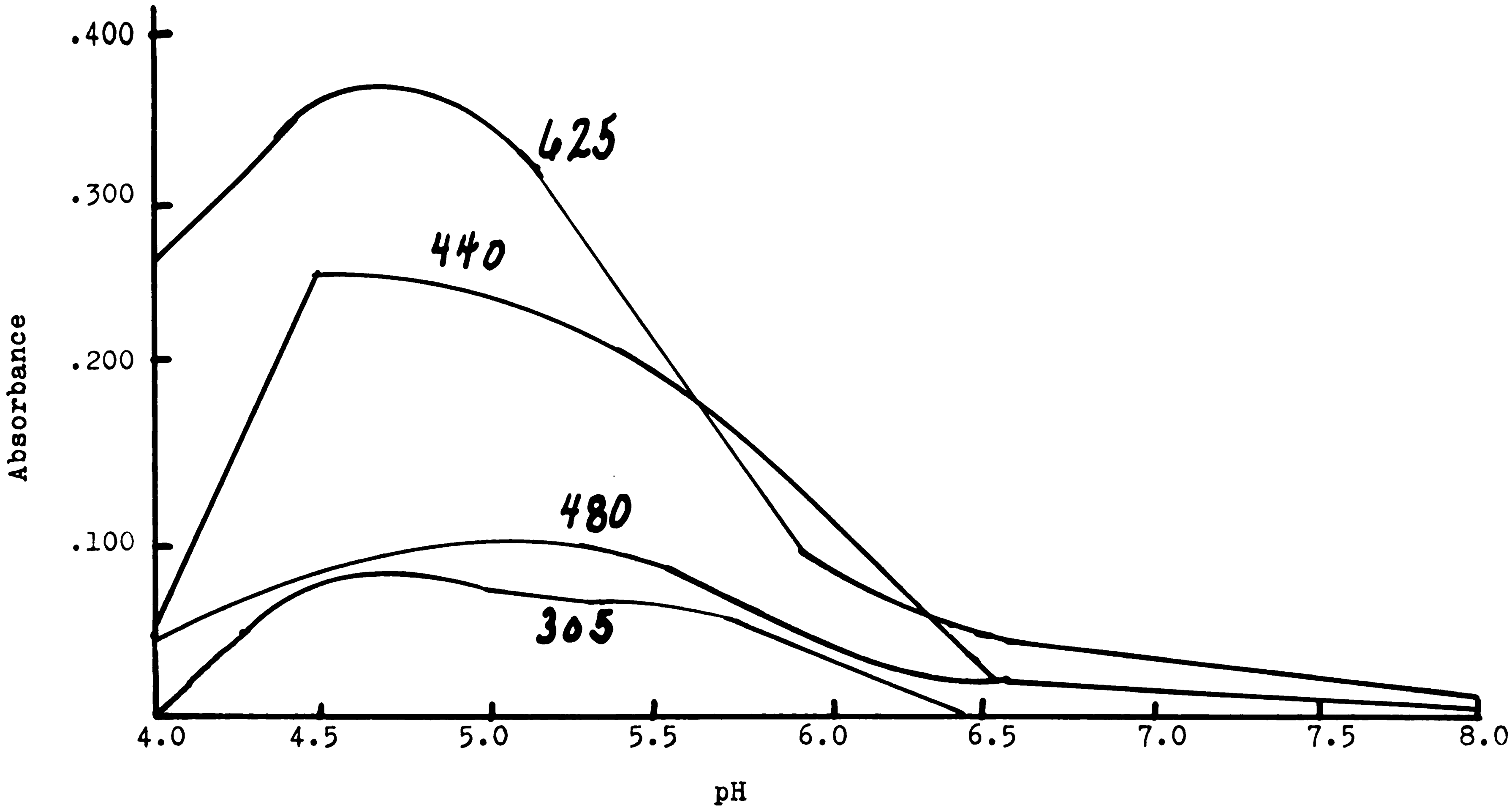

Figure IV. Influence of pH on Activity of Phosphatases Obtained from Sunflower Column II

Note: The absorbance values for the 440 curve have been divided by two.

