PHOSPHOGLYCOLIC ACID PHOSPHATASE

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Ya-shiou L. Yu

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ABSTRACT

PHOSPHOGLYCOLIC ACID PHOSPHATASE

by Ya-shiou L. Yu

Phosphoglycolic acid phosphatase was found in a variety of green leaves. It was partially purified many fold to prove that the enzyme is a specific phosphatase for phosphoglycolic acid. In crude sap from non-green plant tissues such as roots and etiolated leaves, little hydrolysis of phosphoglycolic acid occurred. Also from roots and etiolated leaves the specific phosphatase for phosphoglycolic acid could not be isolated but rather a nonspecific phosphatase was obtained. The phosphatase was not detected in bovine kidney or liver.

During greening of etiolated wheat leaves over several days the specific phosphoglycolic acid phosphatase increased in amount until it was a major phosphatase of the green leaves. From the sap of green Thatcher wheat the ratio of hydrolysis of phosphoglycolic acid, 3-phosphoglyceric acid, and phenolphthalein diphosphate was 1 : 0.2 : 0.4. As isolated from spinach leaves a total of 91% of the phosphoglycolic acid phosphatase was present in the

cytoplasmic fraction. Seven to 8% of the activity was removed from the chloroplast with 0.35 M NaCl. The remaining 1 to 1.5% of the phosphatase activity removable from whole chloroplasts with water was nearly completely specific for phosphoglycolate. Phosphoglycolic phosphatase is considered to function as a means of removing from the chloroplasts reduced carbon as phosphoglycolate formed from carbon dioxide fixation during photosynthesis. In these studies, the phosphatase distribution and activity were consistent with this hypothesis.

Two isolation procedures for the enzyme were investigated. One procedure which involved ammonium sulfate fractionation was satisfactory for the enzyme from tobacco leaves but not for most other plant materials. A new isolation procedure was developed which consisted of acetone precipitation and then fractionation first on DEAE-cellulose and then Sephadex G-50 columns.

Cations were necessary for enzyme activity. The pH optimum was at 6.3. The pH optimum changed to 5.0 when the ammonium sulfate fractionation procedure was used. With DEAE-cellulose fractionation the pH optimum remained at 6.3 for the preparations with maximum specific activity.

An unknown peptide accompanied the phosphatase during the isolation procedure. The peptide was separated from the

phosphatase by Sephadex G-50. The peptide had a molecular weight between 1,000 and 3,000 as estimated by separation on Sephadex. The peptide had an unusually high terminal absorbancy at 260 mμ. The activity of phosphoglycolic acid phosphatase was not affected by the presence or absence of the peptide.

PHOSPHOGLYCOLIC ACID PHOSPHATASE

By

Ya-shiou L. Yu

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INTRODUCTION AND LITERATURE REVIEW

The initial study of a phosphatase was done in 1907 by Suzuki, Yoshimura, and Takaishi (20) who found that rice bran contained an enzyme which split phosphoric acid from phytic acid. Since then many enzymes acting on a variety of phosphate esters have been found both in animal tissues and plant materials. Folly and Kay (5) classified the phosphatases into six groups: phospho-monoesterases; phospho-diesterases; pyropho-diesterases; metaphosphatase; phospho-amidases; and unclassified phosphatases according to the type of substrates. Bamann and Meisenheimer (1) subdivided each group into four subgroups according to their pH optima. The pH optimum for phospho-monoesterases range from 4 to 9. Among these are the alkaline phosphatases which are found in blood, plasma, milk, intestinal mucosa, and bacteria, and have pH optima near 9, have been investigated more extensively. The incorporation of phosphate into alkaline phosphatase and some chemical characteristics of phosphate binding of E. coli alkaline phosphatase were investigated by Schwartz and Lipmann (18). They found that the E. coli alkaline phosphatase reacted with inorganic phosphate which, upon partial acid hydrolysis yielded serine

phosphate and serine phosphate-containing peptides. The acid phosphatases in plant and animal tissues have a pH optima around 5. Recently acid phosphatase of Baker's yeast was reported to have a pH optimum near 3.6 and the enzyme was localized on the surface of the yeast cell (17). Another acid phosphatase catalyzing the hydrolysis of β-glycerophosphate in wheat leaf press juice was found to have an optimum pH of 5.7 (16). The mechanism of phosphatase catalyzed reactions were first investigated by Cohn (2) by using O^{18} labeled water and phosphate. It was found that phosphatases catalyzed the transfer of the phosphoryl group of the ester to the water. The bond between the oxygen adjacent to the carbon and the phosphorus was cleaved ($R\text{-}C\text{-}O\text{-}PO_3^{=}$). An enzyme-phosphate complex is believed to be the intermediate. In general, serine has been found to be involved in the active site (18).

This thesis is about the enzymatic hydrolysis of phosphoglycolate. Glycolic acid and phosphoglycolic acid are among the major products of photosynthesis by plant chloroplasts and are the only significant C^{14}-labeled products excreted by the chloroplasts during <u>in vitro</u> experiments with $C^{14}O_2$ fixation (8, 14, 6). With labeled $C^{14}O_2$ and isolated chloroplasts, Kearney and Tolbert (8) demonstrated that the phosphoglycolate, glycolate, and

glycine were the early products of CO_2 fixation appearing outside the chloroplasts. They assumed that phosphoglycolate was moved out through the membrane. Thus it was suggested that either a kinase, a phosphatase, or both would be involved for phosphorylation and dephosphorylation of the glycolic acid. The finding of a specific phosphatase for phosphoglycolic acid in tobacco leaves by Richardson and Tolbert (15) partially confirmed this hypothesis. This phosphatase was mainly found in the particulate free cell sap and appears to be a constituent of the plant cytoplasm.

Phosphoglycolic acid phosphotase has been shown to be an obligated step in the "glycolate pathway" which was demonstrated and suggested by Tolbert et al. (8, 14, 6). It is postulated that this pathway is the major route for transport of newly formed carbon products of fixed CO_2 from the chloroplasts to the cytoplasm. In this scheme (Fig. 1) phosphoglycolate can cross the chloroplast membrane. This has been shown by observing its secretion by chloroplasts during photosynthesis with $C^{14}O_2$ (8) and by feeding C^{14} labeled phosphoglycolate to chloroplasts (25). However, glycolate-C^{14} when added to a chloroplast suspension will not enter the particles (25). Therefore, the phosphoglycolic acid phosphatase can donate direction to the glycolate pathway by hydrolyzing the phosphoglycolate

which gets out of the chloroplasts to free glycolate which cannot re-enter. It is not known whether the excretion of glycolate is also enzymatically controlled.

The C_2 units have been shown to move out of chloroplast in the form of the phosphoglycolate ions which are dephosphorylated to glycolate and the glycolate then oxidized to glyoxylate as catalyzed by the enzyme glycolic acid oxidase (22, 27). One of two things can then happen to the glyoxylate. It may be converted to glycine which in turn is metabolized via serine and glyceric acid to a sugar phosphate. The details of this route were elaborated by tracer studies (14, 6). On the other hand, glyoxylate-C^{14} can enter the chloroplasts, whereas glycolate cannot (25). Zelitch (27, 28) has shown that chloroplasts contain a $NADPH_2$ specific glyoxylic reductase which could utilize $NADPH_2$ from photosynthetic phosphorylation to resynthesize glycolate. Since photosynthetic phosphorylation also produces ATP, abundant energy would be available for a glycolic acid kinase. The formation of phosphoglycolic acid inside the chloroplast would complete the cycle as depicted in Figure 1.

Some scheme as just elaborated has been proposed for transport of the energy of photosynthesis from the chloroplasts (12, 24). However, many inconsistencies still

