3-P-GLYCERATE PHOSPHATASE IN LEAVES

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3-P-GLYCERATE

ABSTRACT

3-P-GLYCERATE PHOSPHATASE IN LEAVES

by Douglas Dey Randall

A 3-P-glycerate phosphatase was purified from sugarcane (Saccharium officiarum) leaves by a sequence of pH fractionation, acetone and ammonium sulfate precipitations, and chromatography on G-200 Sephadex, phosphocellulose and carboxymethyl Sephadex. The purest preparations were 2530-fold enriched with 4.4% recovery and had a specific activity of 740. The phosphatase hydrolyzed stoichiometrically D-3-P-glycerate to D-glycerate and inorganic phosphate. Between pH 4.5 to 7.0 the phosphatase was stable at 4° and -18° and at room temperatures for brief periods. It was irreversibly inactivated at alkaline pH.

The activity of the purified enzyme was optimal between pH 5.7 and 6.0, but in crude leaf extracts the optimum was at pH 6.3. No requirement for any cofactor was detected. The Michaelis plots for 3-P-glycerate phosphatase activity were hyperbolic. The apparent Michaelis constant for 3-P-glycerate was 0.28 mM. The temperature optimum was about 42° and the enzyme was 50% inactivated in 3 minutes at 50°. The isoelectric point was at pH 6.8.

The enzyme was most active toward 3-P-glycerate. Phosphoenolpyruvate was the only physiological substrate which was hydrolyzed at a rate greater than half the rate for

3-P-glycerate. p-Nitrophenylphosphate was hydrolyzed at 0.66 of the rate with 3-P-glycerate. Of 41 other phosphate esters, none were hydrolyzed at rates more than 0.50 that for 3-P-glycerate. The nucleotidase activity was low. No diesterase activity and limited pyrophosphatase activity were observed. The enzyme did not hydrolyze the C-P bond of phosphonic acid derivatives. The purified enzyme was believed to be free of other phosphatases, since the relative specificity did not change during the last four steps ofpurification. Only one phosphatase was detected by isoelectric focusing, cation exchange columns, and kinetic analysis.

The enzyme activity was not affected by extended dialysis against buffers or 1 mM EDTA, passage through G-25 Sephadex or treatment with nine metal complexing agents. Most divalent cations were partially inhibitory. Zn^{++}, Cu^{++}, Pb^{++} and Hg^{++} at 1 mM inhibited the phosphatase greater than 50%. Na^{+}, K^{+}, NH_4^{+} were without effect. The 3-P-glycerate phosphatase was inhibited by typical phosphatase inhibitors, fluoride, molybdate, L(+)tartrate and arsenate. Iodoacetate, arsensite, and p-chloromecuribenzoate inhibited 13%, 17%, and 24%. Of the large number of metabolites, analogs or related compounds which were examined as possible effectors, dihydroxytartrate, (amino-oxy)acetate, $NaBO_3$, Na_2CO_3, $NaHCO_3$, and $NaBO_3$ partially inhibited. L-α-Alanine and L-asparate at 1 mM stimulated the activity about 10%. Glycidol phosphate was not hydrolyzed and was an irreversible competitive inhibitor. Three derivatives of phosphonic acid stimulated

the enzymatic hydrolysis of 3-P-glycerate by 10%.

From gel filtration and sucrose density centrifugation, the molecular weight of the enzyme was estimated to be in the range of 160,000 to 200,000. On sucrose density gradients aggregation of the protein occurred at low ionic strength, but with 0.25 M KCl in the sucrose one form of the enzyme was obtained. Two polypeptide bands were observed on SDS-polyacrylamide electrophoresis. The molecular weight of the major band was about 51,000 and the minor band 62,000. The molar ratio of the two peptide bands was 3.6:1.

3-P-Glycerate was found in the mesophyll cells of sugarcane leaves. Non-aqueous isolation and density centrifugation procedures indicated the phosphatase was in the cytosol. It was not found in isolated peroxisomes, chloroplasts or mitochondria. In etiolated tissue there was 1/10 the phosphatase activity on a weight basis and 1/4 the activity on a protein basis. Upon exposure to light, the increase in phosphatase activity paralleled the greening of the tissue, such that in 47 hours the activity was 87% that of normal green leaves. The 3-P-glycerate phosphatase activity exhibited a diurnal variation. During days of high sunlight it increased 50% and 60% on a protein and chlorophyll basis, respectively, late in the afternoon and early darkness.

The amounts of 3-P-glycerate phosphatase and P-glycolate phosphatase were determined in 40 plants. Non-CO_2-photorespiring plants with the C_4-dicarboxylic acid cycle, generally had more 3-P-glycerate phosphatase than P-glycolate

phosphatase. The opposite was the case for CO_2-photorespiring higher plants or C_3-pathway plants. The C_3-plants however, had sufficient 3-P-glycerate phosphatase to account for glycerate formation. Algae were typical of C_3-pathway plants, as were most trees and aquatic plants. The non-vascular Liverwort, Marchantia, had more 3-P-glycerate phosphatase than P-glycolate phosphatase.

The amounts of 3-P-glycerate and P-glycolate phosphatases were determined in 15 soybean varieties (Glycine max. L. Merrill) with known net CO_2 fixation rates. The amount of P-glycolate phosphatase correlated positively with increasing CO_2 fixation rates; the amount of the 3-P-glycerate phosphatase correlated inversely with CO_2 fixation. Assays for these enzymes may be useful as an index of photosynthesis potential.

About 30% of the 3-P-glycerate phosphatase activity in spinach leaves was bound to the "starch particles" pelleted through sucrose density gradients. The particulate 3-P-glycerate phosphatase activity had a specific activity 46-fold greater than the activity of crude spinach extracts. The starch particles were so designated because they stain with $KI-I_2$, their density was greater than 2.5 M sucrose, and the phosphatase and reducing sugars were released upon incubation with β-amylase. The phosphatase could also be released from the particles by 0.35 M $MgCl_2$ or extend sonification. Activity was not solubilized by changes in pH, dialysis with or without 1 mM EDTA, repeated freezing and thawing, passage

through French pressure cell or homogenization. The 3-P-glycerate phosphatase solubilized from the particles was further purified 384-fold to a specific activity of 10.8 with a 34% recovery. The pH optimum, the absence of cofactors and the enzyme kinetics of the particulate enzyme were similar to the properties of the soluble phosphatase from sugarcane. A possible role for this phosphatase in regulation of starch formation or degradation is discussed.

Functions of the soluble 3-P-glycerate phosphatase from sugarcane leaves are unknown, but some possibilities were considered. The phosphatase may be involved in (a) regulation of the photosynthetic carbon pathways, (b) serine, glycine and C_1 synthesis as an alternate to or in addition to the peroxisomal-glycolate pathway, (c) starch synthesis, or (d) carbon transport between mesophyll and parenchyma sheath cells.

3-P-GLYCERATE PHOSPHATASE IN LEAVES

By

Douglas Dey Randall

A THESIS

Submitted to
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in partial fulfillment of the requirements
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Department of Biochemistry

1970

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Dedicated to

My wife, Shirley

and our children, Troy and Reyna

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The encouragement and faith of my parents and my wife's parents is appreciated.

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A_λ	Absorbance at wavelength λ
α-KG	α-Ketoglutarate
AMP, ADP, ATP	Adenosine mono-, di-, and triphosphate
Chl	Chlorophyll
CM-	Carboxymethyl-
DEAE-	Diethylaminoethyl-
DHAP	Dihydroxyacetone phosphate
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
f.c.	Foot candles
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
K_m	Michaelis constant
MES	2-(N-morpholine)ethanesulfonic acid
NAD(H)	Nicotinamide-adenine dinucleotide (reduced)
NADP(H)	Nicotinamide-adenine dinucleotide phosphate (reduced)
OAA	Oxaloacetate
PEP	Phosphoenol pyruvate
P-Glycolate	Phosphoglycolate
3-P-Glycerate (3-PGA)	3-Phosphoglycerate
P, P_i, PP_i	Phosphorous, inorganic phosphate, pyrophosphate
RuDP	Ribulose-1,5-diphosphate

S.A.	Specific activity
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
TEAE-	Triethylamino ethyl-
Tris	N-tris(hydroxymethyl)methylglycine

If someone tells me that in making these conclusions I have gone beyond the facts I reply: 'This is true, that I have freely put myself among ideas which cannot be rigorously proved. That is my way of looking at things. Every time a chemist concerns himself with these mysterious phenomena and every time he has the luck to make an important step forward he will be led instinctively to attribute their prime cause to a class of reactions in harmony with the general results of his own researches. That is the logical course of the human mind, in all controversial matters.'