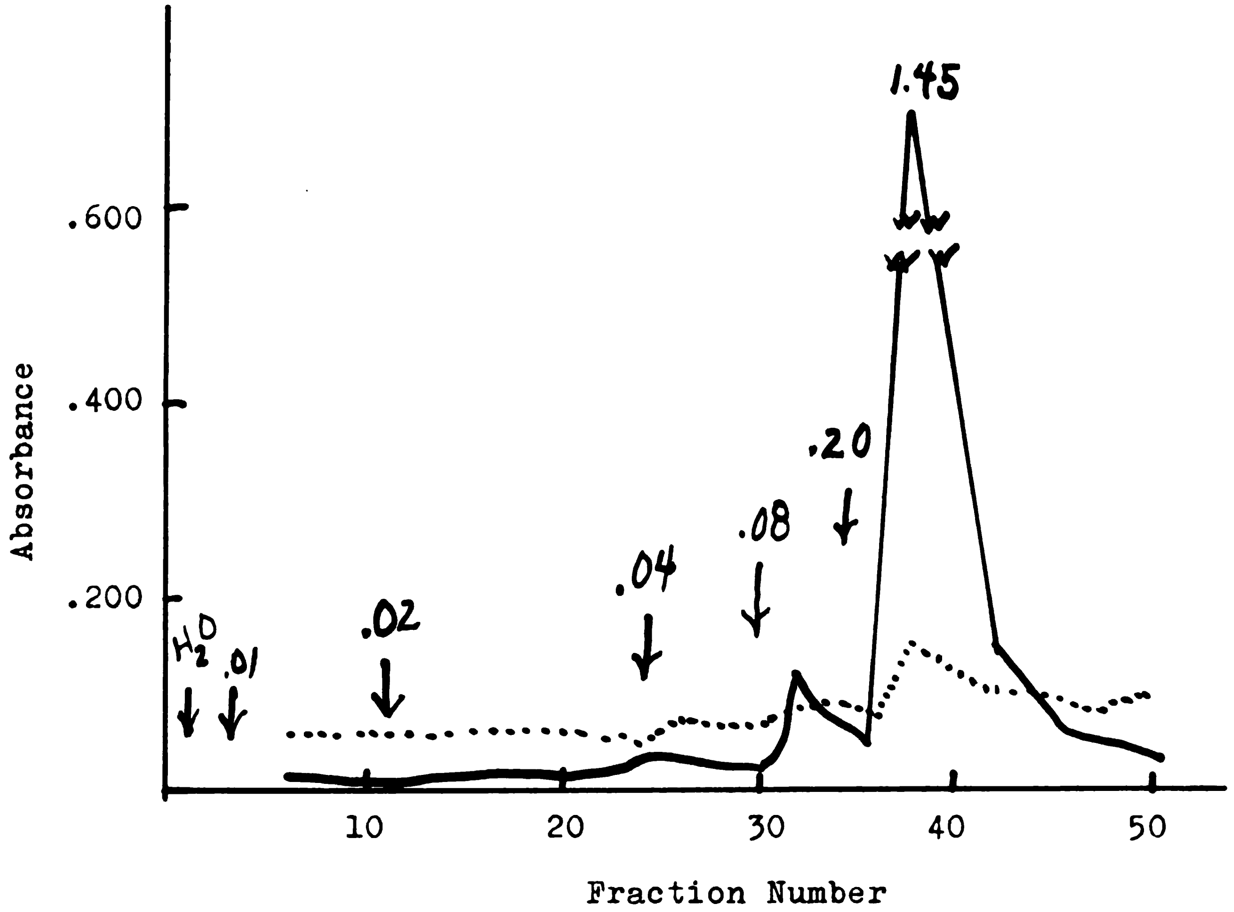

Figure V. Separation of Phosphatase Activity from Wheat Bran on DEAE-Cellulose: Column I

Note: The dotted line represents the absorbance at 280 mμ.

Table VII

Metal Ion Studies on Fractions from Wheat Bran Column I

Peak No.	Optical Density with EDTA	Mg^{++}	Zn^{++}	Mn^{++}	$Mg^{++}+Zn^{++}$
32	0.01	0.035	0.00	0.81	0.015
39	---	0.685	0.205	0.92	0.190

One may suppose the existence of two separate enzyme fractions from this study with little certainty. Further work remains yet to be done in this respect.

It should be noted that on the basis of specific activities (enzyme units per mg. of protein) of the solution put on the column and of tube 32, a 145 fold purification was achieved.

