Phytochemistry, 1967, Vol. 6, pp. 171 to 179. Pergamon Press Ltd. Printed in England

PHOSPHORIC MONOESTER HYDROLASES IN COTYLEDONS OF GERMINATING PEANUT SEEDS (*ARACHIS HYPOGAEA* L.)*

C. T. DUDA, AMOS E. RICHMOND† and JOE H. CHERRY

Horticulture Department, Purdue University, Lafayette, Indiana

(*Received* 20 *May* 1966)

Abstract—A tentative characterization of the phosphatase activity in cotyledons of germinating peanut seeds is presented. Ammonium sulfate precipitation of the crude homogenate yielded a fraction that included most of the phosphohydrolytic activity and indicated the presence of inhibitor(s) specific for P-choline phosphohydrolysis in the crude extract. Assay of the ammonium sulfate fraction with ATP displayed two pH optima. One was at pH 5·3, the other at pH 8·9. Activity toward non-nucleotide P-esters was confined to acid pH. Chromatography of the ammonium sulfate precipitate on a DEAE-Sephadex column resolved four major peaks of phosphohydrolytic activity. Peaks I and II apparently represent orthophosphoric monoester phosphohydrolases (EC 3.1.3.2) of wide substrate specificity at pH 5·0. Peaks III and IV appear to represent 5′-ribonucleotide phosphohydrolases (EC 3.1.3.5) with the unique feature of displaying activity against ATP and ADP over an unusually wide pH range. The activity profile of the DEAE-Sephadex eluate obtained by assaying at pH 5·0 was very similar to that at pH 8·5 suggesting that the phosphohydrolytic activity of a given fraction in both acid and alkaline conditions resided in a single enzyme. Kinetic data supplement the thesis that the four DEAE-Sephadex peaks represent separate enzymes.

INTRODUCTION

WHILE studying the RNA nucleotidyl transferase of peanut cotyledons, we observed pronounced phosphatase activity which interfered with the assay for nucleotide polymerization.[1] Nonspecific acid or alkaline phosphatase (orthophosphoric monoester phosphohydrolase EC 3.1.3.2 and EC 3.1.3.1, respectively) has been isolated from many higher plants[2–5] and animal[6, 7] tissues, as well as from yeast [8] and bacteria.[9, 10] In all cases, the phosphatases exhibited optimal activity either in the pH range from 4·5 to 6·5 (acid phosphatase) or in the pH range from 8·0 to 10·0 (alkaline phosphatase). Since we found that the phosphatase activity extracted from peanut cotyledons exhibited pH optima in each of the

* This investigation was supported by grants from the National Science Foundation, GB-622 and the American Seed Research Foundation and a contract from the U.S. Atomic Energy Commission, COO-1313-12. Journal paper 2752 of the Purdue Agriculture Experiment Station.

† Present address: The Negev Institute for Arid Zone Research, Beer-Sheva, Israel.

Abbreviations used: *p*-NPP, *p*-nitrophenyl phosphate disodium salt; *o*-CPP, *o*-carboxy phenol phosphate; P-choline, phosphocholine chloride Ca; P-serine, *o*-phospho-DL-serine; glucose-6-P, D-glucose 6-phosphate; fructose-1,6-diP, fructose-1,6-diphosphate; Pi, orthophosphate.

[1] A. E. RICHMOND and J. H. CHERRY, *Biochim. Biophys. Acta* **91**, 330 (1964).
[2] B. AXELROD, *J. Biol. Chem.* **167**, 57 (1947).
[3] J. L. YOUNG and J. E. VARNER, *Arch. Biochem. Biophys.* **84**, 71 (1959).
[4] B. K. JOYCE and S. GRISOLIA, *J. Biol. Chem.* **235**, 2278 (1960).
[5] H. J. PRESLEY and L. FOWDEN, *Phytochem.* **4**, 149 (1965).
[6] H. SCHUEL and N. G. ANDERSON, *J. Cell. Biol.* **21**, 309 (1964).
[7] D. P. WALLACH and H. KO, *Can. J. Biochem.* **42**, 1445 (1964).
[8] M. KUNITZ, *J. Gen. Physiol.* **35**, 423 (1952).
[9] L. A. HEPPEL, D. R. HARKNESS and R. J. HILMOE, *J. Biol. Chem.* **237**, 841 (1962).
[10] A. GAREN and C. LEVINTHOL, *Biochim. Biophys. Acta* **38**, 470 (1960).

above pH ranges, phosphohydrolase activity was studied under both acid (pH 5·0) and alkaline (pH 8·5) conditions. The data presented here suggest that the gross phosphohydrolytic activity in the cotyledon homogenate resides in at least four separate enzymes; two of these seem to display equal reaction velocity toward either ATP or ADP under both acid and alkaline conditions.