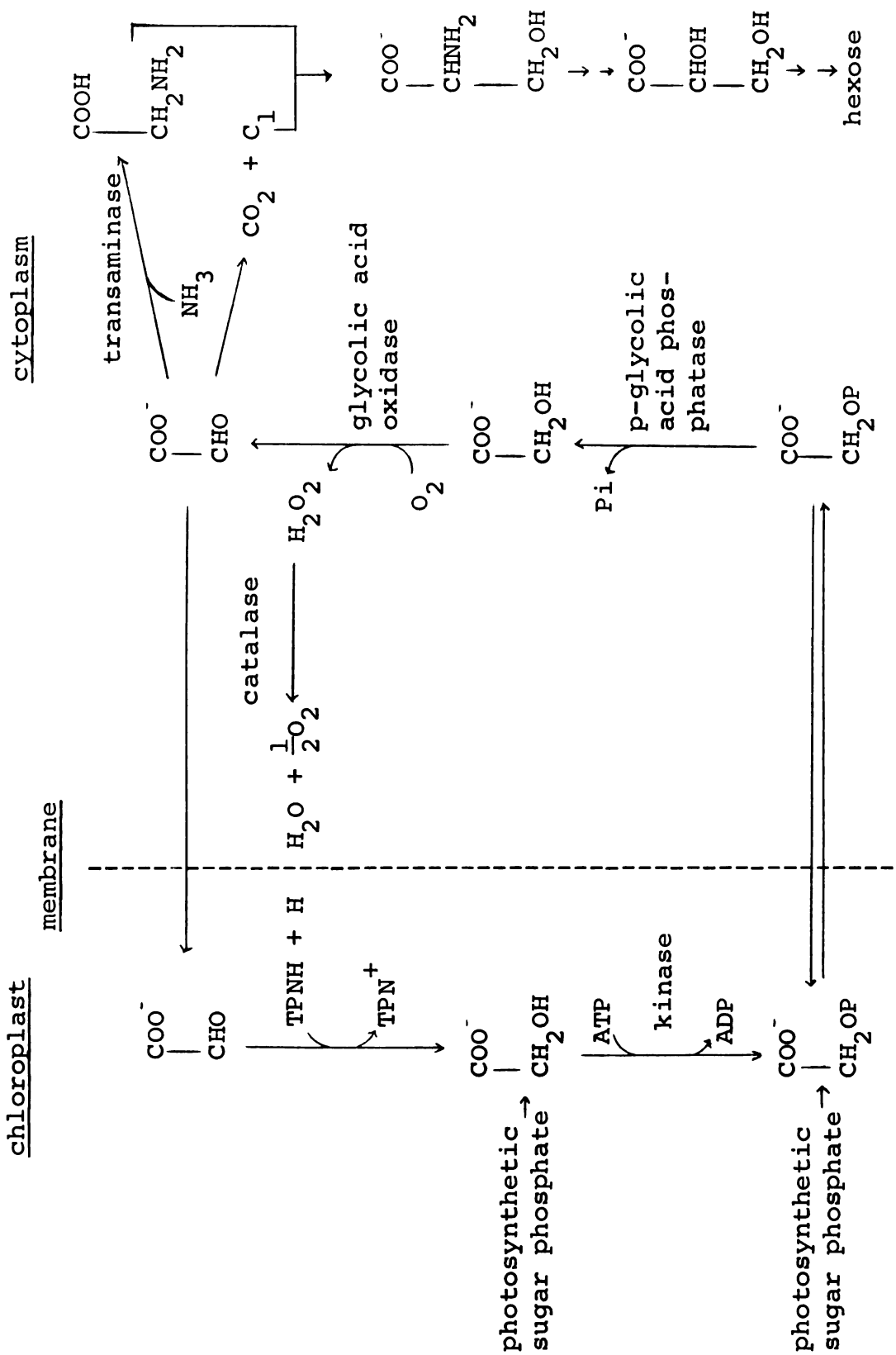

Figure 1. Glycolate pathway.

remain before the scheme can be accepted. A kinase for synthesis of phosphoglycolate has not yet been reported. Most troublesome is the fact that glycolic acid oxidase transfers the energy from the oxidation of glycolic acid to glyoxylic acid outside the chloroplasts to FMN and then to H_2O_2. As now conceived the cycle would only serve to move carbon outside of the chloroplast but the energy is lost because of the mechanism of action of the oxidase. The characteristics and mechanism action of the oxidase are under investigation.

This report is concerned only with one enzyme of the glycolate pathway, namely phosphoglycolic acid phosphatase. It was first detected and isolated about two years ago (15). In order to understand more about this enzyme, further investigations of its properties in relation to its function were necessary. Preliminary experiments showed that crude sap of etiolated corn and albino barley had very little phosphoglycolic acid phosphatase activity. This suggested that the enzyme activity was closely associated with light and the green pigment system of photosynthesis. If it were associated with light, then during greening of etiolated plants there should occur an increase in enzyme activity. It is also possible that the enzyme is activated by its substrate <u>in vivo</u> such as is the case for substrate

activation of glycolic acid oxidase (9, 23). The course of this work was based on the hypothesis that the enzyme was closely associated with photosynthesis and that during the greening of etiolated plants there would be an increase in enzyme activity as the plants were exposed to light prior to harvest. The physiological change and distribution of the enzyme in plant and animal tissues have also been investigated.

MATERIALS AND METHODS

A. Preparation of Substrates

(a) Phosphoglycolic Acid: Phosphoglycolic acid was prepared from phosphoglyceric acid by oxidation with potassium permanganate (7). The product was chromatographically pure and free from phosphoglyceric acid (15). The formula weight of the barium glycolate thus prepared was assumed to be 395. Solution of sodium glycolate were prepared by treatment of 120 mg of the barium salt with 3 ml of Dowex-50, Na^+ form. The filtered solution was adjusted to pH 5 with acetic acid and made to 50 ml final volume. Assay of this solution with phosphoglycolic acid phosphatase indicated a concentration of 2.1 µmole per ml as compared to the theoretical value of 6 µmole per ml. The solution was stable to storage at 10 C.

(b) 3-phosphoglyceric Acid: This chemical was purchased as the barium salt from Calbiochem. The conversion into the soluble sodium salt was also accomplished by the aid of Dowex-50, Na^+ form. The final concentration was 10 µmole per ml. This substrate was stable in solution, and could be kept at 10° C for over a month without extensive hydrolysis.

(c) Phenolphthalein Diphosphate: Phenolphthalein diphosphate, sodium salt, was purchased from Sigma. The substrate was made to a concentration of 10 µmole/ml, pH 5.0. The aqueous solution of this compound was not stable even in the cold as shown by the increasing inorganic phosphate concentration of the solution with time. Therefore, it was prepared fresh at two to three week intervals.

B. Preparation of Columns

(a) DEAE-cellulose Column: DEAE-cellulose columns were prepared essentially as described in Methods in Enzymology (3) with the exception that washings were done with 0.1 N KOH and 0.1 N HCl. These washings were followed by treatment with 0.05 M Tris-acetate buffer, pH 7.2, 0.02 M Tris-acetate buffer, pH 7.0, and finally distilled water. A column 15mm x 120mm required 2 gm of air-dried DEAE-cellulose, which was suspended in 200 ml of 0.02 M Tris-acetate buffer pH 7.2. Columns were packed by gravity alone, and the final washing with 200 ml buffer was done at 4° C with a flow rate of 2.5-3.0 ml per minute. The column was stored in cold room at least two hours before introducing the enzyme.

(b) Sephadex Column: The procedures used in Sephadex chromatography have been described by Pharmacia (13). Both Sephadex G-25 and G-50 were treated by weighing out 20-40 gm

of dry Sephadex and suspending it in approximately 600 ml of 0.02 M Tris-acetate buffer, pH 7.2. After standing for one minute, the suspended fine particles were decanted off. This process was repeated two times for Sephadex G-25, and four times for Sephadex G-50. After the last washing, the suspension was again stirred well and poured rapidly into a column. The columns were packed at atmospheric pressure. For samples of less than 10 ml the column dimensions were 15mm x 150mm. Larger samples required 23mm x 230mm column. When the surface of the settled Sephadex had reached 10 mm beyond the desired height, the excess of suspension was removed by a pipette. Added to the column was 100 ml of 0.02 M Tris-acetate buffer at 4°. The column was kept at 4° for at least two hours before the void volume was determined. The void volume of Sephadex column was measured in two ways: (a) by using bovine hemoglobin; (b) by using diluted India Ink. When the colored liquid was all absorbed on the column, a volume of cold buffer was added. The void volume was the volume of buffer required to move the colored band to the bottom of the column. The determination of the void volume of Sephadex G-50 column by using bovine hemoglobin proved to be unsuccessful because a diffused band was formed. In the second method a 1 to 3 dilution of India Ink filtered once through a separate Sephadex G-50 column

served in place of bovine hemoglobin. Prior filtration of the ink through some old Sephadex G-50 removed particles of ink. After the void volume was determined, the column was again washed with 200 ml of cold Tris-acetate buffer and kept at 4° before the enzyme solution was introduced. For the small columns, the void volume was found to be 11 ml. For the larger column it was approximately 35 ml.

C. Assay and Analysis

(a) Enzyme assay: Enzyme activity measurements were based on the amount of orthophosphate produced during the hydrolysis of a designated phosphate ester. The reaction was initiated by addition of enzyme. A unit of activity was defined as the release of 1 µg of phosphorus in 10 minutes. Inorganic phosphate was determined by the method of Fiske and SubbaRow (4) at room temperature. An adequate volume of enzyme was added to a test tube containing a mixture of 3 µmole $CoSO_4$, 33 µmole Tris-acetate buffer, pH 6.3, and 0.5 ml of the prepared substrate. The final volume after the addition of enzyme was 3 ml. The mixture was incubated at 30°C for 10 minutes. For very dilute enzyme solutions such as DEAE-cellulose fractions and Sephadex fractions, 100 minutes of incubation time was used. The enzymatic reactions were stopped by the addition of 1 ml

of 10% trichloroacetic acid to each test tube. The precipitate was removed by centrifugation and the supernatant was analyzed for inorganic phosphate.

(b) Determination of Protein Concentration: Protein concentration of crude sap and redissolved acetone precipitate were estimated by determination of 260 mμ/280 mμ ratios (26). For the DEAE-cellulose and Sephadex effluents, Lowry's modified Folin-Ciocalteu method with albumin as standard protein solution was used (10).