-- Louis Pasteur, 1857

INTRODUCTION

The last quarter century of research in photosynthesis since Calvin's group envisioned and proposed the "path of carbon in photosynthesis" (1, 2, 3) has seen the elucidation of many of the enzymes, intermediates and products of the pathway (4, 5, 6, 7). The process has subsequently been called "the photosynthetic carbon cycle," or Calvin's cycle, or more recently the C-3 pathway. The kinetic isotope tracer studies allowed the determination of the sequential formation of intermediates and products. Enzymological studies have revealed substrates (NADPH and ATP) which were unlabeled in radiotracer experiments and provided information regarding regulation of the pathway (8, 9, 10, 11, 12). The initial product of the Calvin cycle is the C_3 acid, 3-P-glyceric acid (13). The subsequent metabolism of 3-P-glycerate regenerates the CO_2 acceptor, ribulose-1,5-diphosphate, through a sequence of reactions involving the sugar mono- and diphosphates. This pathway of photosynthetic CO_2 fixation was considered to be ubiquitous in higher plants and algae.

However the C_3-pathway has failed to explain all the products formed during $^{14}CO_2$ incorporation by many photosynthetic organisms. The novel pathway of CO_2 fixation which involves the ferridoxin dependent reductive carboxylation of acetyl-CoA and succinyl-CoA to yield pyruvate and α-keto-

glutarate is termed the reductive carboxylic acid cycle and appears to be limited to two heterotrophic photosynthetic bacteria (14). The very early and rapid incorporation of $^{14}CO_2$ into the dicarboxylic acids, malate and aspartate, of sugarcane and corn was initially ignored, but the persistence of this occurrence stimulated a thorough investigation of the photosynthesis of sugarcane and corn (15, 16, 17, 18). The subsequent radiotracer experiments and enzyme studies have confirmed that a second unique process of CO_2 assimilation termed the C_4-dicarboxylic acid pathway (C_4-pathway) is operative in the tropical and sub-tropical grasses such as sugarcane as well as in other species (Figure 1) (16).

The C_3-pathway of CO_2 assimilation also fails to explain the early and rapid incorporation of $^{14}CO_2$ into glycolate (α-hydroxy acetate) (5, 19, 20). The amount of newly fixed carbon dioxide found in glycolate and the "glycolate pathway" (Figure 2) ranges from 0 up to 92% of the total carbon fixed (21, 22), and is dependent on several physiological and photosynthetic conditions to be cited in the Literature Review. The accumulation of glycolate and its products is observed especially with young or rapidly growing tissue. Thus it seems that glycolate should have a very significant role in photosynthesis.

Glycolic acid is believed to be biosynthesized from some intermediate(s) of the C_3-pathway and its metabolism is closely related to photosynthetic conditions (22). P-Glycolate is believed to be the immediate precursor in the

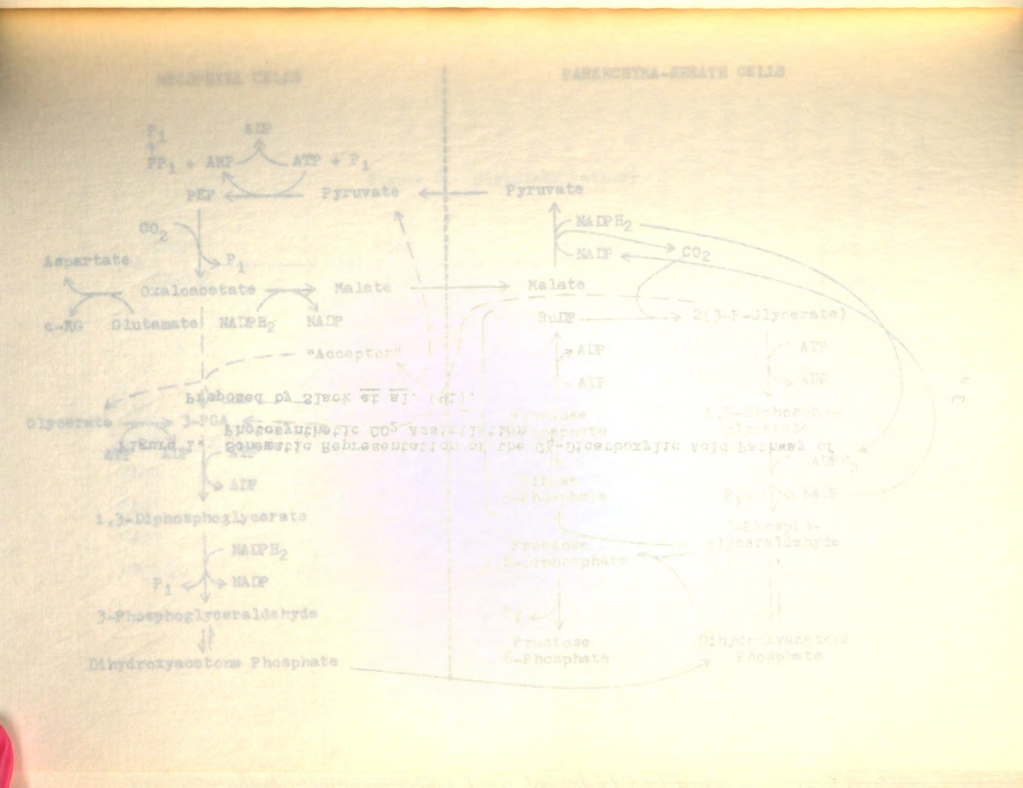

MESOPHYLL CELLS
PARENCHYMA-SHEATH CELLS
P_i
ADP
PP_i + AMP
ATP + P_i
PEP
Pyruvate
Pyruvate
CO_2
$NADPH_2$
NADP
CO_2
Aspartate
P_i
Oxaloacetate
Malate
Malate
α-KG
Glutamate
$NADPH_2$
NADP
RuDP
2(3-P-Glycerate)
"Acceptor"
ATP
ADP
ATP
ADP
Glycerate
3-PGA
Ribulose
5-Phosphate
1,3-Diphospho-
glycerate
ATP
ADP
1,3-Diphosphoglycerate
$NADPH_2$
NADP
P_i
3-Phospho-
glyceraldehyde
Fructose
1,6-Diphosphate
P_i
Fructose
6-Phosphate
3-Phosphoglyceraldehyde
Dihydroxyacetone Phosphate
Dihydroxyacetone
Phosphate

Figure 1. Schematic Representation of the C_4-Dicarboxylic Acid Pathway of Photosynthetic CO_2 Assimilation

Proposed by Slack et al. (41).

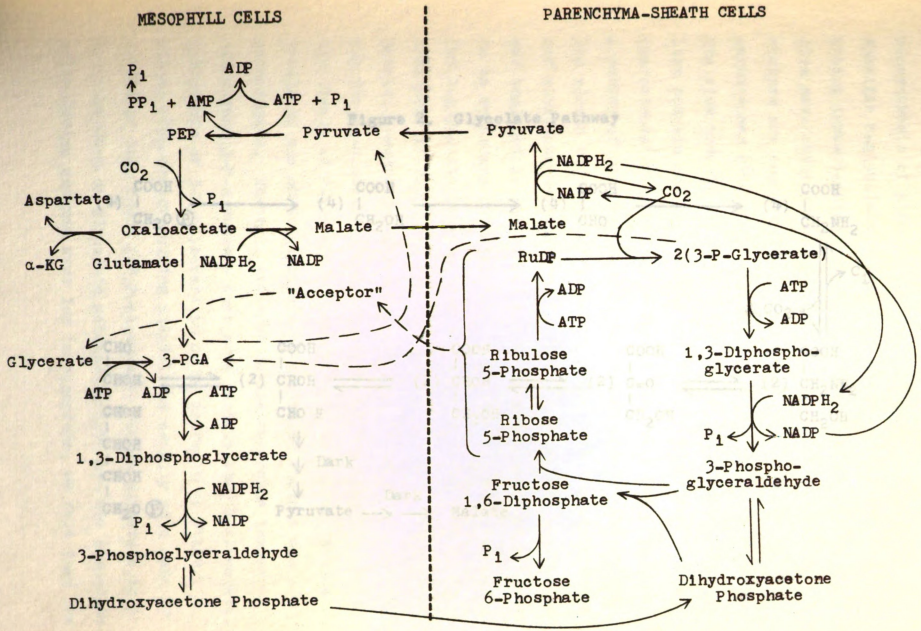
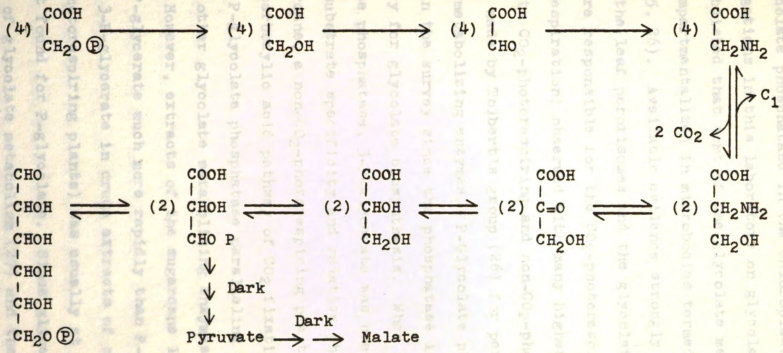