RESULTS

It was of interest to see if phosphatase activity of peanut cotyledons varied with age of the seedling in a manner similar to that of other enzymes found in this tissue.[11] Figure 1 relates

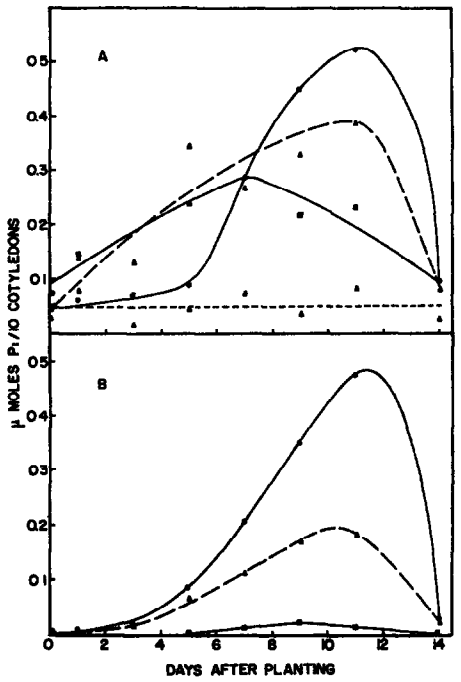

FIG. 1. PHOSPHOMONOESTER HYDROLASE ACTIVITY IN THE COTYLEDONS OF GROWING PEANUT SEEDLINGS.
Crude homogenates were prepared on the days indicated and the standard assay for phosphohydrolase activity was carried out (see methods), with substrates: ●——●, ATP; △——△, ADP; ▲----▲ glucose-6-P; ■——■, *o*-CPP; and □——□, *p*-NPP. A, records the activity assayed at pH 5·0 and B, the activity at pH 8·5.

the phosphomonester hydrolase activity in the crude extract to the age of the seedling. When assayed with ATP or ADP the activity followed the same pattern whether incubated in pH 5·0 or pH 8·5. Activity increased up to the eleventh day and decreased with the rapid decline in fresh weight and the advent of morphological deterioration. The fresh weight of the cotyledons increased up to the seventh day, declining thereafter. By the fourteenth day fresh weight was reduced to about a third of the maximum. It was previously found[11] that within this same period (0–14 days) the total protein content of the cotyledons continuously decreased and that little if any cell division occurred.

[11] J. H. CHERRY, *Plant Physiol.* **38**, 440 (1963).

Effect of pH. The optimal pH of the $(NH_4)_2SO_4$ fraction was dependent on the phosphate ester used as substrate. With ATP the enzyme preparation showed two pH optima, one at pH 5·3 and the other at pH 8·9 (Fig. 2). This was not expected, since previous reports dealing with plant phosphatases recorded only one pH optimum, usually in the acid region. The enzyme preparation did not exhibit two pH optima when other substrates were used: with ADP the pH had no effect on relative reaction velocity between 5·0 and 7·5; with glucose-6-P the pH optimum was 5·6 and with *o*-CPP the optimum was 5·3. Though the phosphohydrolytic activity with ADP decreased in pH above 7·5, approximately half of the maximum activity was still present at pH 8·5. With glucose-6-P low activity was registered in alkaline pH. However, phosphohexoisomerase activity was not determined in any of the enzyme preparations used in these studies.