(c) Inorganic Phosphate Determination: The amount of inorganic phosphate released after enzymic reaction was measured by the method of Fiske and SabbaRow (4). One ml of supernatant was added to a mixture containing 7.4 ml of H_2O, 0.8 ml of 2.5% ammonium molybdate, and 0.4 ml of 10 N H_2SO_4. After mixing, 0.4 ml of the reducing reagent[1] was added. The mixture then stood at room temperature for 15 minutes for color development. The color intensity was measured at 660 mμ in a Coleman spectrophotometer and the results compared with a phosphate standard curve.

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[1]Reducing reagent consisted of 0.5 gm purified 1-amino-2-naphthol-4-sulfonic acid in 195 ml 15% $NaHSO_3$, and 5 ml 20% Na_2SO_3.

D. Source of Biological Materials

Tobacco leaves were obtained from field grown tobacco plants. Spinach leaves were purchased from local supermarkets. Beef liver and kidneys were obtained from a local slaughter house immediately after the animal was killed. Thatcher wheat was grown in a 14" x 24" flat of steam sterilized sand. For etiolated seedlings, the flat after three days germination in the greenhouse was moved to either a specially designed black box, or a box covered by a dark cloth, or a closed cabinet. For best growth, the temperature of the environment was kept between 70-76°F. For the normal green wheat, the flats were held in the greenhouse all the time. Leaves were usually harvested when the wheat was 10-12 days old. All wheat except etiolated wheat was watered in the afternoon and harvested the following morning. Greening of etiolated wheat was accomplished by moving the wheat of proper age into daylight and harvesting at designated times afterward. At night the light source was supplied by two 15 watt cool white Ken-Rad fluorescent bulbs. Immediately after harvesting, wet weight was recorded, and all the preparations of protein solutions thereafter were performed at 0-8°C.

E. Criteria of Enzyme Specificity

Measurements of phosphoglycolic acid phosphatase activity in crude sap or partially purified preparations are complicated by the action of non-specific phosphatases, which are abundant in plant extracts, on the substrate phosphoglycolic acid. Parallel measurements of the rate of hydrolysis of other phosphate esters were necessary in order to indicate the stage of purification of each enzyme preparation. Among many phosphate esters, 3-phosphoglyceric acid and phenophthalein diphosphate were always used because their respective phosphatases showed the greatest tendency of remaining with the phosphoglycolic acid phosphatase during the isolation process from tobacco leaves (15). An increasing ratio of the rate of hydrolysis of the phosphoglycolic acid to the hydrolysis of the other two phosphates would be an index of enzyme purification as well as increasing specific activity. Generally, the data were expressed as a ratio of activity with the unit of phosphoglycolic acid phosphatase activity equaled to one. When the ratio of the rate of hydrolysis of phosphoglycolic acid, 3-phosphoglyceric acid, and phenolphthalein diphosphate change, for example, from 1 : 2 : 7 in crude sap to 1 : 0.6 : 1 in DEAE-cellulose effluent a major separation of the phosphoglycolic acid phosphatase had occurred from the rest of the phosphatases.

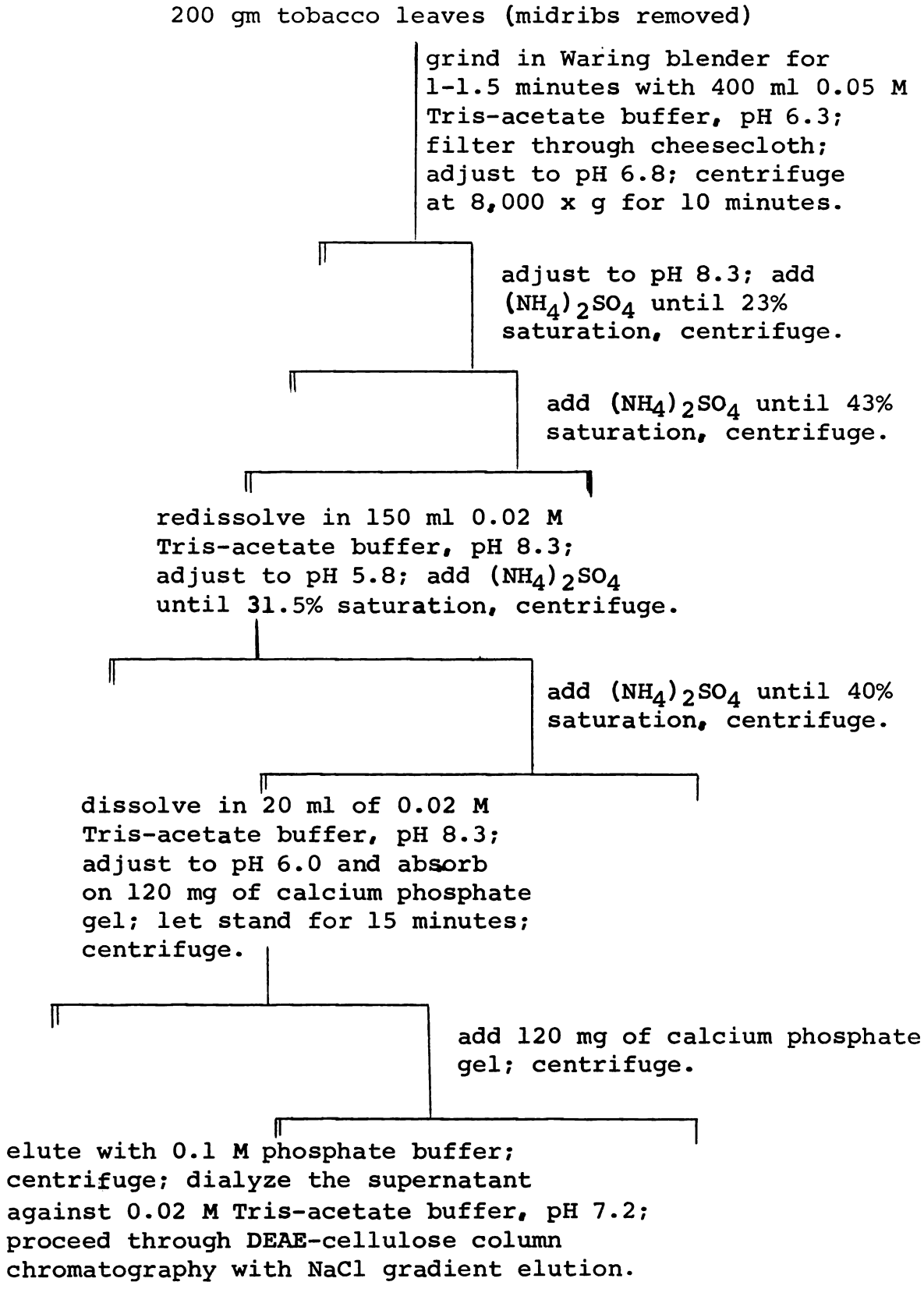
EXPERIMENTAL PROCEDURES AND RESULTS

A. Ammonium Sulfate and Calcium Phosphate Gel Isolation Procedure

Figure 2 illustrates the isolation procedure for phosphoglycolic acid phophatase from tobacco leaves. This is similar to the procedure first worked out by Richardson and Tolbert (15). This procedure was successfully used with tobacco leaves. It involved an ammonium sulfate fractionation procedure followed by absorption and elution from calcium phosphate gel and then purification on DEAE-cellulose column.

Several difficulties with this procedure have been noted. The ammonium sulfate fractionation presented considerable difficulties in that a large part of the total activity was lost by this procedure. Even Richardson and Tolbert (15) reported 55-63% loss under the best conditions. As will be discussed in section B phosphoglycolic acid phosphatase from other plants was even more sensitive to ammonium sulfate fractionation and this fractionation procedure has been abandoned. Of all the plants examined, the phosphoglycolic acid phosphatase from tobacco leaves

Figure 2. Isolation procedure for phosphoglycolic acid phosphatase from tobacco leaves.