MESOPHYLL CELLS
PARENCHYMA-SHEATH CELLS
P_1
ADP
PP_1 + AMP
ATP + P_1
PEP
Pyruvate
Pyruvate
CO_2
P_1
Aspartate
Oxaloacetate
Malate
Malate
α-KG
Glutamate
$NADPH_2$
NADP
"Acceptor"
$NADPH_2$
NADP
CO_2
RuDP
2(3-P-Glycerate)
ADP
ATP
ATP
ADP
Glycerate
3-PGA
Ribulose 5-Phosphate
1,3-Diphospho-glycerate
ATP
ADP
ATP
ADP
Ribose 5-Phosphate
$NADPH_2$
P_1
NADP
1,3-Diphosphoglycerate
3-Phospho-glyceraldehyde
$NADPH_2$
Fructose 1,6-Diphosphate
P_1
NADP
3-Phosphoglyceraldehyde
P_1
Fructose 6-Phosphate
Dihydroxyacetone Phosphate
Dihydroxyacetone Phosphate

Figure 2. Glycolate Pathway



biosynthesis of glycolate, since it is hydrolyzed by a specific P-glycolate phosphatase in the chloroplasts (23, 24). Recent investigations in this laboratory on glycolate metabolism have established that some of the glycolate metabolizing enzymes are compartmentalized in microbodies termed leaf peroxisomes (25, 26). Available evidence strongly supports the view that the leaf peroxisomes and the glycolate metabolism therein are responsible for the CO_2-photorespiration (peroxisomal respiration) observed with many higher plants. A survey of both CO_2-photorespiring and non-CO_2-photorespiring plants was made by Tolbert's group (26) for peroxisomes and glycolate metabolizing enzymes. P-Glycolate phosphatase was included in the survey since the phosphatase is presumed to be necessary for glycolate biosynthesis. When surveying for P-glycolate phosphatase, 3-P-glycerate was used as an indicator of substrate specificity and relative phosphatase levels. Sugarcane, a non-CO_2-photorespiring plant, possessing the C_4-dicarboxylic acid pathway of CO_2 fixation, had low levels of P-glycolate phosphatase paralleling the low levels of the other glycolate metabolizing enzymes and peroxisomes. However, extracts of the sugarcane leaves hydrolyzed 3-P-glycerate much more rapidly than P-glycolate. Hydrolysis of 3-P-glycerate in crude extracts of other plants (CO_2-photorespiring plants) was usually at a rate 25 to 30% of that found for P-glycolate. Since glycerate is a major product of glycolate metabolism (27) and the glycolate metabolizing enzymes were low in sugarcane, we felt that the

rapid enzymatic hydrolysis of 3-P-glycerate might be indicative of a bypass of the glycolate pathway. A 3-P-glycerate phosphatase would also explain the $^{14}CO_2$ incorporation into the carboxyl position of glycerate during photosynthesis giving an isotopic distribution inconsistent with the glycolate pathway (28, 29). The carboxyl-labeled glycerate found during photosynthesis experiments had previously been attributed to inadequate killing procedures of the experiment and was believed to be insignificant, even though in some leaves large metabolically active reservoirs of glycerate were present (7, 28, 29).

The following points suggested that an investigation of a 3-P-glycerate phosphatase would contribute significantly to the understanding of the path of carbon in photosynthesis and photorespiration. (a) Glycolate metabolism and P-glycolate phosphatase activities were low in sugarcane. (b) Enzymatic hydrolysis of 3-P-glycerate by sugarcane extracts was unusually rapid. (c) Sugarcane is a non-CO_2-photorespiring plant with a CO_2 assimilation process differing from the normal Calvin cycle from which glycolate is made. (d) A 3-P-glycerate phosphatase would provide a bypass of the glycolate pathway (negating the need for it), thus bypassing the source of respired CO_2 and O_2 uptake of CO_2-photorespiration and explaining the low level of glycolate metabolism. (e) A 3-P-glycerate phosphatase would explain the appearance of carboxyl-labeled glycerate (and serine) in $^{14}CO_2$ photosynthesis experiments.

The purification and characterization of 3-P-glycerate was undertaken to determine if this enzyme was similar to P-glycolate phosphatase with respect to such parameters as specificity, location and cofactor requirements. Such similarities would support our conclusion that 3-P-glycerate phosphatase was a substitute for P-glycolate phosphatase and in addition provide possible information useful in understanding the function of both phosphatases. Studies directed towards the formation, location, and physiological changes in activity would be useful in delineating whether or not the 3-P-glycerate phosphatase was involved in photosynthetic processes. Studies comparing the distribution of both phosphatases in the plant kingdom should help to elucidate their true function(s).

This thesis describes the study of 3-P-glycerate phosphatase of sugarcane leaves. Procedures for the partial purification of the enzyme and some of its biochemical and physical properties are described. The distribution of this phosphatase, as well as that of P-glycolate phosphatase among many plants, is also presented as well as some physiological studies of its presence in leaves. A study attempting to correlate the relative levels of 3-P-glycerate phosphatase and P-glycolate phosphatase to photosynthetic rates in soybeans is presented. The discovery and partial characterization of a particulate (probably starch grains) 3-P-glycerate phosphatase from spinach leaves is also presented.

LITERATURE REVIEW

The studies on 3-P-glycerate phosphatase are directed ultimately towards determining its function as a part of the photosynthetic process of green plants. Since the source of this enzyme is the photosynthetic tissue of sugarcane, and its substrate is the first or one of the first major intermediates of photosynthetic CO_2 assimilation, the two major pathways of photosynthetic carbon metabolism will be reviewed. The functions to be attributed to the 3-P-glycerate phosphatase require a review of the glycolate pathway and photorespiration. Consideration of the importance of the 3-P-glycerate to the carbon metabolism of the leaf requires a review of 3-P-glycerate metabolism and glycerate formation. Obviously, justice cannot be done to each of these exciting and dynamic research areas without writing a very large book; however, the author feels a brief review of these areas will facilitate the discussion of the studies on 3-P-glycerate phosphatase in leaves.

The reductive assimilation of free atmospheric carbon by photosynthetic organisms through utilization of light energy is summarized by the equation:

$$nCO_2 + 2\,nH_2O \xrightarrow{h\nu} nO_2 + (CH_2O)_n + nH_2O$$

and is termed photosynthesis. This complex process entials converting light energy to chemical energy of excited chlorophyll molecules, converting this chemical energy to reducing equivalents, NADPH and high energy phosphate, ATP, and reducing CO_2 to carbohydrate, using the reducing power and ATP. The "path of carbon in photosynthesis" describes how the ATP and NADPH are used for CO_2 assimilation in green photosynthetic organisms (30).

The C_3-Photosynthetic Carbon Reduction Cycle

Radioautography of chromatographed extracts from the green algae, Chlorella and Scenedesmus, which had been supplied with $^{14}CO_2$ for less than 5 sec during steady state photosynthesis, revealed carboxyl-labeled 3-P-glycerate as the first labeled compound of photosynthesis (13). Since CO_2 is the only source of carbon, the acceptor molecule must be regenerated, thus a cyclic pathway is inherent. Through an elegant series of experiments (13, 31, 32, 34) involving light and dark transitions coupled with a knowledge of metabolic pathways of glycolysis and pentose phosphates, Calvin's group established that the carbon reduction cycle involves (a) the carboxylation of ribulose 1,5-diphosphate to give two molecules of 3-P-glycerate [or 3-P-glycerate and triose phosphate (34)], (b) the reduction of 3-P-glycerate to triose phosphate, (c) the condensation and conversion of triose phosphates to hexose phosphates and pentose phosphates, and (d) the regeneration of ribulose diphosphate

from pentose phosphates and ATP (30). The adequacy of the C_3-pathway as a means of CO_2 fixation was presented by Bassham and Kirk (35) who found that upwards of 85% of the CO_2 fixed by Chlorella could be accounted for by the C_3-pathway and its immediate products. Massive evidence for the C_3-pathway has been accumulating since the pathway was proposed in the early 1950's (34, 38). The isolation of intact spinach chloroplasts capable of CO_2 fixation and carbon cycling at close to in vivo rates has been instrumental in localizing the C_3-pathway in the chloroplasts (36, 37, 38).

The enzymes for the C_3-pathway have been found supporting the radiotracer experiments (10, 11, 30, 38). The nonaqueous isolation and density gradient centrifugation studies by Stocking (39), Smillie (40) and others (41) have provided excellent evidence for the localization of the C_3-pathway enzymes and intermediates in the chloroplasts. Control of the C_3-pathway is manifested through light dependent activation of enzymes, formation of metabolites or changes in the energy charge. Control is also manifested by changes in substrate or cofactor levels (feedback inhibition and homotropic interactions) (12).