Measurements of phosphatase activity under acid conditions (pH 4·5–6·5) with *o*-CPP as substrate indicated little change in activity within a few tenths of a pH unit of the optimum

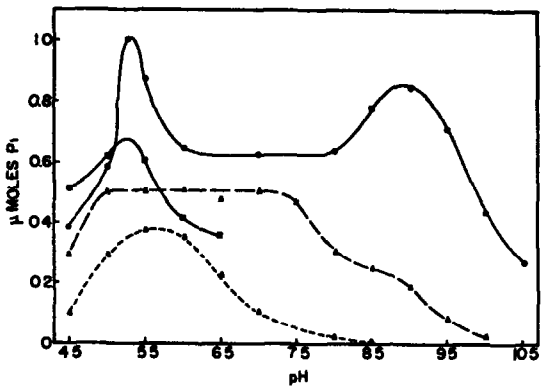

FIG. 2. EFFECT OF pH ON THE RELATIVE REACTION VELOCITY OF THE PHOSPHOHYDROLASE ACTIVITY IN THE $(NH_4)_2SO_4$ FRACTION.

Standard conditions of assay were employed. For the indicated pH ranges 158 µmoles of the corresponding buffer were used: pH 4·5–5·5, acetate; pH 6·0–8·5, Tris maleate; and pH 9·0–10·5, glycine–NaOH. Substrates were ●——●, ATP; △——△, ADP; ▲----▲, glucose-6-P; and ■——■, *o*-CPP.

at 5·3. Similarly, studies under alkaline conditions (pH 7·5–10·5) with *p*-NPP yielded no clear-cut pH optimum. It was therefore felt that pH 5·0 and pH 8·5 would be suitable for determining the degree of phosphatase activity under acid and alkaline conditions, respectively.

Column separation. Figure 3 records the chromatography of the $(NH_4)_2SO_4$ fraction on DEAE-Sephadex. The number of peaks of phosphatase activity detected was dependent upon both the pH of the incubation medium and the substrate. When assayed with ATP at pH 8·5 only two peaks were evident. However, at pH 5·0 four distinct peaks were always obtained. In the case of P-serine (Fig. 3C) assayed at pH 5·0, there appeared three additional peaks of activity which were not present in the other two profiles. No significant activity could be detected at pH 8·5 with P-serine. It is evident from Fig. 3 that when ADP was used as substrate the relative reaction velocity at both pH 5·0 and pH 8·5 reaches a maximum at the same point. This is also true for the last two peaks with ATP as substrate. Further, the points of maximum phosphatase activity assayed with the different substrates coincided quite closely (Fig. 3).

Specificity. It is evident from Table 1 that the phosphohydrolytic activity of the crude fraction did not vary appreciably from that of the $(NH_4)_2SO_4$ fraction, except against P-choline and P-serine assayed at pH 5·0. The phosphohydrolytic activity of the $(NH_4)_2SO_4$ fraction against P-choline and P-serine was 17 and 2·5 times greater, respectively, than that of the crude fraction. We feel that the large increase in activity with P-choline indicates the presence of a specific inhibitor(s) for P-choline hydrolysis in the crude extract. This lack of activity could not be relieved by dialysis (at 0° for 48 hr).

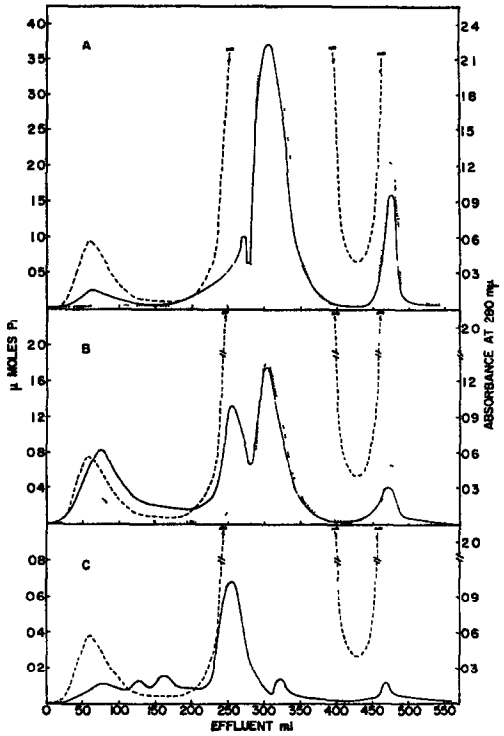

FIG. 3. DISTRIBUTION OF PHOSPHOHYDROLASE ACTIVITY OF THE $(NH_4)_2SO_4$ FRACTION AFTER PASSAGE THROUGH DEAE-SEPHADEX (SEE METHODS).