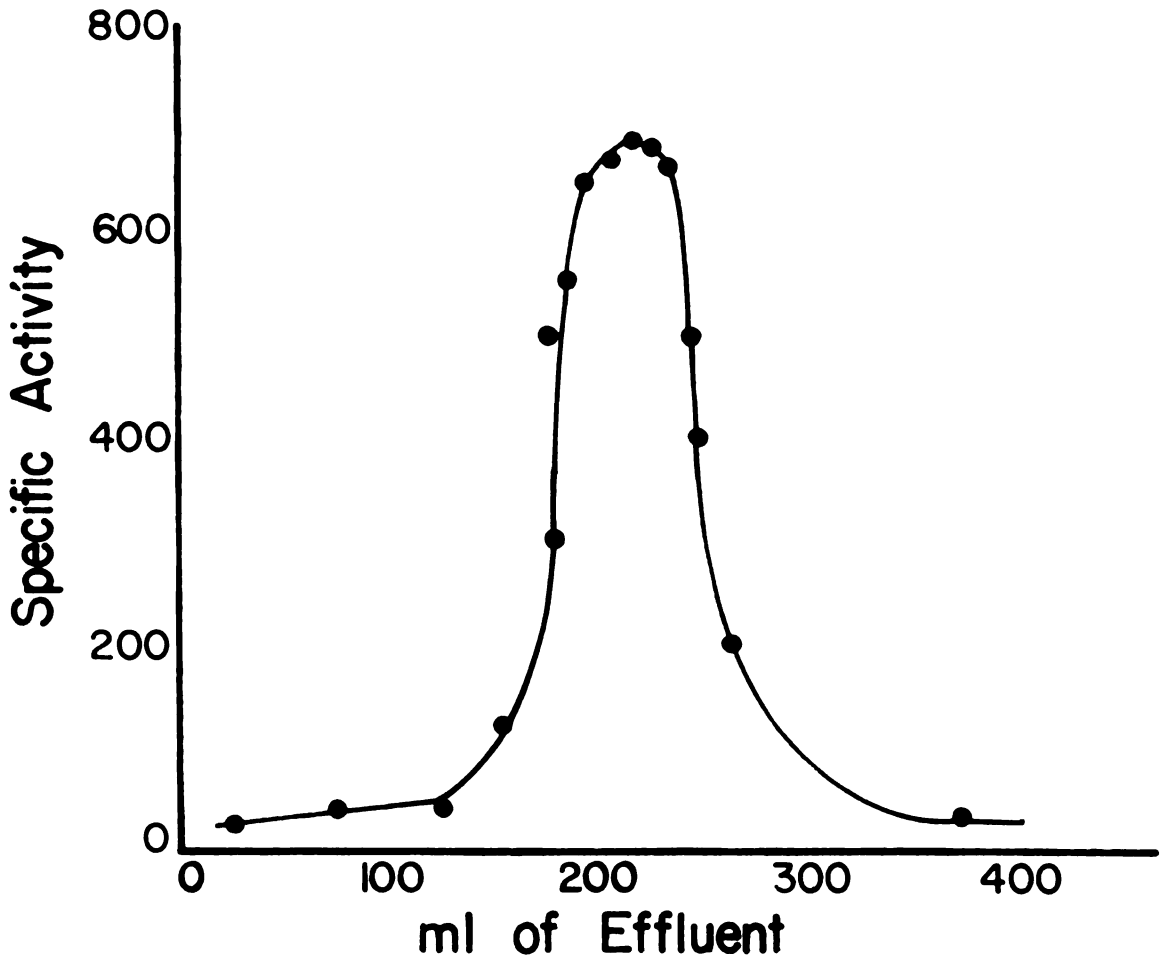
was the most stable to ammonium sulfate fractionation. In my experience the bulk of the enzyme activity was precipitated between 31.5-40.0% salt saturation. Richardson and Tolbert (15) had used 23-32% saturation with ammonium sulfate to precipitate the enzyme.

The calcium phosphate gel step as used by Richardson and Tolbert (15) also caused considerable difficulty. Commercially prepared calcium phosphate gel (Sigma) failed to adsorb any significant amount of phosphoglycolic acid phosphatase regardless of the amount of gel used or the method of preparing the enzyme. When calcium phosphate gel was prepared here and used the absorption on the gel and elution by phosphate buffer could be confirmed. However, it was always extremely difficult to remove by dialysis all of the phosphate gel and buffer from the eluted enzyme. It appeared as if phosphate were bound to the protein after this treatment. Exhaustive dialysis never completely lowered the inorganic phosphate background in the enzyme preparation as eluted from phosphate gel, to a sufficiently low value to permit extremely sensitive enzyme assays.

When the calcium phosphate gel absorption procedure was omitted, the NaCl elution of DEAE-cellulose column chromatography of dialyzed redissolved ammonium sulfate precipitate gave one peak (Fig. 3). The majority of the

Figure 3. NaCl gradient elution pattern from DEAE-cellulose column of tobacco leaves.

The gradient device which was used consisted of two vessels, one mounted above the other. The upper vessel contained 0.4 M NaCl in 0.02 M Tris-acetate buffer, pH 7.2. The constant volume of the lower vessel was 500 ml. At the start the lower vessel contained 0.02 M Tris-acetate buffer, pH 7.2, and no NaCl.

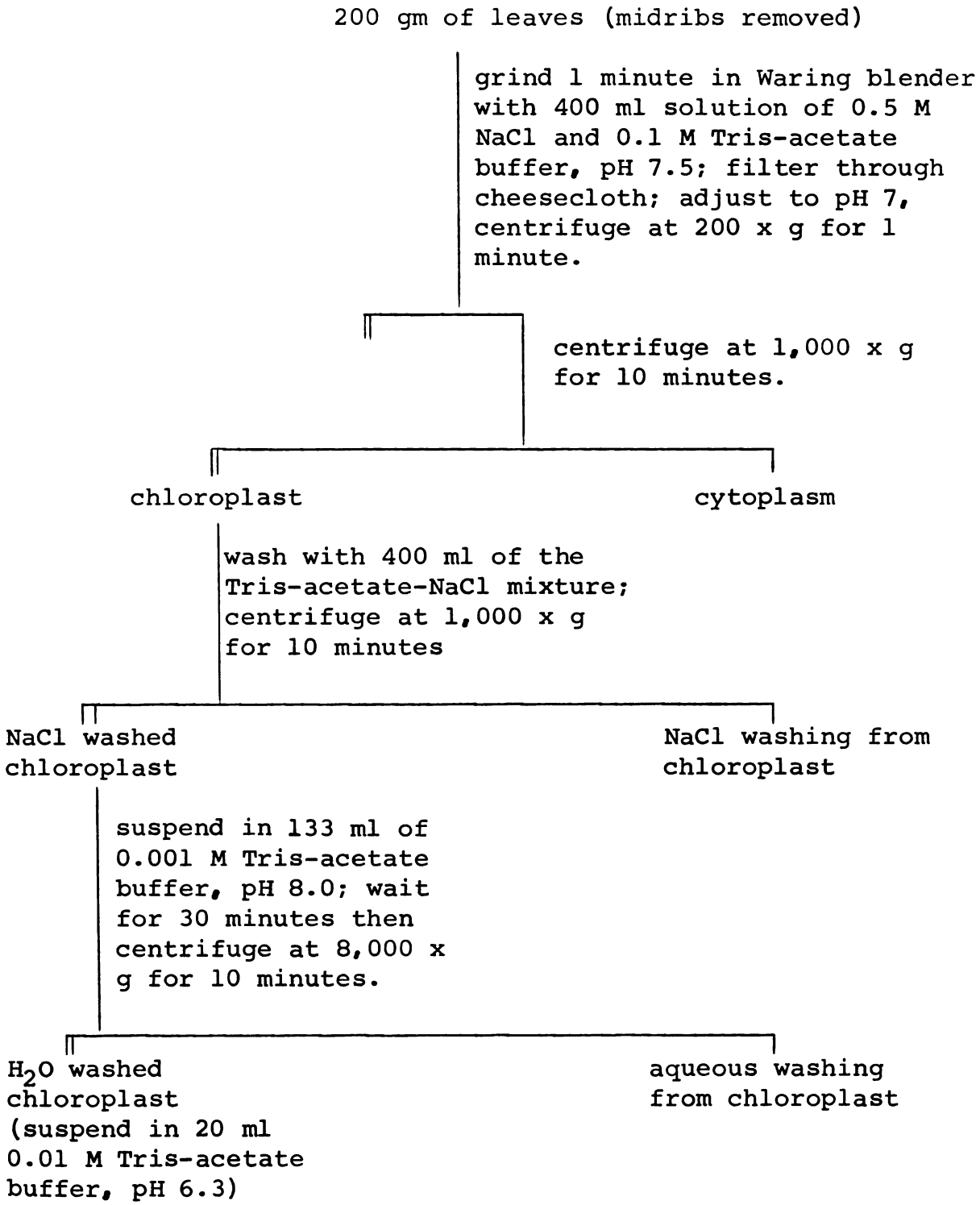

800
600
400
200
0
Specific Activity
0
100
200
300
400
ml of Effluent

enzyme came off between 180-230 ml of effluent which corresponded roughly to 0.12-0.14 M NaCl concentration. However, there was always a trace of enzyme activity, which hydrolyzed the phenolphthalein diphosphate and which persistently came off with the phosphoglycolic acid phosphatase. The 3-phosphorglyceric acid phosphatase activity was lost. This was also true in isolating the enzyme from green wheat and corn.

B. Distribution of Phosphoglycolic Acid Phosphatase in Chloroplast and Cytoplasm of Spinach Leaves

The procedure for separation of chloroplasts from cytoplasm of spinach is summarized in Figure 4. Two hundred gm of washed, fresh spinach, with midribs removed were ground with 400 ml of 0.5 M NaCl in 0.1 M Tris-acetate buffer, pH 7.5, in a Waring blender for one minute at full speed. The suspension was adjusted to neutral pH and centrifuged at 200 x g for 1 minute to remove cell debris, nuclei, etc. The supernatant was again centrifuged, this time at 1,000 x g for 10 minutes to separate the chloroplasts from the cytoplasm. The supernatant thus obtained represented the cytoplasm and was saved for enzymic assay. The residue was washed with 400 ml of the Tris-acetate-NaCl solution and centrifuged as before. The washing was discarded and

Figure 4. Isolation procedure of chloroplast from cytoplasm.



the precipitate was suspended in 133 ml (2/3 of original wet weight) of 0.001 M Tris-acetate buffer, pH 8.0. The chloroplasts were subjected to this hypotonic treatment for 30 minutes, and they were then centrifuged at 8,000 x g for 10 minutes. Both the pale yellow clear supernatant and the precipitate, resuspended in 20 ml of 0.01 M Tris buffer, pH 6.3, were assayed for enzyme activity.