The function of the C_3-cycle is to provide carbon compounds to the plant. Although the chloroplast is capable of making all the major types of compounds, there are some priorities necessary in the photosynthetic products in order for the plant to be able to survive. Except for the formation

of glycolic acid, some amino acids and certain carbohydrates which will be discussed later in the review, sucrose and starch (α-glucan) are the major immediate products in the photosynthetate. Sucrose and starch represent a sink or reservoir for carbohydrate produced from the energy and reducing power provided by the photochemical event. Both are made in the chloroplast (42, 43, 44). Sucrose is the chief carbohydrate transported elsewhere in the plant (44). Chloroplast starch or assimilatory starch is used during low light periods or darkness for biosynthesis and energy. Starch synthesis is regulated by intermediates of the C_3-pathway, 3-P-glycerate and fructose-6-phosphate, at the ADP-glucose pyrophosphorylase level (12).

The C_3-pathway has been found in numerous higher plants, green algae and photosynthetic bacteria. The C_3-pathway was assumed to be ubiquitous, until evidence for the reductive carboxylic acid cycle in some photosynthetic bacteria was recently reported (14), and a second major pathway (C_4-pathway discussed below) was proposed for higher plants (16). The belief that the C_3-pathway was the universal pathway was partially due to geography and choice of plant material. Most research was done in the temperate geographic zones, using easily manipulated tissue such as spinach, soybeans, and green algae. These plants give perfect examples of the C_3-pathway. It is almost incredible as one reads the literature of photosynthesis with the present information that 99% of the earlier photosynthesis

research was done using a limited number of temperate zone plants. Corn and sugarcane, which are extremely important economic crops, and should have been studied, were too difficult to use in photosynthesis experiments because of their tough leaf tissue and consequently were assumed to possess the C_3-pathway of CO_2 assimilation. Sugarcane and corn have an exceptional capacity for producing dry matter (45) and are capable of photosynthesis rates much greater than most other plants (46, 47).

The C_4-Dicarboxyl Acid Pathway of Photosynthesis

Tarchevskii and Karpilow (50) in 1963 and Kortshak, Hartt and Burr (15) in 1965 noted that aspartate and malate were the first compounds labeled during $^{14}CO_2$ experiments with corn and sugarcane.* Hatch and Slack extended these experiments and added oxaloacetate to the list of initially labeled compounds (17). Using sugarcane, corn and sorghum leaves, radiotracer photosynthesis studies by Hatch and Slack showed that:

(a) Incorporation of ^{14}C into oxaloacetate, aspartate and malate was linear with time, light dependent and occurred first during $^{14}CO_2$-fixation (17, 18, 51).

(b) The ^{14}C labeling into C-4 of dicarboxylic acids was faster than the C-1 labeling of 3-P-glycerate (51).

*Calvin's group back in the 50's had similar data, but ignored it! (Personal communication from one who was present.)

(c) The ^{14}C in C-4 of the dicarboxylic acids became the C-1 of 3-P-glycerate in a light dependent reaction, and then flowed sequentially through dihydroxyacetone-P, the hexose phosphates, and the pentose phosphates to sucrose and α-glucan in a manner consistent with their role as intermediates in the fixation cycle (51).

(d) ^{14}C accumulated in RuDP when leaves fixing $^{14}CO_2$ were placed in CO_2-free air (51), thus suggesting it was the CO_2 acceptor from the C-4 of the dicarboxylic acid or an immediate precursor.

With the above evidence Hatch and Slack proposed the cyclic C_4-dicarboxylic acid pathway in Figure 1. The mode of transferring the C-4 of the dicarboxylic acids to C-1 of 3-P-glycerate is uncertain at this time. The enzymes for all the reactions except the "transcarboxylase" reaction have been established as being present in adequate amounts (41, 52, 53, 54, 55).

The enzymes found include three enzymes unique to the C_4-pathway of photosynthesis, phosphoenol pyruvate (PEP) carboxylase for the primary carboxylation reaction (52); pyruvate, P_i dikinase to regenerate the primary CO_2 acceptor, PEP (54, 56); and NADP-malate dehydrogenase to reduce oxaloacetate (41).

Attempts to isolate an enzyme catalyzing the proposed transcarboxylation have been unsuccessful (16, 57). Andrews (54) proposes that the transcarboxylation process is the

coordinated result of malic enzyme (52, 55) and RuDP carboxylase. The oxaloacetate made by the PEP carboxylase would be converted to malate by the NADP-malate dehydrogenase. The malate would be decarboxylated in the presence of RuDP carboxylase by malic enzyme (NADP-malate dehydrogenase decarboxylase). The CO_2 (possibly enzyme bound) would be refixed by the RuDP carboxylase to yield 3-P-glycerate. The RuDP carboxylase activity, which was initially reported as being too low to have an important role in the C_4-pathway, has been found to be adequate and to possess even better kinetic parameters than the enzyme in C_3-pathway plants (54, 58).

Location of C-4 Pathway

Corn, sugarcane and other C_4-pathway plants differ from C_3-pathway plants in that they contain two morphologically distinct types of chloroplasts. Roades and Carvalho (48) and Brown (119) noted that panicoid grasses had two different types of chloroplast containing cells. These chloroplasts were of two distinct types with differing starch storing capacities (48, 49), but the significance of this to the photosynthetic process in these plants was not considered until recently when the C_4-pathway was thoroughly investigated. The two types of chloroplasts have been designated mesophyll chloroplasts and bundle (parenchyma) sheath chloroplasts by their presence in these cells. The bundle sheath chloroplasts contain the assimilatory starch. Using non-aqueous isolation and density

fractionation techniques, Slack and associates (41, 59) were able to separate bundle sheath chloroplasts and mesophyll chloroplasts by their differences in starch content. This permitted him to distinguish enzymes of mesophyll chloroplasts from bundle sheath chloroplasts and also chloroplastic enzymes from cytosol. This same procedure could be used to localize labeled intermediates produced in $^{14}CO_2$ experiments. The results of these experiments are summarized in Table I.

All the enzymes of the regular C_3-pathway of photosynthesis plus malic enzyme are located in the bundle sheath chloroplasts (Table I), while the enzymes of the C_4-pathway are localized in the mesophyll cells. According to Andrews (54), the fact that malic enzyme (high activity only in C_4-pathway plants) was located in the bundle sheath chloroplasts supports the proposal that it is part of the transcarboxylation mechanism. It would also provide half of the reducing power needed to convert 3-P-glycerate to triose phosphates, since there appears to be a low level of reducing power in the bundle sheath cells (57).

The ^{14}C labeled intermediates of the C_4-pathway were located in the plastids with their respective enzymes. 3-P-Glycerate and DHAP were distributed fairly evenly between the two types of chloroplasts (41).

In summary the investigators in Australia have provided the following evidence for the C_4-pathway of photosynthesis in a large number of plants (57, 60).

Table I. Location of Enzymes of the C_4-Dicarboxylic Acid Pathway Between Two Types of Chloroplasts (16, 41).

Mesophyll Chloroplasts	Bundle Sheath Chloroplasts	Both Chloroplasts
PEP-Carboxylase	FDP Aldolase	3-P-Glycerate kinase
NADP-Malate dehydrogenase	Alkaline FDP phosphatase	NADP-3-P-Dehydrogenase
Aspartate-aminotransferase	Ribose-P-isomerase	Triose phosphate isomerase
Pyruvate, P_i dikinase	Ribulose-P-kinase	
Adenylate kinase	RuDP-Carboxylase	
Pyrophosphatase	Malic enzyme (dicarboxylase)	
Glycerate kinase		

(a) The labeling of the primary carboxylation products were light dependent and showed the necessary kinetics of labeling.

(b) The ^{14}C flowed sequentially through the proposed cycle.

(c) The labeled intermediates were found in the chloroplasts.

(d) The enzymes necessary were present in adequate amounts and were in the plastids.

(e) The enzymes were synthesized during the biogenesis of the chloroplasts in corn plants.

(f) The pathway operates in a cyclic manner to regenerate the CO_2 acceptor moiety.

It has been mentioned above that there are certain morphological, physiological, and environmental correlations between which plants have the C_3- or C_4-pathway.

Leaf Anatomy and Chloroplast Structure

Three sub-families of Gramineae, Panicoideae, Aristoideae and Chloridoideae, have their leaf vascular bundles sheathed by large, chloroplast-containing, parenchyma sheath cells which are surrounded in a radial fashion by mesophyll cells (with small and numerous chloroplasts) (48, 49). The chloroplasts of the bundle sheath cells accumulate considerable starch. Dicotyledons such as the Amaranthus of the Amaranthaceae and some Atriplex of the Chenopodiaceae possess the same leaf anatomy (61). Without exception,

plants with these features that have been investigated have the C_4-pathway. For intragenera exceptions such as Panicum of the Panicoideae in which the C_4-pathway was missing and only the C_3-pathway was present, the bundle sheath cells lack developed chloroplasts (57).

The chloroplasts of the mesophyll cells have the familiar grana stacks. Chloroplasts of the bundle sheath cells in the Panicoids are without grana but grana are present in the bundle sheath chloroplasts of some other C_4-pathway plants. The two types of cells of the C_4-pathway plants are connected by numerous plasmadesmata (62). A well developed peripheral reticulum linked directly to the bounding membrane of the chloroplasts was also noted by Laetsch (62) and others (63) and it has been suggested that this peripherial reticulum may interconnect the two types of chloroplasts (57, 62).