Phytase studies, that is the ability of these enzyme fractions to catalyze the hydrolytic release of inorganic phosphate from phytic acid, have been undertaken but yield little conclusive evidence for the presence of this activity in either of the phosphatase peaks. One cannot be sure if the activity measured is actually phytase activity or merely experimental error in the readings for the activity measured is very low.

D. Wheat Bran Column II

A second attempt to fractionate a wheat bran extract has been carried out, but with poor results. As Figure VI indicates there is little separation of activity. This fractionation was carried out by means of a gradient elution technique which did not give completely satisfactory results. More work remains to be done on this fractionation.

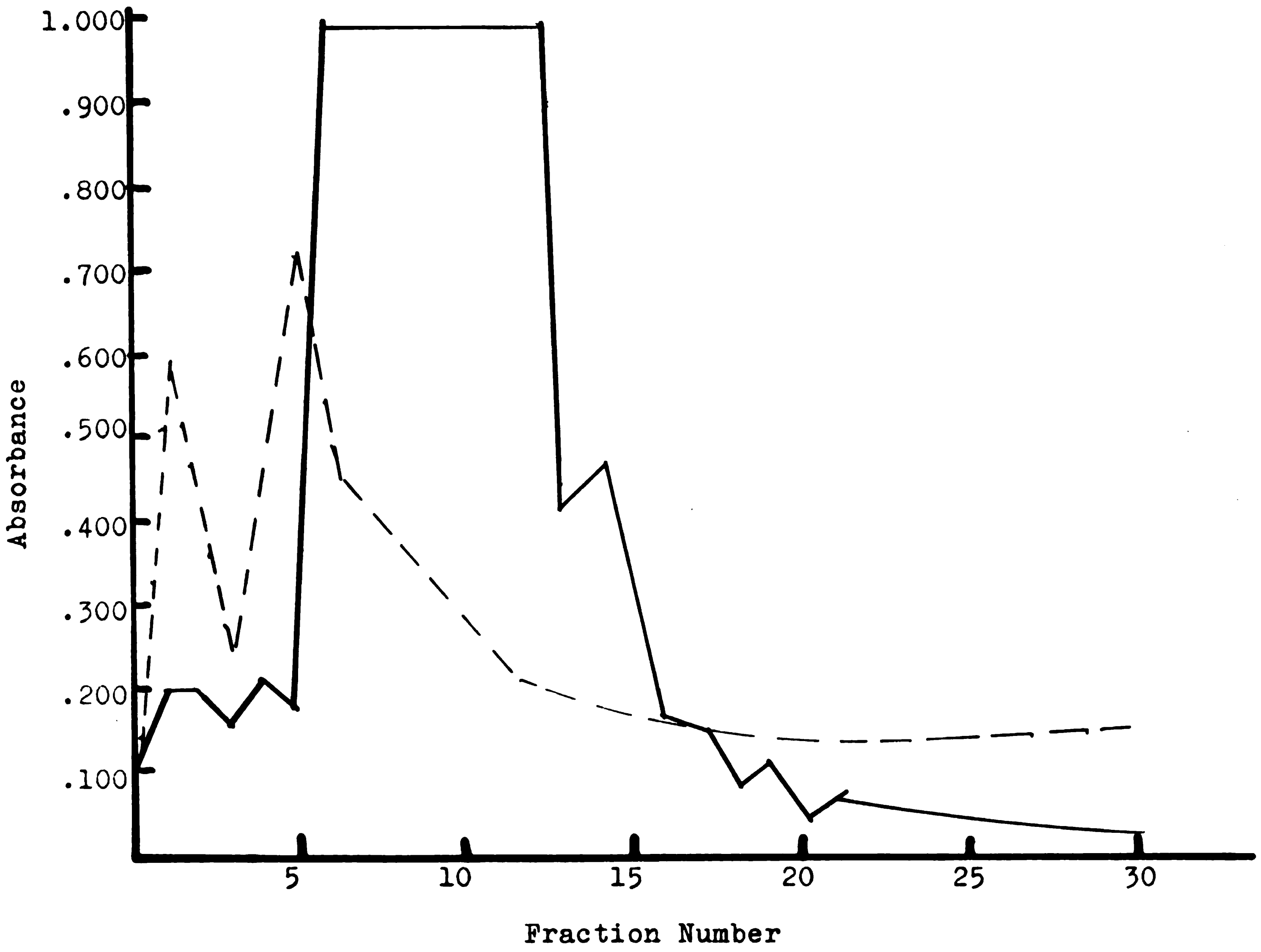

Figure VI. Separation of Phosphatase Activity from Wheat Bran on DEAE-Cellulose: Column II

Note: The absorbance values for the activity curve have been divided by two; the dotted line shows absorbance at 280 mμ.