The results are summarized in Table 1. It was found that about 91.5% of the phosphoglycolic acid phosphatase activity was in the cytoplasm and about 1.5 was in the aqueous washing from the chloroplast fraction. The NaCl washing of the chloroplast released about 7 to 8% of enzyme. However, the specific activity of the enzyme in chloroplasts was three times greater than that in the cytoplasm. These data emphasize the large amount of phosphoglycolic acid phosphatase activity in spinach. The ratio in the crude sap was 1 : 0.3 : 0.3 : which is similar to the ratio in crude sap from green wheat leaves (see Section C).

Several interpretations of these results are possible. The low percentage of the total phosphoglycolic acid phosphatase activity with the chloroplast fraction suggests that the phosphatase is a cytoplasmic enzyme. In this case the activity with the chloroplasts would be

Table 1. Distribution of enzyme in chloroplast and cytoplasm.

Enzyme	Substrate*	Total unit of activity	% of activity	Specific activity	Ratio**
cytoplasm	PGA	78,300	91.5	15.1	1
	3-PGA	21,600			0.26
	PPD	27,000			0.34
aqueous washing from chloroplast	PGA	1,625	1.5	46	1
	3-PGA	100***			0
	PPD	62***			0
NaCl washing from chloroplast	PGA		7	--	
	3-PGA	--			
	PPD	--			
H_2O washed chloroplast	PGA	625***	0.5	0.8	1
	3-PGA	0			0
	PPD	3,750			6

*PGA; phosphoglycolic acid, 2.1 µmole per ml.
3-PGA; 3-phosphoglyceric acid, 10 µmoles per ml.
PPD; phenolphthalein diphosphate, 10 µmole per ml.

**The ratio of rate of hydrolysis.

***The O.D. of these samples when analysis for inorganic phosphate were between 0.005 to 0.01 which may not be significant.

attributed to adsorption on the chloroplast surface. This interpretation supports the hypothesis in the introduction that the phosphoglycolic acid is excreted by the chloroplast and hydrolyzed in the cytoplasm.

The isotonic washing from the isolated chloroplast, designated aqueous washing from chloroplast in Fig. 4,

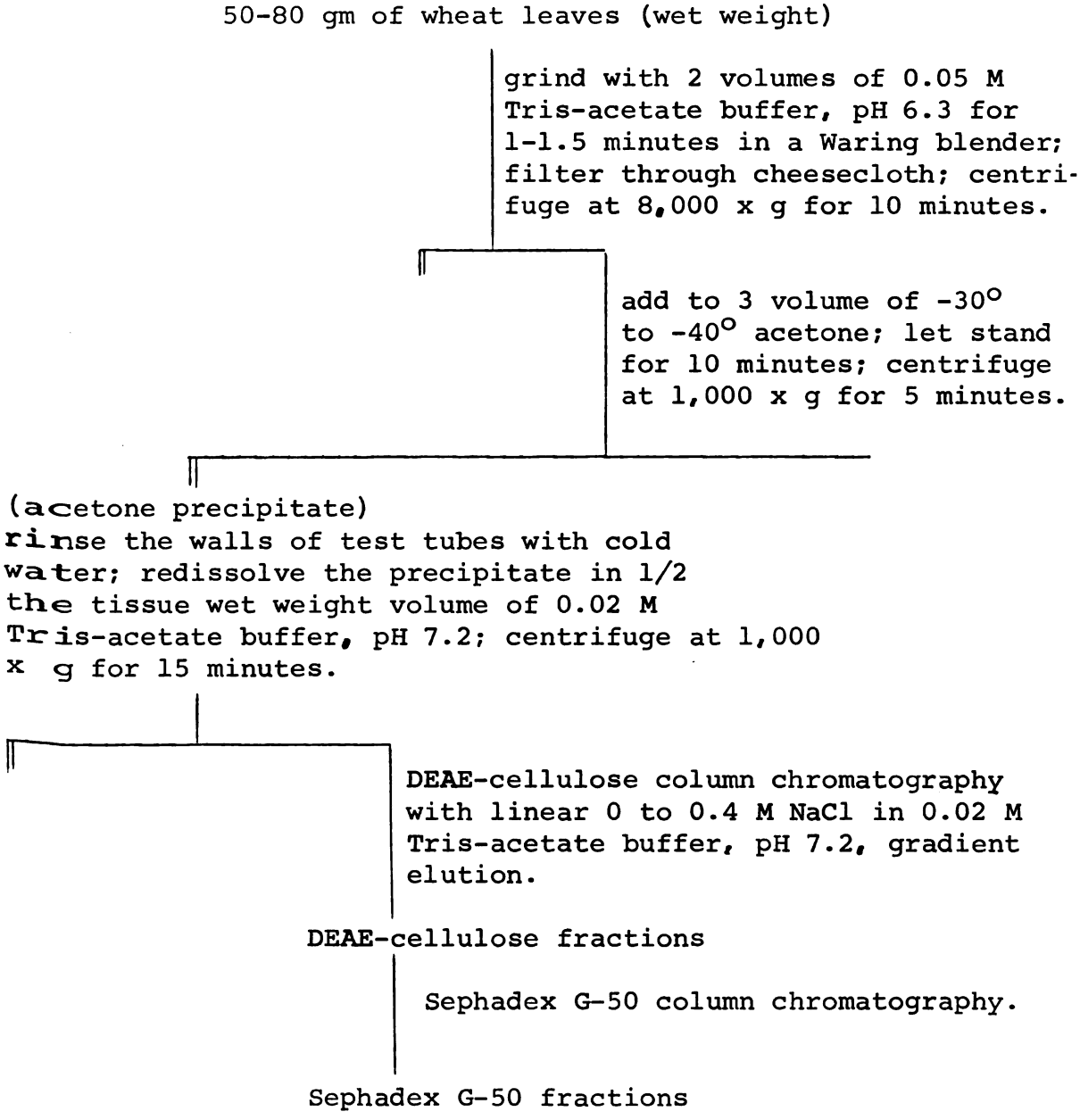
contained significant amounts of only the phosphatase for phosphoglycolate. This fact suggests that phosphoglycolic acid phosphatase might be specifically but lightly bound by chloroplasts. Further studies with this system might be valuable to help visualize the protein orientation about the chloroplast particles *in vivo*.

C. Isolation of Phosphoglycolic Acid Phosphatase from Wheat

The ammonium sulfate and calcium phosphate gel isolation was unsatisfactory as described in Part A. In addition this procedure could not be used with plant tissues other than tobacco leaves. Many failures were experienced particularly with wheat leaves, spinach, and alfalfa leaves. Precipitation by ammonium sulfate led to enzyme inactivation and less than 30% recovery when either alkaline (pH 8.3) or acid (pH 5.8) conditions were used. The enzyme inactivation caused by dialysis was also large (30-70%). Therefore, it was necessary to develop another enzyme isolation procedure which is described in Figure 5.

The enzyme in the crude leaf extract was stable and no pH adjustment of the sap was necessary when 0.05 M Tris-acetate buffer, pH 6.3 was used in grinding. Time of grinding varied from 1 to 1.5 minutes depending on the size of the

Figure 5. Isolation procedure for phosphoglycolic acid phosphatase from wheat leaves.



sample. The homogenate was centrifuged at 8,000 x g for 10 minutes at 4°C. The supernatant thus obtained was added rapidly with stirring into three volumes of precooled (−30° to -40°) acetone. The cooling of acetone was accomplished by the aid of a dry-ice acetone bath, but no dry ice was added to the acetone with the protein. After standing for 15 minutes the precipitate was collected by centrifugation at 1,000 x g for 5 minutes. The acetone was removed by decantation and by washing the side of the inverted centrifuge tube with cold water before the precipitate was redissolved in 0.02 M Tris-acetate buffer, pH 7.2 and recentrifuged. At least 50 to 80% of the enzyme activity was recovered and often nearly complete recovery was achieved. The enzyme activity in the redissolved acetone precipitate retained 75% of its activity over a 10 day period when stored at 0°-4°C and it could also be stored at -20°C.

The phosphate gel step was eliminated and instead the enzyme was further purified by chromatography on DEAE-cellulose and Sephadex columns. Usually 200 mg of protein solution was added to a column prepared from 2 gm of DEAE-cellulose. The protein concentration was estimated by the determination of 260 mμ/280 mμ absorption of light. After the introduction of enzyme solution the column was

washed with 600 ml of the same Tris-acetate buffer to remove the loosely adsorbed protein. The column was then eluted with a gradient of 0-0.4 M NaCl in 0.02 M Tris-acetate buffer, pH 7.2 as described in Fig. 3 footnote. Fractions of 10-12 ml were collected and assayed for enzyme activity and measurement of optical density at 260 mμ and 280 mμ. Protein concentration was also measured by Lowry's method (10). The NaCl gradient elution patterns from DEAE-cellulose were similar to that shown in Fig. 3. Fractions with highest specific activity were found to be in the fractions of 180-220 ml of effluent which corresponds roughly to 0.12 to 0.135 M NaCl concentration. It was necessary to add cobalt ionsto restore complete enzyme activity at this degree of purity (15). This fact is shown in Table 2.