Photosynthesis Rates

Photosynthesis rates of C_4-pathway plants are high, averaging 60-70 mg CO_2 per dm^2 per hour as opposed to 5-30 mg CO_2 per dm^2 per hour for C_3-pathway plants. The photosynthesis of C_3-pathway plants in air is saturated at less than 50% of full sunlight while photosynthesis of the C_4-pathway plants remains unsaturated even at full sunlight (46).

CO_2 Compensation Point and Oxygen Inhibition of Photosynthesis

The CO_2 compensation point is the CO_2 concentration at which the rate of photosynthesis equals the rate of carbon dioxide released by respiration. C_4-pathway plants show a CO_2 compensation point of less than 5 ppm CO_2 (64) and can reduce the CO_2 concentration of a closed system nearly to zero (65). C_3-Pathway plants show a finite value in the 15-150 ppm CO_2 range, however at low O_2 pressures their compensation point approaches that of C_4-pathway plants in air (65).

Photosynthetic CO_2 fixation in plants with the C_3-pathway is inhibited by O_2 at its concentration in air (21%) (67). This inhibition decreases with decreasing oxygen concentrations (67) and has been attributed to CO_2-photorespiration which will be discussed below with glycolate metabolism. In the C_4-pathway plants there is no effect of oxygen on rates of net photosynthetic CO_2 fixation (65).

Environmental Correlations

Plants with the C_4-pathway inhabit high sunlight areas of the world, such as grasslands, open forests and arid or semi-arid regions (49, 57). The inter- and intragenera exceptions to the C_4-pathway distribution support this correlation. The Panicum of the Panicoideae inhabits the shaded rain-forest floor and is a C_3-pathway plant instead of a C_4-plant as are most other panicoids (57). Bamboo occurs in high sunlight and tropical regions but is a C_3-plant (57).

However, the habitat of bamboo receives adequate rainfall distribution over the whole year. C_4-Pathway plants would definitely have an advantage in areas of frequent or annual drought. Their more efficient net CO_2 fixation (no loss of CO_2 from photorespiration) permits much shorter periods of open stomata from which vital water would transpire.

The examples of the above correlations are plentiful and such data are increasing. There is no taxinomic evidence for the C_4-pathway in primitive plants or algae. The C_4-pathway plants are located among the most highly evolved plant species.

The fundamental differences between the C_4-pathway and the C_3-pathway are the reactions involving the incorporation of CO_2 into 3-P-glycerate, the location of the carboxylation reactions, the correlation between the C_3-pathway and CO_2 photorespiration, the lack of the CO_2 photorespiration in C_4-plants, and possibly the stoichiometry of the pathways with regard to ATP and $NADPH_2$ requirements which has yet to be resolved (16, 57). The major, quantitative end products of both pathways are sucrose and assimilatory starch.

In both the C_3- and C_4-pathway, 3-P-glycerate is a major early product of photosynthesis which is then photosynthetically reduced to sugars. What we believe to be the reasons for its hydrolysis by a specific phosphatase have already been presented. First it seems necessary to understand the properties of this phosphatase and some of its physiological characteristics. This is the objective of this thesis.

Glycolate Pathway

Any consideration of compounds resulting from photosynthesis certainly must include the glycolate pathway. The glycolate pathway describes the metabolic sequence from P-glycolate and glycolate through glyoxylate, glycine, serine and hydroxypyruvate to glycerate (22, 27). Glycolate and the intermediates of the pathway (Figure 2) are rapidly labeled during $^{14}CO_2$ photosynthesis experiments using C_3-pathway plants, however this ^{14}C incorporation into glycolate and products cannot be explained by the C_3-carbon reduction cycle. The mode of glycolate formation during CO_2 fixation in photosynthesis remains unknown although 10 to 90% of the total carbon may be passing through it (21, 22, 69). The kinetics of the ^{14}C accumulation in glycolate indicate that it is formed from an intermediate of the C_3-pathway (5). The immediate precursor of glycolate is believed to be P-glycolate formed in the chloroplast (22) which is hydrolyzed by a specific P-glycolate phosphatase located in or on the chloroplast (23). Glycolate and the immediate products formed from short time (4-11 sec) photosynthesis experiments with $^{14}CO_2$ are uniformly labeled and its metabolites should subsequently be uniformly labeled. 3-P-Glycerate and glycerate are carboxyl-labeled at these times. Feeding experiments with glycolate-1-^{14}C produced glycine-1-^{14}C, serine-1-^{14}C and glycerate-1-^{14}C; with glycolate-2-^{14}C produced glycine-2-^{14}C, serine-2,3-^{14}C and glycerate-2,3-^{14}C and hexose-1,2,5,6-^{14}C; with serine-3-^{14}C yielded glycerate-

3-^{14}C and hexose-1,6-^{14}C (27, 71, 72, 73).

Glycolate formation is definitely linked to photosynthetic conditions. It is favored by low CO_2 concentration, high O_2 concentrations (20%) and high light intensity (21, 22). Inhibition of photosystem II and manganese deficiencies curtail glycolate formation (22, 74). Several of the enzymes of the glycolate pathway are absent or greatly reduced in activity in etiolated tissue (75) and develop during greening (76, 77). However the manner in which glycolate is biosynthesized is a major unresolved part of photosynthetic carbon metabolism. This in turn contributes to the fact that the function of glycolate metabolism is unknown. Although for 20 years glycolate and its metabolism have been the subject of intense research by several laboratories, its role and function in the metabolism of plants have never been established. The glycolate pathway is gluconeogenic, ultimately producing sucrose in the light but yielding malate in the dark (22). Glycolate contributes to the serine pools of the plant (29) but as will be discussed later, is not the only source of serine.

Glycolate metabolism appears to be responsible for photorespiration. Photorespiration occurs in the light and is usually measured as light dependent CO_2 evolution or increased O_2 uptake. The rate of photorespiration has been estimated at 5 times the dark respiration (78) and is inhibited by inhibitors of glycolate oxidase (79). The O_2 uptake of photorespiration is attributed to glycolate

oxidation by glycolate oxidase (80), and the CO_2 released is attributed to the direct enzymatic conversion of two glycines to one serine. This gas exchange is the reverse of that which occurs during photosynthesis. During the last 4 years most of the glycolate metabolizing enzymes (Figure 3) have been found compartmentalized in leaf microbodies, called peroxisomes (25, 26). Thus glycolate metabolism has been designated as photorespiration or peroxisomal respiration. The glycolate pathway in peroxisomes and photorespiration is still gluconeogenic with glycerate and CO_2 as major products. Glycolate is itself a major product of the C_3-pathway of photosynthesis; it is the substrate for photorespiration (peroxisomal respiration) and it is a major source of glycerate, hexose phosphate and sucrose (27) in C_3-pathway plants. Electron micrographs indicate a close physical association between chloroplasts, peroxisomes and microbodies (81). It has been estimated that peroxisomal enzymes are capable of as much carbon metabolism as there is CO_2 fixed during photosynthesis in the chloroplasts (82). It seems rather a waste to respire 25% of the newly fixed carbon, thus reducing the net photosynthetic CO_2 fixation and efficiency. However, no judgment is legitimate until the functions of glycolate formation and peroxisomal respiration are established. A perusal of the glycolate pathway (Figures 2 and 3) in C_3-pathway plants reveals that a 3-P-glycerate phosphatase would be capable of bypassing the glycolate pathway, eliminating the O_2 uptake, the CO_2 evolved and still produce