Void volume of buffer was allowed to pass through the column before fractions were collected. Phosphatase activity of alternate tubes was detected with the standard assay, using ATP (A), ADP (B) and P-serine (C) as substrates. With the latter phosphate ester the incubation period was extended to 30 min. ——, assay carried out at pH 5·0; ·····, assay carried out at pH 8·5 and – – – –, absorbance at 280 mμ.

Assay of the DEAE-Sephadex peaks indicated that they differed greatly in hydrolytic capacity against various phosphate esters (Table 1). At pH 8·5 no detectable hydrolysis of ATP by DEAE Peaks I and II was observed while DEAE Peaks III and IV exhibited a high degree of activity at this pH. At pH 5·0, Peaks I and II exhibited a much higher degree of phosphohydrolase activity than Peaks III and IV with AMP, glucose-6-P, fructose-1,6-diP and P-serine. However, of these four esters only AMP and P-serine are hydrolysed to any extent at pH 8·5. Table 1 also shows that virtually all the phosphohydrolytic activity at pH 5·0 against P-choline and AMP resided in Peak I.

Table 2 records a comparison of the phosphohydrolytic activity of a given peak with ADP to the activity with another ester at the same pH; similar ratios would indicate identical enzymes. In fact the ratios demonstrate wide variability between the four peaks. A comparison of the phosphohydrolase activity of Peak II at pH 5·0 with ADP as substrate to the

TABLE 1. PHOSPHOMONOESTER HYDROLASE ACTIVITY ON SEVERAL PHOSPHATE ESTERS AT VARIOUS STAGES OF PURIFICATION†

	Fraction				DEAE Peaks							
	Crude		30–70% $(NH_4)_2SO_4$		I		II		III		IV	
Substrate	pH 5·0	pH 8·5	pH 5·0	pH 8·5	pH 5·0	pH 8·5	pH 5·0	pH 8·5	pH 5·0	pH 8·5	pH 5·0	pH 8·5
	μmoles Pi/hr/mg protein											
ATP	16·6	15·0	19·8	17·2	12·0	0	20·0	0	45·0	46·2	21·2	21·8
ADP	14·0	7·0	12·2	7·4	55·6	7·2	33·2	2·4	20·6	24·0	7·6	9·6
AMP	7·0	1·4	6·2	1·8	36·4	15·8	9·0	0·6	1·2	0·2	1·2	0·4
Glucose-6-P	2·6	0·4	5·0	0·2	13·0	0	14·4	0	0·8	0	1·4	0·2
Fructose-1, 6-diP	*	*	*	*	20·4	0	22·6	0·2	2·4	0·6	1·8	1·8
P-Choline	0·6	0·2	10·6	1·6	2·2	0	0·8	0	0	0	0·2	0
P-Serine	1·4	0·4	3·6	0·2	15·2	4·0	10·6	0	0·6	0	1·2	0·2
o-CPP	7·4		9·2		30·0		26·8		5·6		5·4	
p-NPP		0·6		0·8		1·0		1·4		0·4		0·4

* Not assayed.
† Standard conditions of assay were used.

TABLE 2. COMPARISON OF THE PHOSPHOHYDROLASE ACTIVITY WITH ADP TO THE ACTIVITY WITH OTHER PHOSPHATE ESTERS†

	DEAE Peaks							
	I		II		III		IV	
Ratio	pH 5·0	pH 8·5	pH 5·0	pH 8·5	pH 5·0	pH 8·5	pH 5·0	pH 8·5
ADP/ATP	4·6	*	1·7	*	0·5	0·5	0·4	0·4
ADP/AMP	1·5	0·5	3·7	4·0	17·2	120·0	6·3	24·0
ADP/Glucose-6-P	4·3	*	2·3	*	25·8	*	5·4	48·0
ADP/Fructose-1,6-diP	2·7	*	1·5	12·0	8·6	40·0	4·2	5·3
ADP/P-Serine	3·7	1·8	3·1	*	34·4	*	6·3	48·0
ADP/*o*-CPP	1·9		1·2		3·7		1·4	

* No activity at pH 8·5.
† The data from Table 1 were used for these calculations.

corresponding value for ATP discloses that the latter phosphate ester was hydrolyzed twice as fast as the former. However, a similar comparison with Peak I demonstrates that the hydrolytic activity on ADP was 4·6 times higher than that on ATP. A comparison with AMP (i.e. ADP/AMP) at pH 8·5, yielded even larger differences between the four peaks. The high figure obtained for this ratio in Peak III simply reflects the small degree of hydrolytic activity obtained with AMP.