The pH optimum for enzyme purified through the DEAE-cellulose and Sephadex column was found to be 6.3 as shown in Figure 6. This is in contrast to a pH optimum of 5.0 for the same phosphatase prepared from tobacco leaves by $(NH_4)_2SO_4$ fractionation. The pH optimum of the tobacco enzyme in the crude state was also 6.3 and Co^{++} ion was not necessary for activity. However, after $(NH_4)_2SO_4$ precipitation and calcium phosphate gel treatment Co^{++} was needed for activity and the pH optimum had changed to

5.0. Perhaps this deleterious $(NH_4)_2SO_4$ procedure partially altered the protein enough to change the pH optimum.

Table 2. Effect of Co^{++} ions on phosphoglycolic acid phosphatase activity at different stages of purification from green wheat.

Enzyme preparations*	Total unit of activity	
	With Co^{++}	Without Co^{++}
crude sap	2,560	2,600
redissolved acetone precipitate	1,920	1,400
DEAE-cellulose fraction	2,000	0
Sephadex G-50 fraction	2,040	0

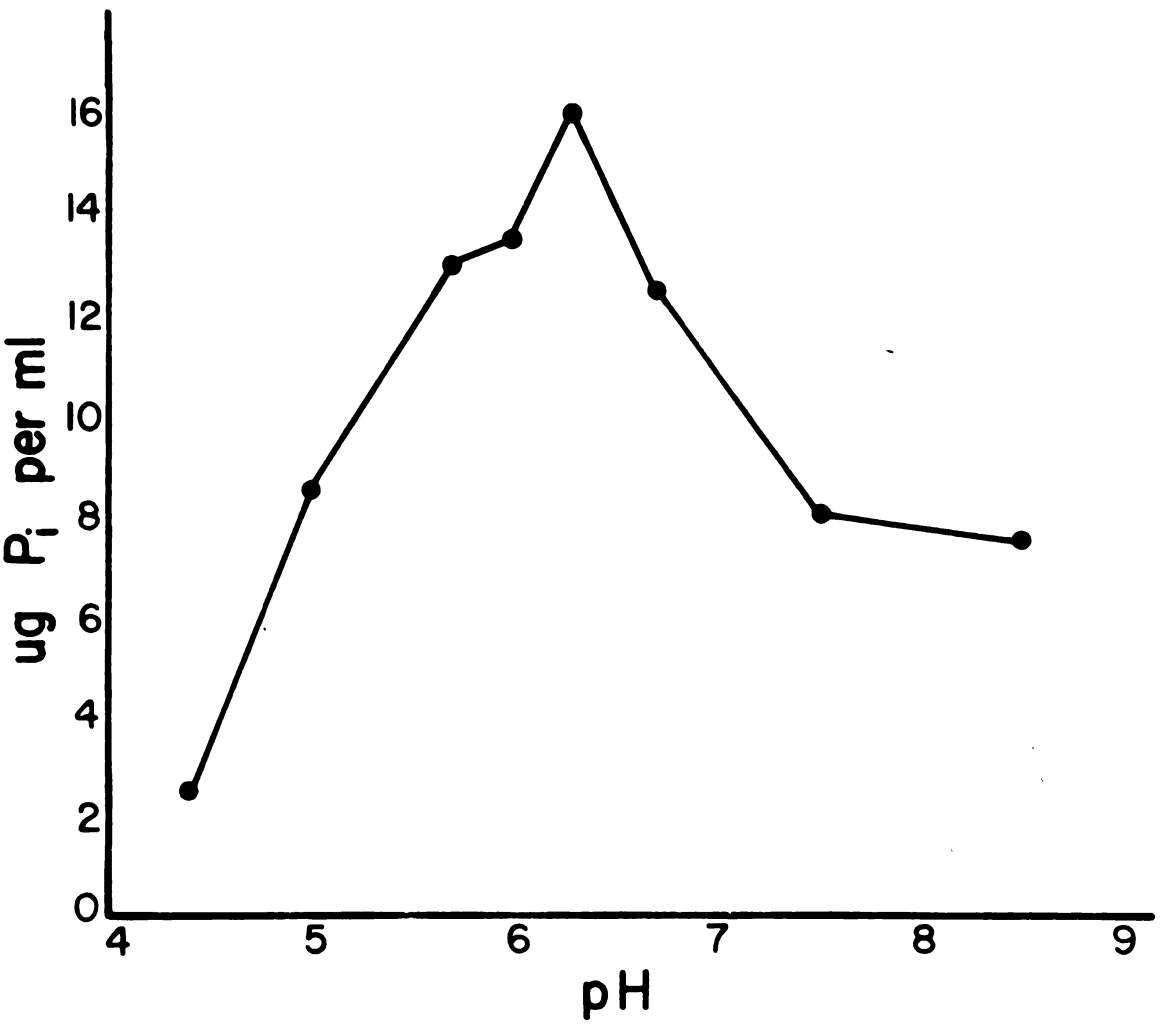
*Separate preparations.

D. Formation of Phosphoglycolic Acid Phosphatase During Greening

Kearney and Tolbert (8) have indicated that phosphoglycolic acid phosphatase may be of special significance in tissues which are capable of photosynthesis. If so the enzyme should be present only in green tissues. A study was made, therefore, of the enzyme in roots and in etiolated wheat where it should be absent. Further the increase in activity of the enzyme was studied when the etiolated plants

Figure 6. pH optimum of the partially purified enzyme obtained from Sephadex G-50 separation of DEAE-cellulose effluent.

Reaction mixture contained the following in a total volume of 3.0 ml; 1 ml of enzyme, 1 µmole of phosphoglycolic acid, 100 µmole of Tris-acetate buffer at indicated pH, and 3 µmole of Cobalt sulfate.


ug P_i per ml
0
2
4
6
8
10
12
14
16
4
5
6
7
8
9
pH

were exposed to light for specified time intervals prior to harvest. The results are summarized in Table 3. Stepwise purification showed that the enzyme from green leaves, after DEAE-cellulose column chromatography, had the highest specific activity and the most favorable ratio for hydrolysis of phosphoglycolic acid in comparison to the other two phosphate esters. The specific activities of the DEAE-cellulose purified enzyme from roots, etiolated leaves, and the etiolated wheat with 12 hours of greening were about the same while that of the etiolated wheat with 24 hours of greening reached the same level as that of the green wheat. The gradual increase in total units of enzyme activity per gram of the etiolated wheat and the changing ratio of the enzyme activity toward phosphoglycolic acid hydrolysis were related to the length of time for greening of etiolated plants. Root tissue contained an insignificant amount of active enzyme in the crude sap, although the specific activity of the DEAE-cellulose preparation from the root was the same low value as from the etiolated plants.

In the crude sap and the acetone precipitate of root and etiolated wheat leaves the rate of hydrolysis of phosphoglycolic acid was hardly significant in comparison with that of phenolphthalein diphosphate. There were few units of activity and the ratio of phosphatases activity

Table 3. Comparative enzyme activity of green leaves, illuminated and non-illuminated leaves, and roots of wheat plants.

Hours of light prior to harvest	crude sap			acetone precipitate			DEAE-cellulose	
	ratio*	unit per gm**	spec. act.***	ratio*	unit per gm**	spec. act.***	ratio*	spec. act.***
green	1:0.2:0.4	900-1,500	25	1:0.3:0.3	800	160	1:0.1:03	400
24	1:0.7:7	300-400	6	1:0.6:1	230-300	30-40	1:0.6:1	300-400
12	1:0.6:9	300-330	5	1:0.8:3	190-200	20	1:0.5:1.2	60-140
0	1:1:9	110-150	2-3	1:1:(1.5-4.5)	100-150	14	1:0.6:(1-2.4)	60-95
root	1:3:6	25	2.4	1:2:3	16	9	1:1:1.7	66-88

*Ratio of rate of hydrolysis of phosphoglycolic acid, 3-phosphoglyceric acid, and phenolphthalein diphosphate.

**Units of activity per gm wet weight for hydrolysis of phosphoglycolic acid.

***Specific activity of phosphoglycolic acid phosphatase.

greatly favored phenolphthalein diphosphate. But in green plants phosphoglycolic acid was rapidly hydrolyzed while the hydrolysis of phenolphthalein diphosphate and 3-phosphoglyceric acid were comparatively slow. These data suggest that the enzyme was largely inactive or absent in roots and etiolated wheat leaves but very active in green wheat leaves. The phosphatase activity measured in root and etiolated plants may be partially catalyzed by non-specific enzymes which could hydrolyze phosphoglycolic acid and which were not associated with photosynthesis. This suggestion is made because the DEAE-cellulose purification of the enzyme from root or etiolated leaves never favored phosphoglycolic acid hydrolysis. A complete proof of the non-existence of the specific phosphatase in root or in etiolated wheat leaves is not possible at present because a more highly purified enzyme preparation has not yet been obtained. However, the results described have confirmed the hypothesis that the phosphoglycolic acid phosphatase is active only in plant tissues which are capable of photosynthesis.