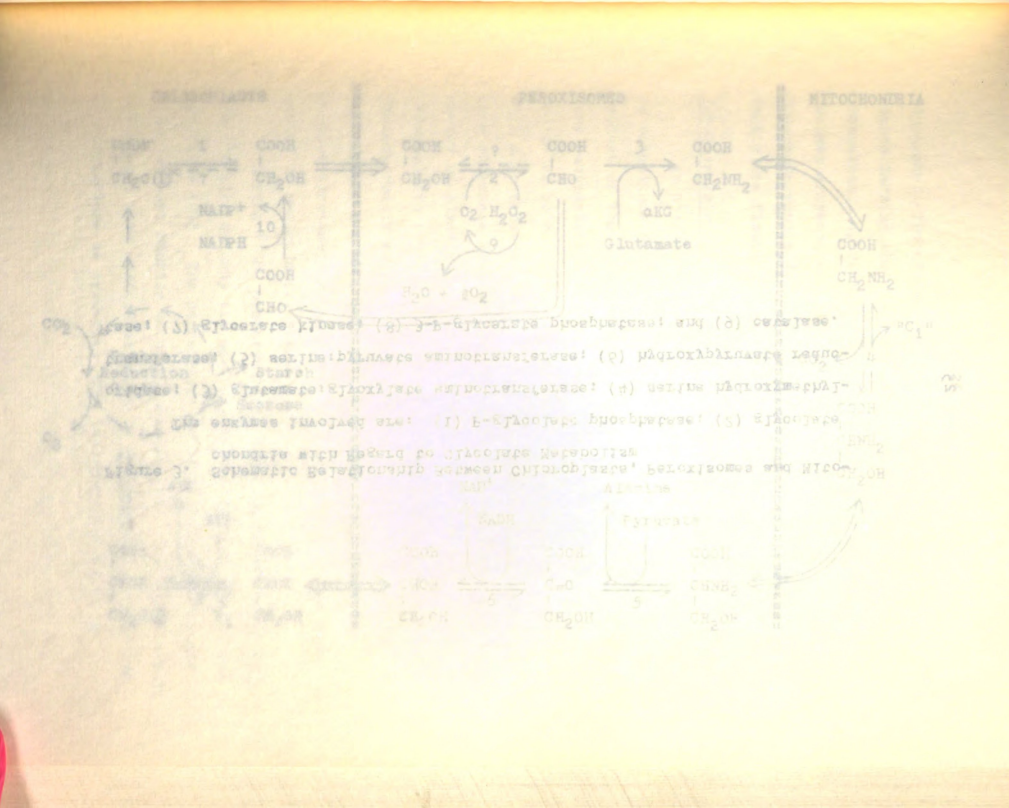
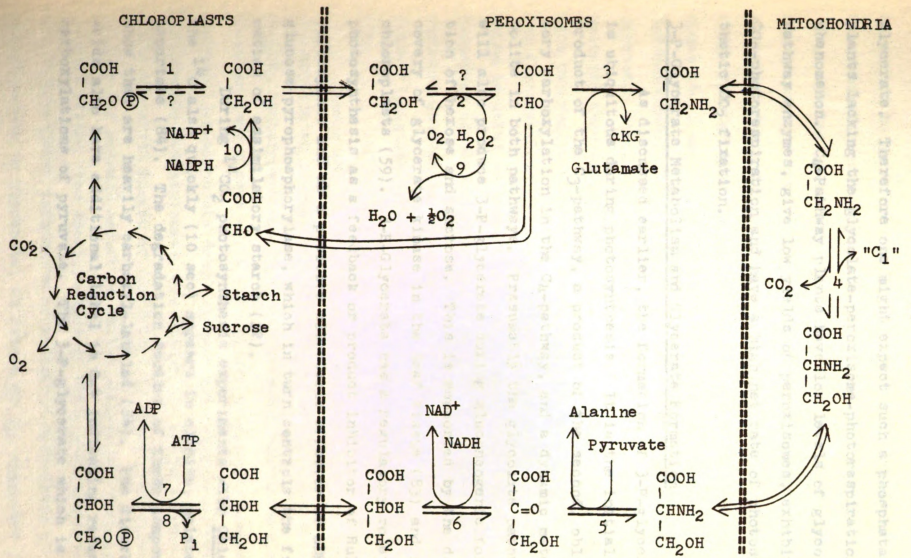

CHLOROPLAST
PEROXISOME
MITOCHONDRIA
COOH
CH2OH
CHO
CH2NH2
NADP+
NADPH
Glutamate
αKG
O2 H2O2
H2O + ½O2
CO2
Starch
Reduction
Oxidation
Alanine
Pyruvate

Figure 3. Schematic Relationship Between Chloroplasts, Peroxisomes and Mitochondria with Regard to Glycolate Metabolism

The enzymes involved are: (1) P-glycolate phosphatase; (2) glycolate oxidase; (3) glutamate:glyoxylate aminotransferase; (4) serine hydroxymethyltransferase; (5) serine:pyruvate aminotransferase; (6) hydroxypyruvate reductase; (7) glycerate kinase; (8) 3-P-glycerate phosphatase; and (9) catalase.


CHLOROPLASTS
PEROXISOMES
MITOCHONDRIA
COOH
CH2O(P)
1
?
CH2OH
NADP+
NADPH
10
CHO
CO2
Carbon Reduction Cycle
Starch
Sucrose
O2
ADP
ATP
7
8
P1
CHOH
CH2O(P)
CH2OH
2
O2 H2O2
9
H2O + ½O2
3
αKG
Glutamate
CH2NH2
"C1"
4
C=O
6
NAD+
NADH
Alanine
Pyruvate
5
CHNH2

glycerate. Therefore one might expect such a phosphatase in plants lacking the glycolate-peroxisome-photorespiration phenomenon. C_4-Pathway plants have low levels of glycolate pathway enzymes, give low yields of peroxisomes, exhibit no CO_2-photorespiration and have a high net rate of photosynthetic CO_2 fixation.

3-P-Glycerate Metabolism and Glycerate Formation

As discussed earlier, the formation of 3-P-glycerate is ubiquitous during photosynthesis. It is the initial product of the C_3-pathway, a product of the second, obligatory carboxylation in the C_4-pathway, and a dynamic metabolite in both pathways. Presumably the glycolate pathway will also produce 3-P-glycerate during gluconeogenic formation of hexose and sucrose. This is supported by the discovery of glycerate kinase in the leaf tissue (83) and in chloroplasts (59). 3-P-Glycerate has a regulatory role in photosynthesis as a feedback or product inhibitor of RuDP carboxylase and as a positive allosteric effector of ADP-glucose pyrophosphorylase, which in turn controls the formation of assimilatory starch (12).

During $^{14}CO_2$ photosynthesis experiments with Chlorella the ^{14}C also quickly (10 sec) appears in alanine, malate and aspartate (84). The degradation studies of these compounds show they are heavily carboxyl-labeled (84). The dicarboxylic acids also have additional label in C-4 indicating reductive carboxylations of pyruvate. The 3-P-glycerate which is

converted to pyruvate through PEP provides substrate for tricarboxylic acid cycle and acetyl-CoA for lipid synthesis.

Serine is also labeled very early in $^{14}CO_2$ photosynthesis experiments. Serine is rapidly turning over and is an intermediate in glycolate metabolism. Hess and Tolbert (29) reported that serine was carboxyl-labeled at short times (4-11 sec) in tobacco, but less carboxyl-labeled than 3-P-glycerate. By 30 seconds the serine was uniformly labeled. Since glycine and glycolate (possible serine precursors) were uniformly labeled they concluded that there were two routes of serine synthesis, but that serine from the glycolate pathway predominated. Chang and Tolbert (28) using isolated spinach chloroplasts and $^{14}CO_2$ reported the formation of carboxyl-labeled serine. The reports of carboxyl-labeled serine suggest that serine is a product of either 3-P-glycerate or glycerate from 3-P-glycerate hydrolysis.

Cheung et al. (83) found that enzymes for the non-phosphorylated pathway of serine synthesis (glycerate dehydrogenase, hydroxypyruvate:L-alanine transaminase) were highest in green leaf tissue while the enzymes of phosphorylated pathway (3-P-glycerate dehydrogenase and P-hydroxypyruvate: L-glutamate transaminase) were highest in seeds, although there was still enough of the latter in green leaf tissue to account for a small amount of serine synthesis. Carboxyl-labeled serine from short periods of $^{14}CO_2$ photosynthesis can be considered indicative of enzymatic hydrolysis of 3-P-glycerate to glycerate, as Rabson et al. suggested (27).

Glycerate Formation

Glycerate has been reported many times to be rapidly labeled in photosynthesis experiments but its mode of formation has not been established. Since glycolate is uniformly labeled in $^{14}CO_2$ photosynthesis, the glycerate that arises from the glycolate pathway would be uniformly labeled (27). When leaves were fed specifically labeled glycolate, glycine and serine the resulting labeling patterns were consistent with direct formation of glycerate from serine (27). Glycerate arising from 3-P-glycerate during $^{14}CO_2$ photosynthesis should be carboxyl-labeled. Bassham (85) and Heber (86) have shown that the chloroplast and cytosol pools of 3-P-glycerate are very rapidly equilibrated and the chloroplasts of C_3-plants are very permeable to 3-P-glycerate in either direction (87). Data from Slack et al. (41) indicates that the 3-P-glycerate in corn was distributed in both parenchyma and bundle sheath cells. Thus it is possible to suggest that enzymatic hydrolysis of 3-P-glycerate could occur in the chloroplasts or the cytoplasm and still be carboxyl-labeled at short times in $^{14}CO_2$ fixation experiments. The appearance of carboxyl-labeled glycerate on radioautographs of photosynthesis products has often been explained as an artifact due to enzymatic hydrolysis of 3-P-glycerate during the killing procedure (6). This explanation was supported by Ullrich (88) who reported that there was considerable phosphatase action occurring during the killing procedure with 80% boiling ethanol. Mortimer (89) reported experiments

with soybeans which suggested there was considerable *in vivo* hydrolysis of 3-P-glycerate but that the 80% boiling ethanol killing procedure was quite adequate. Aronoff (90) reported carboxyl-labeled glycerate from soybean which was more carboxyl-labeled than 3-P-glycerate after 5 sec. This might be indicative that some of the 3-P-glycerate was hydrolyzed before it had any chance of being metabolized through the C_3-pathway.