In summary, it may be concluded that three distinct phosphatases have been separated after one pass on a column of DEAE cellulose and that these enzymes have very little, if any, phytase activity. Preliminary results indicate at least two different phosphatases are present in the extracts of wheat bran. Phytase activity of these enzymes, if present at all, is at a very low level.

EXPERIMENTAL

Reagents

Sunflower seeds (*Helianthus giganteum*), a product of the Olds Seed Co. graciously supplied by Dr. Dalton Allen

Diethylamino ethyl cellulose from Eastman Organic Chemicals (abbreviated: DEAE)

p-Nitrophenyl phosphate, sodium; (Σ 104 grade) from Sigma Chemical Co.

Wheat bran, commercial grade

Molybdate Reagent 2 (A solution of ammonium molybdate in 3 normal sulfuric acid) from the Hartman-Leddon Company

Vermiculite, Terra-Lite brand from the Zonolite Co.

Tris (hydroxy methyl) aminomethane from the Sigma Chemical Co. under their trade name of Sigma 7-9 (abbreviated: tris)

p-Methyl amino phenol sulfate produced by the Eastman Kodak Co. under the trade name of Elon

Preparation of Crude Tissue Extracts

The sunflower seeds were soaked overnight in water, then planted in Vermiculite and harvested after time intervals varying from 3 to 7 days. The two inch seedlings, after removal of the roots, were homogenized in 0.04 molar tris buffer, pH 7.5 for 2 minutes in a Waring Blendor and centrifuged for 30 minutes at 5° C at 40,000 RCF, filtered through glass wool (to yield a cloudy yellow solution) which was then further fractionated. If the seedlings were allowed to grow to a height of six inches the supernatant solution after centrifugation was a clear greenish yellow.

The commercial wheat bran was extracted by homogenizing a sample in 10 volumes of water in a Waring blendor for five minutes, stirring the resulting material overnight with a magnetic stirrer, filtering through cheesecloth and then through glass wool. The solution was then centrifuged at 8,000 RCF at 5° C to yield a clear, light yellow supernatant. This solution was then adjusted to 80% ammonium sulfate saturation and centrifuged for 10 minutes at 4,000 RCF. The resulting pellet was dissolved in less than 20 ml. of water and dialyzed overnight against water at 0° C.

Routine Phosphatase Assay Conditions

3.0 ml. acetate buffer (pH 5.0, 0.1 molar)
1.0 ml. p-nitrophenyl phosphate, sodium salt (50 mg./100 ml.)
0.1 ml. $MgCl_2$ (0.1 molar)
0.5 ml. enzyme solution
Incubated the mixture for one hour at 35° C, then added 5.4 ml. NaOH (0.1 molar) to stop the reaction.

Read at 400mu using a Beckman Model B spectrophotometer.

Note: For assay of alkaline phosphatases, 3.0 ml. acetate buffer was changed to 3.0 ml. of glycine buffer, pH 9.6.

Enzyme Purifications

A. Ammonium Sulfate Fractionations

The solution after centrifugation was adjusted to 1/3, 2/3, and complete saturation with ammonium sulfate. Each of the precipitates was spun down at 3,000 RCF for 20 minutes at 5° C and the resulting pellets suspended in 0.005 molar phosphate buffer, pH 7.0 and frozen. The clear yellow supernatant solution from the 100% saturation procedure was assayed for both acid and alkaline activity and showed no measurable activity. Then each of the dissolved precipitates as well as the 100% supernatant was dialyzed against 0.005 molar phosphate buffer, pH 7.0 at 5° C for sixty hours. Each of the solutions was then assayed for acid and alkaline activity by the standard assay method. The 0-33% saturation fraction seemed to be the most active fraction and contained only 4% nucleic acid. It was refractionated to give precipitates corresponding to 0-10%, 10-20%, and 20-30% saturation. Again the precipitates were suspended in 0.005 molar buffer and dialyzed and stored in the refrigerator. Each fraction was then read at 280 and 260mu and a standard assay for phosphatase

activity was run. Another preparation was carried out and the 0-10%, 10-20% and 20-33% fractions were split into two portions, one of which was dialyzed and the other was not. Each of the eight fractions as well as the original solution was read at 280 and 260mμ. Again each of these fractions was checked for acid and alkaline activity by the standard assay procedures.