The mechanism for formation of the specific phosphatase for phosphoglycolate during greening of the etiolated plant has been considered. The same situation occurred for glycolic acid oxidase (22, 23). A proenzyme for glycolic acid

oxidase exist in etiolated plant which can be activated *in vivo* or *in vitro* by excess substrate (19). The *in vitro* activation by glycolate must be done immediately after grinding the tissue and before the temperature rises to 30°. Similarly then the possibility was tested that glycolate or phosphoglycolic acid might activate *in vitro* the phosphoglycolic acid phosphatase from an inactive protein precursor. Etiolated wheat leaves were homogenized in the presence of phosphoglycolate or glycolate, but no increase in phophatase activity for phosphoglycolate hydrolysis was observed (Table 4). *In vivo* tests were not run because of insufficient phosphoglycolate.

Table 4. Effect of glycolic acid and phosphoglycolic acid to phosphoglycolic acid phosphatase activity.

Substrate added in grinding	Specific activity	Total activity
none*	1.96	1960
glycolate**	2.02	2080
phosphoglycolate***	2.05	1950

*15 gm of etiolated wheat was homogenized with 5 ml of 0.1 M Tris-acetate buffer pH 6.5, in a chilled motar for 7 minutes and centrifuged as usual to prepare a crude sap for enzyme assay. 3 µmole of cobalt ions were added in assay system.

**Same as in control except used 1 ml of 0.2 M glycolate and 4 ml of buffer.

***Same as in control except used 2 ml of 2.1 µmole phosphoglycolic acid and 3 ml of buffer.

E. Unknown Peptide

During the course of phospholycolic acid phosphatase isolation from wheat, the protein concentration was determined by the optical density ratio at 260 and 280 mμ. However, these readings for the DEAE-cellulose fractions with the highest enzyme activity usually had such an abnormally high 260 mμ reading that very unreliable protein values were obtained (Table 5). In fact, the fraction with maximum phosphatase activity had such a high 260 mμ reading that the optical density method for protein determination gave negative values. Nevertheless, protein was present as determined by Lowry's method (Table 5). The material in the enzyme preparation which gave the high 260 mμ absorbancy was present in both etiolated wheat and in green wheat (Table 6). However, the unknown material was not observed in an enzyme preparation from tobacco leaves. Therefore, the protein determinations by the 260 mμ/280 mμ were valid in the original publication on this phosphatase when Richardson and Tolbert (15) isolated the enzyme from tobacco leaves.

The unknown material could be partially separated from the enzyme activity by Sephadex G-25 (Table 7). Sephadex G-25 of 100-250 mesh passes molecules of molecular

Table 5. Comparison of protein measurement by 260 mμ/280 mμ and by Lowry's method.

DEAE-cellulose fractions with high enzyme activity*	mg protein per ml			
	260 mμ	280 mμ	by 260mμ/280 mμ	by Lowry's
1	0.136	0.158	0.14	0.065
2	0.169	0.181	0.15	0.065
3	0.425	0.223	0.03	0.063
4	0.449	0.208	----	0.063
5	0.276	0.184	0.08	0.065
6	0.151	0.161	0.15	0.065

*Samples used were fractions collected from a DEAE-cellulose column of a 24 hour greening etiolated wheat.

Table 6. Optical density at 260 mμ of DEAE-cellulose effluents from wheat.

Hours of illumination of etiolated wheat	Optical density at 260 mμ	mg of prot. per ml by Lowry's	Specific activity*
0	0.590	0.060	90
12	0.384	0.050	120
24	0.449	0.063	380
green wheat	0.869	0.030	400

*Specific activity of phosphoglycolic acid phosphatase.

Table 7. Isolation of the unknown peptide by Sephadex G-25 column chromatography.

Fractions	O.D. 260 mμ	Spec. act.*	mg prot. per ml**
1	0	0	0
2	0.078	37	0.043
3	0.226	107	0.030
4	0.352	0	0.008
5	0.147	0	0.005
6	0.041	0	0.005

8 ml of DEAE-cellulose effluent was chromatographed on a Sephadex G-25 column. 0.02 M Tris-acetate buffer, pH 7.2 was the eluant. Following the void volume successive fractions of 3 ml were collected.

*Specific activity of phosphoglycolic acid phosphatase.

**Lowry's method.

weight larger than 4,000 while holding back by trapping molecules of molecular weight less than 1,000. As indicated in Table 8 the unknown substance came off the column slightly after the enzyme activity but considerable overlapping of the enzyme and unknown occurred.

Complete separation of phosphoglycolic acid phosphatase and the unknown with high 260 mμ absorbancy was accomplished by Sephadex G-50 column chromatography (Table 8). Sephadex G-50 will hold back molecules of molecular weight less than 3,000. With Sephadex G-50 the unknown substance as determined by O.D. at 260 mμ came off the column free of phosphoglycolic acid phophatase activity. A determination

Table 8. Isolation of the unknown peptide by Sephadex G-50 column chromatography.

Fractions	O.D. 260 mμ	Spec. act.*	mg prot. per ml**
original sample	0.436	256	0.047
1	0.036	290	0.023
2	0.043	192	0.035
3	0.040	192	0.035
4	0.020	45	---
5	0.015	0	---
6	0.114	0	---
7	0.235	0	0.007
8	0.328	0	0.010
9	0.286	0	0.008

The original sample was the DEAE-cellulose fraction of the etiolated wheat exposed to light 24 hours prior to harvest.

30 ml of DEAE-cellulose effluent was chromatographed on a Sephadex G-50 column. 0.02 M Tris-acetate buffer, pH 7.2 was the eluant. Following the void volume successive fractions of 10 ml were collected.

*Specific activity of phosphoglycolic acid phosphatase.

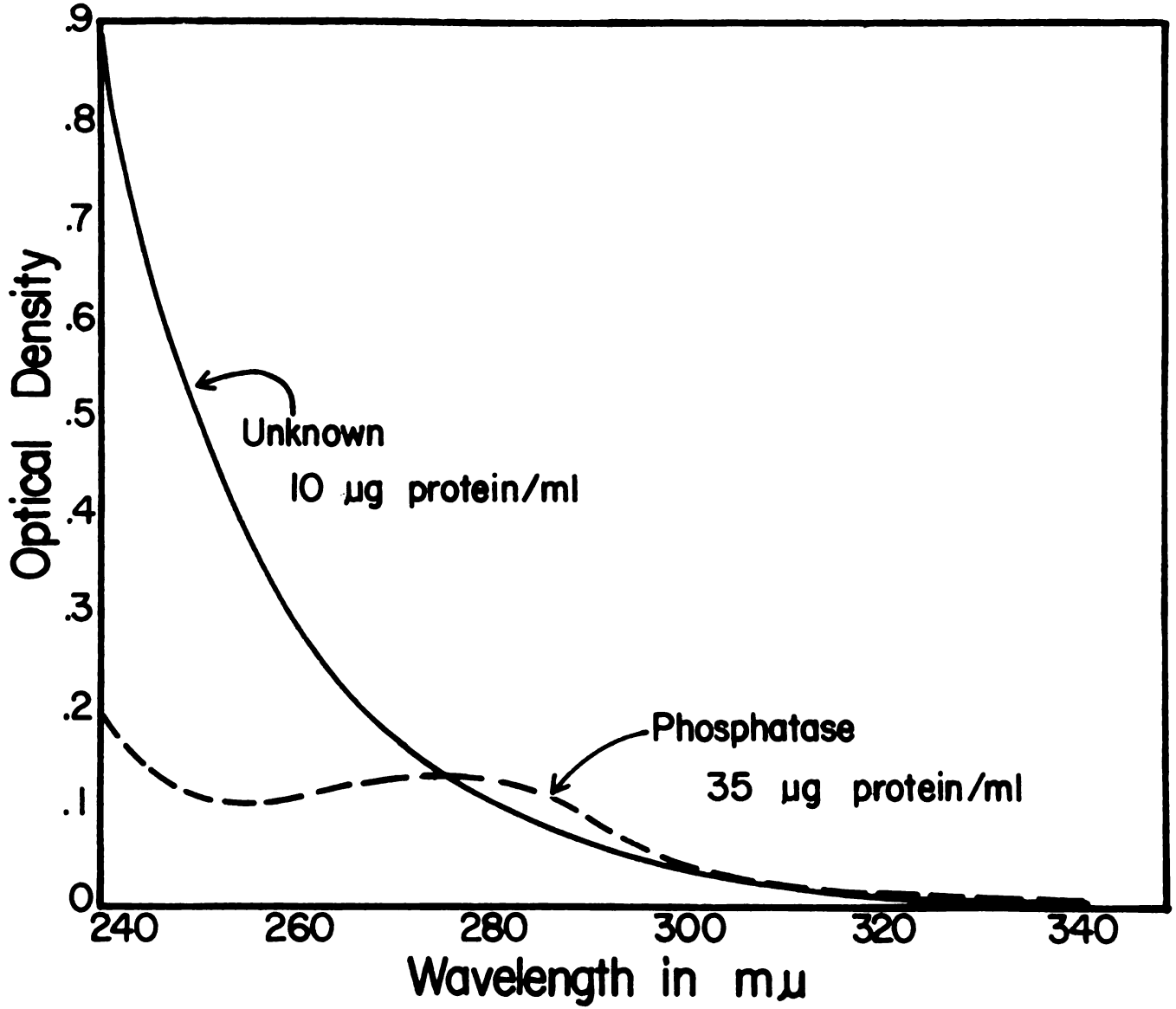
**Lowry's method.

of protein in the unknown was possible by the Lowry's method which indicated a value of 10 μg protein per ml. This small amount of protein gave a very high O.D. reading at 260 mμ. The phosphoglycolic acid phosphatase activity came through the Sephadex G-50 column immediately after the void volume of effluent was collected. There was no loss of activity and in fact the milligram of protein per milliliter decreased and the specific activity of the

enzyme increased due to the removal of the unknown protein factor. Preliminary investigations about this material with high 260 mμ absorbancy have indicated that it might be a low molecular weight peptide. Since the substance was precipitated by cold acetone and redissolved in aqueous buffer, it has the physical properties of a water soluble protein. Since it was eluted off the DEAE-cellulose column with the phosphatase, it might either be associated with the enzyme or have a charge distribution with relationship to size which were equivalent to the enzyme. Thus, the unknown substance might be a cofactor or inhibitor, tightly or loosely bound to the phosphatase, or it could be just another protein not associated with the enzyme at all.