Rabson *et al.* (27) found carboxyl-labeled serine and glycerate with $^{14}CO_2$ photosynthesis experiments with leaves from soybean, coleus, corn, peppermint and barley after 20 seconds in steady state fixation experiments. Corn had the most carboxyl-labeled glycerate. The carboxyl-label of glycerate in corn was 20 times that of the α and β carbons, while in the other plants it was only 2 to 5 times. However, the glycerate and serine were not as carboxyl-labeled as 3-P-glycerate, and it was suggested that there were two sources of glycerate, one from 3-P-glycerate by phosphatase action and the second coming from the glycolate pathway. At times of 60 seconds or greater, serine became uniformly labeled, while glycerate remained carboxyl-labeled, thus indicating serine formation from the glycolate pathway was dominant.

Hess and Tolbert (29) found considerable glycerate (4-10% of total $^{14}CO_2$) formed during $^{14}CO_2$ experiments with tobacco. The glycerate and 3-P-glycerate were carboxyl-labeled through 30 seconds with the same percent distribution

in the carbons of both. At the same times glycolate and glycine were uniformly labeled. Serine was slightly carboxyl-labeled at 4 and 11 seconds and was uniformly labeled by 30 seconds. The specific activity of the ^{14}C in the carbon atoms of glycerate was much less than that of 3-P-glycerate or glycolate. Such results would possibly indicate a large reservoir of glycerate. The equilibration of the ^{14}C in glycerate was as rapid as in 3-P-glycerate indicating a very active reservoir of glycerate. Had the glycerate been an artifact of the killing procedure the ratio of the amount of free glycerate to 3-P-glycerate would be constant, but this definitely was not the case with tobacco (29). Thus it would appear that most of the glycerate was the result of *in vivo* enzymatic hydrolysis of 3-P-glycerate.

Bidwell *et al.* (91) using chloroplasts from the large marine alga *Acetabularia*, found that glycerate was labeled after about 3 minutes of $^{14}CO_2$ photosynthesis. At 3 minutes the ^{14}C incorporation into 3-P-glycerate was about half maximal, while incorporation into glycolate was about 1/3 maximal. The glycerate pool was 1/8 the glycolate and glycine and serine pools, and 1/6 the 3-P-glycerate pool. After the pools were saturated and the lights were removed, there was an immediate (~15 sec) 80% increase in 3-P-glycerate, which then returned to a steady state level 20% above the predarkness level. Glycerate in turn increased 60% within 2 minutes of darkness followed by a very slow rate

of decline. At the peak the radioactivity of the glycerate pool was 5% of the total soluble ^{14}C. The investigators were unable to explain the glycerate formation. Only 2% of the total ^{14}C was outside of the chloroplast.

The amount of glycerate in leaf tissue is quite significant. Palmer (92) reports that 0.5 to 1.5% of the dry weight of tobacco leaves is glycerate. Bruin (74) found 10-28% of the total $^{14}CO_2$ fixed by the C_4-plants pigweed, corn and sugarcane during steady state photosynthesis experiments to be in glycerate.

In summary, glycerate is an early carboxyl-labeled compound in $^{14}CO_2$ photosynthesis experiments in all plants studied. The glycerate pool is significant and highly active. The kinetics of glycerate formation and labeling indicate that it is formed by quickly hydrolyzing 3-P-glycerate. Without considering a phosphatase, the action of known metabolic sequences and data cannot account for all the glycerate formation in plants, however very little data is available for C_4-plants.

Acid Phosphatases from Leaves

Acid phosphatases from a number of plant tissues have been reported (93-113) but in only a few cases have the studies involved green leaf tissue (23, 93, 99, 102, 106, 112). Very few investigations present evidence for specificity or sufficient purification to indicate whether or not one enzyme was responsible for the activity observed.

This review will be limited to acid phosphatase of green leaf tissue.

The only specific acid phosphatase from leaves that has been reported thus far is P-glycolate phosphatase (23, 24, 76). It is ubiquitously distributed in green leaves (this thesis) and has been isolated from spinach (76), tobacco (23) and green algae (Randall and Tolbert, unpublished results). The enzyme from tobacco was shown to be specific for its substrate with optimal activity at pH 6.3, but under some conditions divalent cations lowered the optimum to about pH 5. The enzyme required a divalent cation with Zn^{++}, Mg^{++}, and Co^{++} providing the highest activity and EDTA completely inhibited the enzyme. Citrate, isocitrate or cis-aconitate at 10^{-3} M were required for enzyme stability (114). The enzyme showed typical phosphatase inhibition by fluoride, but no inhibition by L(+)tartrate. Inhibition by cysteine, glutathione and p-chloromecuribenzoate indicated a sensitive sulfhydryl or disulfide bond on the enzymes. P-Glycolate phosphatase was formed during biogenesis of the chloroplast (76) and was localized on or in the chloroplasts of C_3-plants (70, 76). It has been proposed that the enzyme functions as a part of a permease or gives direction to glycolate excretion from the chloroplast. P-Glycolate phosphatase may be the link between the site of glycolate biosynthesis (chloroplasts) and the site of glycolate metabolism (peroxisomes), thus performing a very specific function (115).

Another acid phosphatase(s) requiring divalent cations has been reported by Boroughs (93), who found that at least two phosphatases (two pH optimums) which hydrolyzed p-nitrophenylphosphate were present in leaves of sugar beet and tobacco. All of this phosphatase activity could be eliminated by acid dialysis or addition of chelators. The enzyme activity could be restored most effectively by addition of Cu^{++}.

Aside from the P-glycolate phosphatase the most extensive study on an acid phosphatase in leaf tissue has been presented by Shaw (112) on a 300-fold purified, tobacco leaf enzyme. Optimal activity was at pH 5.6, it did not require divalent cations, was unaffected by sulfhydryl reagents and was located in the cytoplasm. Fluoride, arsenate, molybdate, and phosphate were inhibitors. The enzyme was not very specific, for it had equal activity towards, ATP, ADP, PP_i, NADP, 3-P-glycerate, 2-P-glycerate, PEP, phenyl phosphate and p-nitrophenylphosphate. The enzyme was slow to hydrolyze 5'-nucleotides in comparison to 3'-nucleotides, and it did not exhibit diesterase activity towards RNA or DNA. The carbohydrate esters were hydrolyzed quite rapidly (about 2/3 of the rate for ATP) and non-specifically. The relative specificity was fairly unchanged during the purification.

Roberts has reported on wheat leaf acid phosphatases in a series of investigations (96-98, 101-104) and concluded that there could be as many as eleven phosphatases based on

various levels of inhibition by 17 inhibitors. A typical example was a β-glycerol acid phosphatase, requiring no cations and operating optimally at pH 5.7. The phosphatases were reported as being non-specific, but no data were presented with regard to the various substrates. The second type of leaf phosphatase that Roberts reported was a 5'-nucleotidase, which also possessed phosphotransferase activity (104). No characterizations of the enzymes were presented which would indicate function or location. Again most of the studies were performed with p-nitrophenylphosphate as substrate, which is rapidly hydrolyzed by most phosphatases except P-glycolate phosphatase.

Plant seedlings have much higher acid phosphatase levels than fully developed plants or leaves. An acid phosphatase from seedlings of dwarf beans has been reported (106) which was relatively non-specific (hydrolysis of 3-P-glycerate, FdiP, and glucose-1-P was 1/10-1/8 that of p-nitrophenylphosphate), did not require cations, and had a pH optimum of 5.3 to 6.3. The enzyme hydrolyzed 3'-nucleotides but had little activity towards 5'-nucleotides and had no diesterase activity. An acid phosphatase purified 1000-fold from lupine seedlings (99) has also been reported. This phosphatase had pyrophosphatase and monoesterase activity, but did not hydrolyze 5'-nucleotides, polymetaphosphates or nucleic acids. The rate of hydrolysis of sugar phosphates was very non-specific and almost as high as for p-nitrophenyl phosphate. It was inhibited by Cu^{++}, Zn^{++}, and Co^{++} and was

slightly stimulated by Mn^{++} and Mg^{++}.

Pierpont (97, 98) isolated an acid phosphatase from pea seedlings which was very nonspecific, hydrolyzing poly- and metaphosphates, nucleic acids, nucleotides and other monoesters. The enzyme activity was optimal between pH 5 to 6, and required no cations and showed typical fluoride and molybdate inhibition.

Acid pyrophosphatase from spinach leaves which would hydrolyze PP_i, ATP, ADP but few sugar phosphates was reported by Forti (107), but no function for this enzyme was proposed. Forti *et al.* (105) also isolated and characterized a nucleotidase from pea leaves which hydrolyzed 3'- and 5'-nucleotides and pyrophosphate linkages. This enzyme hydrolyzed PEP as rapidly as *p*-nitrophenylphosphate but was very inactive toward β-glycerolphosphate, glucose-6-P and 2-P-glycerate. The enzyme very actively converted NADP(H) to NAD(H), and speculation exists for this hydrolysis as a regulator mechanism. The enzyme activity was optimal at pH 5.9, required no cations (stimulated by EDTA), and possessed a K_m of 3×10^{-4} M for ATP and 9×10^{-5} M for PEP.