B. DEAE-Cellulose Column Separations

Sunflower Column I

An extract of six-inch sunflower seedlings was prepared as outlined above. Ammonium sulfate was added to 70% saturation and the precipitate was collected by centrifugation for 20 minutes at 9750 RCF at 0° C. The pellet was dissolved in distilled water and after dialysis against running distilled water overnight, the solution was read at 280 and 260mμ (after a 1:500 dilution):

$$\frac{280\text{m}\mu}{260\text{m}\mu} = \frac{.280}{.290} = 0.97 \text{ or } 106 \text{ mg. protein/ml.}$$

(The values for protein concentration were obtained from tables by Warburg and Christian (54).) Approximately 50 mg. (½ ml.) was put onto a 12 mm. diameter column containing 1.25 g. DEAE, and eluted with a discontinuous concentration gradient of tris buffer (pH 7.5) according to the following program:

Eluent	Tubes
50 mls. water	1-9
50 mls. 0.01 molar tris	10-17
50 mls. 0.02 molar tris	18-30
50 mls. 0.04 molar tris	31-42
50 mls. 0.08 molar tris	43-49
150 mls. 0.20 molar tris	50-82 (stopped overnight)
50 mls. 0.50 molar tris	83-93
50 mls. 1.00 molar tris	94-98
50 mls. 1.00 molar tris	99-112 (pH 10.6)

The entire procedure was carried out in the cold room at 2° C.

Each of the resulting fractions (approximately 10.0 mls.) was read at 280 and 260mμ and every second tube was assayed according to the routine assay method for both acid and alkaline activity.

A modified procedure was used subsequently on the fractions eluted with the 0.50 molar, and the 1.0 molar tris buffers at pH 7.5, as well as the fractions eluted with the pH 10.6 buffer.

Modified procedures:

<u>0.50 molar</u>

2.75 mls. buffer (0.1 molar acetate pH 5.0)
0.25 mls. HCl 1.0 molar
0.5 mls. enzyme solution
1.0 mls. p-nitrophenyl phosphate at zero time

<u>1.0 molar</u>

2.5 mls. buffer (0.1 molar acetate pH 5.0)
0.5 mls. HCl 1.0 molar
0.5 mls. enzyme solution
1.0 mls. p-nitrophenyl phosphate at zero time

Incubated all tubes ½ hour at 35° C, then added to all tubes

2 mls. NaOH (0.1 normal)
3.5 mls. water to give a total volume of 10.0 mls.

Read on a Beckman Model B spectrophotometer at 400mμ.

In view of the results of these assays, tubes 84, 88, 92, 96, 100, 106, and 109 were dialyzed against running distilled water overnight and re-assayed according to the routine procedure for both acid and alkaline activity.

Sunflower Column II

A second crop of sunflower seedlings was prepared and found to give $\frac{280m\mu}{260m\mu} = \frac{1.15}{1.44}$ (for a 1:100 dilution). Therefore, the original solution contained 69 mg. protein/ml. Approximately 15 mls. of solution (1 g.) were applied to a similar DEAE column which was then eluted with tris buffer pH 7.5 of increasing concentration as follows:

Eluent	Tubes	
1500 mls. 0.02 molar tris	1-129	(123 Let stand overnight)
1500 mls. 0.04 molar tris	130-288	(263 Let stand overnight)
2000 mls. 0.08 molar tris	289-508	(379 Let stand over weekend) (499 Let stand overnight)
1000 mls. 0.20 molar tris	509-610	(519, 587, 596 Let stand overnight)
1000 mls. 1.00 molar tris	611-706	

The resulting fractions were assayed by taking a one ml. aliquot from each of five consecutive tubes and combining them. From this combined solution a one ml. sample was taken for determination of acid activity and protein concentration.

Wheat Bran Column I

An extract from wheat bran was prepared by taking a portion of the filtered solution previously described and adding ammonium sulfate to 80% saturation. The resulting precipitate was dialyzed against running distilled water at 5° C overnight. A ½ ml. aliquot of the resulting solution was diluted to 50 mls. and read at

$\frac{280m\mu}{260m\mu} = \frac{.104}{.087} = 1.20$ Therefore, there is 98 mg. protein/ml.