Influence of substrate concentration on enzyme activity. In order to determine K_m and V_{max} the velocity of peak phosphatases was determined at various concentrations of substrate (0·5, 1·0, 2·5, 5·0 and 10·0 mM). The data were plotted on a double-reciprocal axis (Lineweaver–Burk plot[12]) and the values obtained are shown in Table 3. ADP was not suitable for kinetic studies of Peak I because of its high activity with AMP which affected the reaction rate as the ADP was progressively hydrolyzed. Similarly, all attempts to determine K_m and V_{max} of the four DEAE peaks with ATP were complicated by their high activity against ADP. Table 3 shows that values for K_m and V_{max} at either pH are quite different for each peak. Further, if the peaks contained the same enzyme one would expect that the ratio of the K_m value at pH 5·0 to that at pH 8·5 would be the same for each peak. With use of the values from Table 3 this ratio varies from 0·34 to 2·0, amplifying the differences in substrate affinities of the DEAE four peaks.

TABLE 3. MICHAELIS–MENTEN CONSTANT (K_m) AND MAXIMUM VELOCITY (V_{max}) FOR THE DEAE-SEPHADEX PEAKS*

DEAE Peak	Substrate	$K_m \times 10^3$ pH 5·0	$K_m \times 10^3$ pH 8·5	$V_{max} \times 10^4$ pH 5·0	$V_{max} \times 10^4$ pH 8·5
I	AMP	1·5	4·4	1·9	0·6
II	ADP	5·8	2·9	3·5	1·7
III	ADP	2·2	5·6	1·2	2·3
IV	ADP	2·2	2·7	0·5	0·6

* V_{max} is expressed as moles per liter per min.

Heat liability. The heat inactivation pattern for the individual DEAE peaks shown in Fig. 4 was obtained by assaying each peak with the substrate with which it showed highest activity (refer to Table 1). Phosphatase from each of the four peaks was not inactivated by exposure to 50° for 5 min, but a sharp decline occurred when the enzyme was exposed to a temperature a few degrees higher. A decrease in activity was observed for all four peaks, when the heated material was left at room temperature for 24 hr. Except for Peak II the regeneration pattern was essentially the same as the pattern obtained immediately after the heat treatment.

Loss of half the original activity occurred at 61°, 58°, 53° and 56° for Peaks I, II, III and IV, respectively. The drop in activity with increasing temperature did not occur at the same rate for the enzyme in each peak. In the region 50–55°, Peak I enzyme lost 32% of its original activity while Peak III enzyme lost twice this proportion (70%). After 50° the phosphatase activity of Peaks II and IV, unlike that of Peaks I and III, decreased linearly with temperature. The protein of Peaks I and II appears to be less sensitive to heat denaturation than that of Peaks III and IV: since at 65° Peaks I and II retained 33 and 20% respectively, of original activity and almost no activity was observed for Peaks III and IV.

The heat lability of the phosphohydrolytic activity reported here is much greater than that of the acid phosphatase of pea seed cotyledons,[3] where the enzyme was not inactivated by exposure to 70° for 5 min. However, the acid phosphatase purified from wheat germ by

[12] H. LINEWEAVER and D. BURK, *J. Am. Chem. Soc.* **56**, 658 (1934).

Joyce and Grisolia[4] showed a much lower resistance to heat, since 5 min of heating at 50° destroyed about 75% of the activity.