Some properties of the unknown substance can be deduced from the relatively pure material from the Sephadex G-50. Its absorbancy does not change when heated for 10 minutes in a boiling water bath. From the pore size of the Sephadexes G-25 and G-50, the molecular weight of the unknown material is estimated between 1,000 and 3,000 and probably closer to 1,000. The absorption spectra of the unknown, as shown in Figure 7, is that of a substance with very high terminal absorbancy beginning at 260 mμ. Also shown in Figure 7 is the absorbancy spectra of phosphoglycolic acid phosphatase from Sephadex G-50. The spectra of the

Figure 7. Absorption spectra of Sephadex G-50 separated fractions.


Optical Density
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
Unknown
10 µg protein/ml
Phosphatase
35 µg protein/ml
240
260
280
300
320
340
Wavelength in mµ

phosphatase is typical for protein with a peak at 280 mμ from the aromatic amino acids and a terminal absorbancy beginning at 240 mμ. The extremely high 260 mμ terminal absorbancy for the unknown is not due to the presence of excess peptide because the total protein present was very small (Table 9). Rather the high terminal absorbancy at 260 mμ suggests that it may be a peptide with -S-S- bonds.

The function of the unknown peptide is not known. In order to study the effect of the unknown substance on phosphoglycolic acid phosphatase activity, the two fractions after separation on Sephadex G-50 were assayed for enzyme activity separately and when combined. No change in activity of the phosphatase was observed in the presence or absence of the unknown peptide (Table 9).

Table 9. Enzyme assay of Sephadex G-50 effluent.

Fraction* from Sephadex	Specific activity of P-glycolic acid phosphatase	Ratio of rate of hydrolysis of P-glycolate, 3-PGA, and pehnolphthalein di-P
8	0	---
2	290	1:0.18:0.4
8 + 2	290	---

Reaction mixture contained 0.5 ml of effluent(s), 0.5 ml of 0.02 M Tris-acetate buffer, pH 7.2, 1 ml of 0.1 M Tris-acetate buffer pH 6.3, 3 μmole of cobalt sulfate, and 0.5 ml of the substrate. The final volume was 3 ml.

*See Table 8 footnote.

F. Phosphoglycolic Acid Phosphatase Activity in Animal Tissues

Crude minces of beef liver and kidney were prepared by grinding 50 gm of the fresh tissue in a Waring Blendor with 100 ml of 0.05 M Tris-acetate buffer, pH 6.3 at full speed for 1.5 minutes. The homogenate was centrifuged at 8,000 x g for 10 minutes. The supernatant was assayed for enzyme activity against four substrates, namely phosphoglycolic acid, 3-phosphoglyceric acid, phenolphthalein diphosphate, and glucose-6-phosphate. The results are shown in Table 10. In both tissues, no enzymatically catalyzed hydrolysis of phosphoglycolic acid and 3-phosphoglyceric acid was found. The hydrolysis of phenolphthalein diphosphate was very slow in comparison with the hydrolysis of glucose-6-phosphate. The results combine with the finding in wheat roots and etiolated wheat leaves support the assumption that the phosphoglycolic acid phosphatase is only present in green plant tissues and that the enzyme is associated with the photosynthetic process.

Table 10. Phosphoglycolic acid phosphatase in bovine liver and kidney.

Source of enzyme	Substrate	Total activity in 50 gm	Unit activity per gm
liver	PGA	0	0
	3-PGA	0	0
	PPD	8,800	176
	G-6-P	59,400	1,188
kidney	PGA	0	0
	3-PGA	0	0
	PPD	8,640	175
	G-6-P	43,200	864

Reaction mixture contains 0.1 ml of crude sap, 0.5 ml of substrate, 0.0001 mole Tris-acetate buffer, pH 6.3, final volume was 3 ml. After addition of enzyme, the mixture was incubated at 37° for 10 minutes before enzymic reaction was stopped by 1 ml of 10% TCA.

PGA: phosphoglycolic acid, 2 μmole per ml.
3-PGA: 3-phosphoglyceric acid, 10 μmole per ml.
PPD: phenolphthalein diphosphate, 10 μmole per ml.
G-6-P: glucose-6-phosphate, 10 μmole per ml.

DISCUSSION

Two isolation procedures were used for phosphoglycolic acid phosphatase. One procedure had been developed by Richardson and Tolbert (15) with enzyme from tobacco leaves and involved $(NH_4)_2SO_4$ fractionation and then elution with phosphate buffer after adsorption on calcium phosphate gel. This procedure would not work with other leaves because of severe inactivation by the $(NH_4)_2SO_4$ fractionation. In addition, removal by dialysis of all residual phosphate after the absorption on calcium phosphate gel was extremely difficult.

Another procedure for isolation of the specific phosphoglycolic acid phosphatase was developed which involved acetone precipitation, chromatography on DEAE-cellulose column and the Sephadex G-50 treatment. Throughout this procedure the enzyme from several tissues was stable. Furthermore, the pH optimum remained at 6.3 even though cobalt ion was necessary for enzyme activity. After the ammonium sulfate fractionation procedure the pH optimum had changed to 5.0 which was similar to that of an acid phosphatase. The pH optimum of phosphoglycolic acid

phosphatase in the crude sap was 6.3. After purification of the enzyme by the DEAE-cellulose procedure the pH optimum remained at 6.3 which is thus the apparent true optimum. The change to pH 5.0 by ammonium sulfate fractionation is indicative of alteration in the protein superstructure and should be further investigated.

The phosphatase required cobalt ion for activity after DEAE-cellulose separation. This was thoroughly investigated by Richardson and Tolbert (15) who found that other ions such as Mg^{++} and Mn^{++} were less efficient. However, in their studies the protein had an altered pH optimum from the isolation procedure. Perhaps with the acetone precipitation method a different order of reactivity with metal ions might be found, but this was not investigated.

The rate of hydrolysis of phosphoglycolic acid by crude sap from etiolated wheat leaves and roots was 5 to 10% of the rate from green leaves. Upon purification of phosphatase activity from root or etiolated leaves the enzyme was able to hydrolyze other substrates as well or better than phosphoglycolate. On the other hand, the hydrolysis of phosphoglycolate by green sap was extremely dynamic and more rapid than with most other phosphate esters. With purification of the enzyme specificity for phosphoglycolate increased. Thus, the amount and specificity of the enzyme

in green tissues seem highly significant. The pH optimum is also unusual for phosphatase, which generally function at either acid (pH 5) or alkaline (pH 9 to 10) range. In fact, the only other well studied phosphatase with a pH optimum at 6.4 is erythrocyte phosphatase (11).

The phosphoglycolic acid phosphatase enzyme increased in activity during greening of the plant. This increase in activity has also been found to occur with other enzymes specifically associated with photosynthesis. Thus glycolic acid oxidase is inactive in etiolated plants but increases in activity during greening (9, 22, 23). Ribulose diphosphate carboxylase and other enzymes associated with the photosynthesis carbon cycle increase in activity during development of a green leaf (19). The increase in activity of phosphoglycolic acid phosphatase seemed relatively slow in that at least 24 hours of light were necessary for formation of substantial amounts of enzyme and several days were necessary before the phosphatase may have been fully active. The green plant is capable of photosynthesis after 8 to 12 hours of light. The mechanism of activation of the phosphatase is not known. In the case of glycolic acid oxidase a proenzyme was converted to the holoenzyme (9). However, the slow activation of the phosphatase suggests <u>de novo</u> synthesis of the enzyme.

As discussed in the introduction, the glycolate pathway may function for transport across the chloroplast membrane. Phosphoglycolate can move across the membrane but glycolate cannot. Thus the phosphatase on the outside of the membrane shifts the flow of glycolate out of the chloroplast. A year ago the glycolic acid oxidase enzyme was also reported in kidney (21). This animal tissue also has a major role to perform in transport. It seems possible that a glycolate system might also participate there as well as in chloroplasts. However, no phosphoglycolic acid phosphatase could be found in bovine kidney or liver. Therefore, if a glycolate system were present in the kidney it would be modified from that in the chloroplast.

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