One-half ml. of the dialysate was then put on a 12 mm. diameter column containing 0.5 gm. DEAE which had been prewashed with 0.1 molar tris, pH 7.5, to neutral effluent which required about 250 ml. The column was then eluted with tris buffer pH 7.5 according to the following scheme:

Eluent	Tubes
10 mls. water	0-1
50 mls. tris 0.01 molar	2-10
100 mls. tris 0.02 molar	11-23
50 mls. tris 0.04 molar	24-29
50 mls. tris 0.08 molar	30-35
150 mls. tris 0.2 molar	36-50

Assays were run on the fractions for both acid and alkaline activity and protein concentration.

Wheat Bran Column II

A second identical column was prepared through which was passed 10 mls. of the dialysate (equivalent to 60 mg. of protein). The column was then eluted with tris buffer pH 7.5 by the gradient elution technique starting with 125 mls. of water in the single mixing flask and adding 0.5 molar buffer. Each fraction was assayed for acid phosphatase activity as well as protein concentration.

Characterization of the Various Enzyme Fractions

A. Metal Ion Studies

Assays were run on some of the peak tubes from the Sunflower Column II fractions to see if any two or more of the peaks represented identical enzymes. Five of the tubes near and including the peak tube were combined and used as the enzyme source. Assays were then run according to the routine assay procedure except that various metals were substituted for magnesium, such as the chlorides of zinc, manganese, and the disodium salt of ethylene diamine tetraacetic acid (EDTA). Any precipitates that were formed were spun down before the tubes were read.

Similar experiments were carried out on the peak tubes from the wheat bran fractionations.

B. Temperature Optima

Studies on the temperature optima of several peaks from the second fractionation of sunflower seedling extracts were run. Routine assay procedure was followed except that instead of incubation at 35° C for one hour, assays were run for one-half hour at 20°, 30°, 40°, 50°, 55°, 60°, 65°, and 70° C.

C. pH Optima

Studies on the pH optima of four of the fractions from the second fractionation of sunflower seedlings were also undertaken. Buffers at each 0.5 pH unit were used from 4.0 to 9.0 in the routine assay scheme. The buffers, each 0.1 molar, were succinate from pH 4.0 to 7.0, and tris from pH 7.5 to 9.0.

D. Phytase Studies

A further attempt at characterization was also made by studying the phytase activity (that is the ability of the enzyme fractions to split phosphoric acid from phytic acid). The assay (a determination of inorganic phosphate) was run as follows:

3.8 mls. acetate buffer (0.1 molar, pH 5.0)
0.4 mls. phytic acid solution
0.1 mls. $MgCl_2$ (0.1 molar)
0.2 mls. water
0.5 mls. enzyme sample

An aliquot of 1.0 ml. was taken at zero time and at one hour. These samples were immediately added to tubes containing 7 mls. of water and 1 ml. of Molybdate Reagent 2. Color was produced upon the addition of 1 ml. of bisulfite-Elon reducing solution (1% Elon-3% sodium bisulfite) and allowed to develop for 20 minutes before being read at 660mμ in the Beckman Model B spectrophotometer. The readings were referred to a standard curve for determination of amount of phosphate released. These assays were run on the solution prepared for addition to Sunflower Column II as well as both the wheat bran preparations. Phytase assays were also run on every fifth tube from the first wheat bran fractionation.

The phytic acid used in the assay was prepared by adding a sample of barium phytate to IR 120 (H). The resin removed the barium giving soluble phytic acid. The solution was then assayed for total phosphate and inorganic phosphate according to the following procedure (which is described in Carter, "Experimental Biochemistry", Second Reprint, page 66 (55):

Total phosphate:

Two mls. of the sample and 0.5 mls. of 10 normal sulfuric acid are heated to 130°-160° C for thirty minutes and cooled. Then 2 drops of 30% H_2O_2 are added and the mixture is again heated to 130°-160° C for 15 minutes. After cooling, 2 mls. of water are added and the resulting solution is heated to boiling in a water bath for 10 minutes and cooled. The assay for inorganic phosphate can now be run on this solution and the amount of total phosphate determined by comparison to a standard curve.

Inorganic phosphate:

Run assay according to above procedure.

The difference between total phosphate and inorganic phosphate can be used as a measure of the concentration of phytic acid in the sample.

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