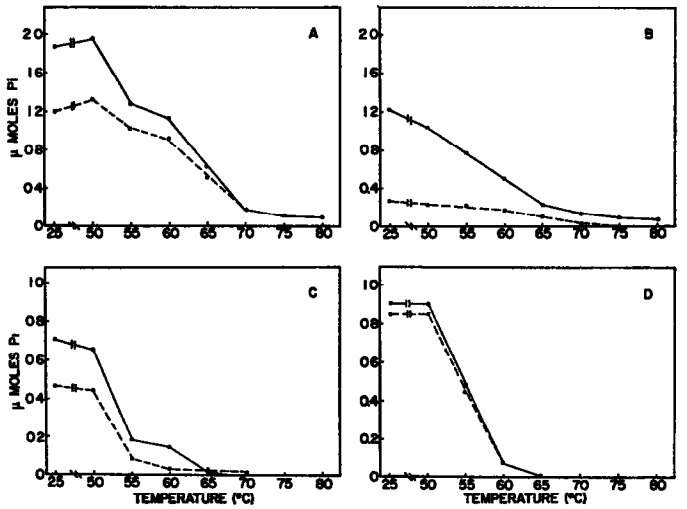

FIG. 4. EFFECT OF TEMPERATURE ON PHOSPHOHYDROLASE ACTIVITY IN THE DEAE PEAKS (REFER TO FIG. 3).

An aliquot of the eluate from each peak together with 68 μmoles, Tris buffer, pH 8·2, 4 μmoles $MgCl_2$ and 6 μmoles NaCl in a final volume of 0·6 ml, was held at the indicated temperature for 5 min and then rapidly cooled to room temperature. Phosphatase activity was determined under standard conditions of assay immediately after cooling, ●——● and after storage at 23° for 24 hr, ●- - - -●. Peaks I(A) and II(B) were assayed at pH 5·0 with ADP, Peak III at pH 5·0 with ATP (C) and Peak IV at pH 8·5 with ATP (D). Amount of protein used in the heated mixture was 0·558 mg, 0·940 mg, 0·230 mg and 1·566 mg for A, B, C and D, respectively.

DISCUSSION

The data presented in this paper suggest that the gross phosphomonoesterase activity in the cotyledons of germinating peanut seeds is due to at least four different enzymes. The presence of a mixture of phosphatases in plant[13, 14] and animal[6] organs has been reported previously. In both types of tissue maximum activity was confined to the acid range. Likewise, Rogers and Reithel succeeded in separating five acid phosphatases from *E. coli*.[15] Uniquely, cotyledons of germinating peanuts contain both acid and alkaline phosphatase activity, the pattern of development during germination being similar for both activities (as assayed at pH 5·0 and pH 8·5, respectively).

The significance of the activity profile (Fig. 3) obtained for the ammonium sulfate fraction after passage through DEAE-Sephadex is twofold. Maximum phosphohydrolase activity assayed with ATP and ADP at pH 5·0 chromatographed with maximum activity at pH 8·5. This suggests that the hydrolytic activity in both acid and alkaline media resides in a single enzyme. Furthermore, it is evident that the enzyme displayed low substrate specificity, since maximum activity against ATP, ADP and P-serine was contained in the same fraction. It was previously found[16] that at alkaline pH acid phosphatase from *E. coli* tended to polymerize

[13] H. BOROUGHS, *Arch. Biochem. Biophys.* **49**, 30 (1954).
[14] D. W. A. ROBERTS, *J. Biol. Chem.* **219**, 711 (1956).
[15] D. ROGERS and F. J. REITHEL, *Arch. Biochem. Biophys.* **89**, 97 (1960).
[16] V. B. HOFSTERN and J. PORATH, *Acta Chem. Scand.* **15**, 1791 (1961).

and form complexes with other proteins. Such polymerization or complex formation would not readily account for the distribution of phosphatase in the DEAE-Sephadex column reported here, because of the great variation in the degree of substrate specificity exhibited by the various peaks. The variability in the values of K_m and V_{max}, and the heat inactivation observed for the four peaks agrees with the conclusion that the peaks represent separate enzymes.

The data in Table 1 allow a tentative characterization of the phosphomonoesterase activity contained in the four DEAE-Sephadex peaks. Perhaps the easiest one to classify is Peak II, which clearly represents an acid phosphatase (EC 3.1.3.2) capable of hydrolyzing a variety of substrates. Peaks III and IV exhibited high activity with ATP and ADP and, except for *o*-CPP, the activity on the other substrates tested is low. Therefore, these two peaks represent 5'-nucleotidases (EC 3.1.3.5) with a unique low specificity to pH, being equally active at either pH 5·0 or pH 8·5. The classification of Peak I is rather difficult. In general it may be said to represent an acid phosphatase, since the phosphohydrolytic activity at pH 5·0 is greater than the activity at pH 8·5. However, at pH 8·5 this peak exhibits the interesting property of being able to hydrolyze AMP and ADP but not ATP.

EXPERIMENTAL

Nucleotides and glucose-6-P[1] were purchased from the Sigma Chemical Company. The other phosphate esters, with the exception of fructose-1,6-diP and *o*-CPP, were obtained from Calbiochem. The former ester was obtained from Nutritional Biochemical Company and the latter from Biochemical Research Corporation.

DEAE-Sephadex A-50 (medium), lot number TO 4330, was purchased from the Pharmacia Fine Chemicals Inc. (New Jersey). All other chemicals were obtained from the Fischer Scientific Company.

Protein was estimated by the method of Lowry *et al.*[17] Samples were prepared for analysis by precipitating the protein with 50 vol of cold 10% trichloroacetic acid, washing the precipitated protein twice with cold acid and dissolving the washed precipitate in 0·1 N NaOH. Recrystallized bovine serum albumine was used as standard. Pi was measured by the method of Allen.[18]

Assay. The standard assay for phosphatase activity consisted of the following components mixed to a final volume of 1·0 ml: 158 μmoles buffer (acetate, pH 5·0 or Tris-maleate, pH 8·5) 10 μmoles of $MnCl_2$, 2·5 μmoles substrate and 0·1 ml of enzyme preparation. After 15 min incubation at 35° the reaction was stopped by adding 10 ml of 1 M $HClO_4$. Insoluble material was removed by centrifugation and aliquots of the supernatant were tested for Pi. In the enzymic assays the activity varied linearly with enzyme concentration. Specific activity is expressed as micromoles phosphate ester hydrolyzed per hour per mg protein, under the conditions of the assay indicated above.

Fractionation. Peanut seeds (*Arachis hypogaea* L.) were sown in wet artificial soil (1:1 mixture of Perlite:Vermiculite) and grown in darkness and high relative humidity at 28 ± 1° for 8 days. Cotyledons excised from seedlings of uniform height were homogenized by means of a VirTis homogenizer in the cold with an equal amount (wt./vol.) of cold 0·01 M Tris buffer, pH 7·5. Alternate half-minute grinding periods at high speed and low speed were employed for a total of 4·5 min. The homogenate was strained through cheesecloth and then

[17] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[18] R. J. L. ALLEN, *Biochem. J.* **34**, 858 (1940).

centrifuged at 10,000 × *g* for 30 min in order to remove cellular debris and free fat (all centrifugations were carried out at 0°). The debris- and fat-free supernatant will be regarded as the crude extract. The crude extract was brought to 30% saturation with solid $(NH_4)_2SO_4$ and stirred for 5 min in an ice bath. It was then centrifuged at 5000 × *g* for 10 min and the supernatant decanted, brought to 70% saturation with respect to $(NH_4)_2SO_4$ and recentrifuged. The resulting pellet was collected and suspended in 0·01 M Tris buffer, pH 7·0. This formed the $(NH_4)_2SO_4$ fraction.

Chromatography. Twenty grams of DEAE-Sephadex A-50 (medium) were allowed to swell in 2 l. of cold distilled water for 1 week (fine suspended material was periodically decanted). The Sephadex mixture was then brought to room temperature, the excess liquid decanted and the Sephadex washed several times with 0·02 M Tris-maleate buffer, pH 8·0. The Sephadex suspension was deaerated under vacuum for 30 min.

A column (5 × 28 cm) was prepared from the washed and deaerated Sephadex and left to pack under gravity for 12 hr. A circle of miracloth was placed on the surface of the packed Sephadex. An aliquot of the $(NH_4)_2SO_4$ fraction (containg 470 mg of protein) was added to the column and the protein eluted (at 23°): first with 275 ml of 0·02 M Tris-maleate buffer, pH 8·0, followed by 275 ml of the same buffer made 2·0 M with NaCl. Consecutive 5 ml fractions were collected and absorbance at 280 mμ was determined with a model DU Beckman spectrophotometer. Phosphatase activity was determined by the standard conditions of assay at pH 5·0 and pH 8·5 with ATP, ADP and P-serine as substrates (Fig. 3). The contents of the peak tubes were pooled and the volume was reduced some tenfold with Aquacide 1.