A perusal of the literature on acid phosphatases in leaves leads me to conclude that there are few generalities about the acid phosphatases. Thus far, most of the studies have been quite incomplete, but indicative of several different enzymes. Most have pH optimum between 5.0 and 5.9 and are non-specific with respect to substrate. P-Glycolate phosphatase is an exception. It is substrate specific and

has a pH optimum of 6.3. A divalent cation requirement for some, but not all, of the leaf phosphatase has been shown. This thesis adds another acid phosphatase to the list of those isolated from plant leaves, and the studies indicate it to be different from those reviewed above.

MATERIALS AND METHODS

Plants

Sugarcane, *Saccharium officiarum* (variety C.I. 41-223) was grown continuously in the greenhouse from stem nodes which were obtained originally from L. P. Hubert of the United States Sugarcane Field Station, Canal Point, Florida. Leaf tissue for enzyme purification was from plants 10 to 15 feet in height and 3 to 18 months old. New plants were propagated regularly from stem nodes. The sugarcane and other plants were grown in regular greenhouse mix with additional sand and were treated with Hoagland's nutrient solution on a regular basis. Etiolated tissue was grown in controlled environment chambers in vermiculate and Hoagland's nutrient solution.

The plants used in the survey investigations are listed in Table XVI. Most of these plants were grown in the greenhouse and were 3 to 6 weeks old when leaves were taken for experiments. Other plants were obtained from specific cultures or gardens on campus. *Chlorella pyrenoidosa* (Warburg strain) was grown as described by Hess (116). Cultures of *Chlamydomonas reinhardtii* were from E. B. Nelson. The extracts of aquatic plants (*Elodea densa* and *Sagittaria*) were donated by R. Donaldson.

The leaf tissue from soybean (*Glycine max* L. Merrill)

varieties Chippewa , Wayne, Hark, Harvasoy, Grant, Amsoy, Clark 63, Corsoy, Richland, Scott, Kent, Lee, Kanrich, Hawkeye, and Seneca, were obtained from the experimental plots of the USDA Regional Soybean Research Laboratory at Urbana, Illinois.

Light intensity was measured in foot candles with a Weston Model 756 Illumination Meter.

Chemicals

The chemicals and biochemicals used during the research for this thesis were reagent grade and for the most part the most pure available. Heavy metal salts of any of the biochemicals were exchanged to appropriate counter ion before use.

Aldrich Chemical Co.

Chromotropic acid

αα-Dipyridyl

8-Hydroxyquinoline-5-sulfonic acid

8-Hydroxyquinoline

2-Pyridylhydroxymethane sulfonic acid

Dihydroxytartaric acid

Dihydroxymaleic acid

Isonicotinyl hydrazide

O-,m-Phenanthroline

Worthington

Catalase

β-Amylase

Nutritional Biochemicals Corp.

β-Alanine

Citric acid

DL-Serine

Sodium azide

Hydroxypyruvate (Li)

Glutathione

Carbamyl phosphate

5'-Citydylic (Na)

5'-Guanylatic (Na)

Sodium Pyruvate

Phosphoserine

5'-Uridylate (Na)

Oxalic acid

General Biochemical Co.

Glycolic acid

P-Glycolic acid

Calbiochem

L-Aspartate

L-Alanine

L-Cysteine

Malic acid

Malonic acid

Phytic acid

Thioglycolate

meso-Tartaric acid

L(+)Tartaric acid

L(-)Tartaric acid

DL-Glyceraldehyde-3-phosphate

Phosphohydroxypyruvate

Phosphochloine

Oxaloacetic acid

Glycolaldehyde phosphate

Creatine phosphate

2-P-Glycerate

Fructose-6-phosphate

cis-Aconitic acid

Iso-Citric acid

DL-α-Hydroxybutyrate

Sigma

DL-Glycerate

L-Ascorbic acid

Cacodylic acid

p-Chloromercurobenzoic acid

Glycylglycine

Dihydroxyacetone phosphate

Trizma base

Glyoxylate

NAD, NADP, NADPH, NADH

Phosphoenol pyruvate

Pyridoxal-5'-phosphate

Dithioethreitol

bis-p-Nitrophenyl phosphate

p-Nitrophenyl phosphate

Bovine serum albumin

Phenolphathalein diphosphate

α- and β-Glycerol phosphate

Glucose-6-phosphate

Fructose-1,6-diphosphate

Ribulose-1,5-diphosphate

Ribose-5-phosphate

AMP, ADP, ATP

Eastman Organic Chemicals	Merk
(Amino-oxy)acetic acid	Barbital
Diethyldithiocarbamic acid	General Aniline and Film
Iodoacetic acid	Polyclar AT
p-Methylaminophenol sulfate	

Protein, Chlorophyll and Reducing Sugar

The method of Lowry et al., a modified Folin-Ciocaltau method (117), was used whenever sufficient protein sample was available. Lowry proteins on a semi-microscale were run on samples of 0.10 ml with 1.0 ml Lowry reagent C and 0.10 ml Folin-Phenol reagent. The phenol reagent used was a 1:1 dilution with H_2O of 2.0 N Phenol Reagent (Fisher Scientific Co., Fairhaven, N. J.). The absorbance of protein reaction was measured at 660 nm. The protein standard was crystalline bovine serum albumin.

For determining protein content of samples when loss of enzyme was undesirable, the 260:280 ratio method was used with the formula (118):

$$\text{mg protein} \times \text{ml}^{-1} = 1.45\ (A_{280}) - 0.74\ (A_{260})$$

The absorbance at 280 nm on Beckman Model DU with a 1.0 cm light path was used for obtaining protein profiles of column effluents.

Chlorophyll determinations were by the procedure of Arnon (119).

Determination of reducing sugar was by the method of Nelson (120).

Phosphate Determinations

Method A: The standard determination of inorganic phosphate was a modified method of Fiske and Subbarow (121). To a 1.0 ml aqueous phosphate solution ideally containing 3 to 30 µg phosphorous was added 8.0 ml molybdate reagent (50 ml 10 N N_2SO_4 plus 100 ml 2.5% (w/v) $(NH_4)_6MO_7O_{24}\cdot 4H_2O$ plus 650 ml H_2O) and 1.0 ml elon reducing agent (3 g of $NaHSO_3$ plus 1 g p-methylaminophenol sulfate, "elon," in 100 ml H_2O) (114). After 20 minutes at room temperature the absorbance was measured at 660 nm on a Coleman Jr. Spectrophotometer. A standard curve was made using 0 to 40 µg P as KH_2PO_4 per ml (Fischer certified reagent).

Method B: Inorganic phosphate was also determined by an iso-butanol-benzene extraction procedure (114). To a 2.0 ml aliquot of solution, containing no more than 8 µg of phosphorous, 0.5 ml of 6% (w/v) $(NH_4)_6MO_7O_{24}\cdot 4H_2O$ in 2 N H_2SO_4 was added. The mixture was vortexed for 5 sec and allowed to stand for 5 minutes. To this solution was added 2.5 ml iso-butanol-benzene (1:1 v/v) and the mixture vortexed for 45 seconds. After the phases had separated, the absorbance at 310 nm of a 1.0 ml aliquot from the upper phase plus 10 µl absolute alcohol to dissolve the remaining H_2O was read in a Beckman Model DU and compared with a standard curve.

Method C: A microphosphate determination (122) was also used which was 8 times more sensitive than the method of Fiske and Subbarow (Method A). To 0.30 ml of solution with less than 0.04 μmole phosphate was added 0.7 ml ascorbic acid-ammonium molybdate reagent (1 part 10% ascorbic acid, plus 6 parts 0.42% (w/v) $(NH_4)_6MO_7O_{24}\cdot 4H_2O$ in 1 N H_2SO_4). After a 20 minute incubation at 45° the absorbance at 820 nm was measured with a Beckman Model DU.

Glycerate Determinations

Glycerate was determined by the method of Bartlett (123). To a 0.2 ml sample containing between 0.01 to 0.30 μmoles glycerate was added 5.8 ml 0.01% chromatropic acid (10 mg recrystallized 4,5-dihydroxy, 2,7-naphthalene-disulfonic acid per 100 ml concentrated sulfuric acid). The reaction mixture was mixed thoroughly and placed in boiling water for exactly 30 minutes and then cooled abruptly in an ice bath to room temperature. The absorbance at 695 nm was measured with a Beckman Model DU and compared against a standard curve.

Enzyme Assays

Standard assay for 3-P-glycerate phosphatase: A reaction solution containing 12.5 μmoles 3-P-glycerate, 100 μmoles sodium cacodylate buffer, pH 5.9, and enough H_2O to bring the final volume to 0.75 ml was equilibrated to 30° in a conical test tube. The reaction was initiated with enzyme unless otherwise stated. The enzyme reaction

was run for 10 minutes and was terminated by the addition of 0.25 ml of 10% (w/v) trichloroacetic acid (TCA). For crude or low purity enzyme, the denatured protein was removed by centrifugation. To determine the amount of phosphate hydrolyzed from the substrate, phosphate determinations were performed on the TCA treated reaction mixture (0.5 ml aliquots for crude or low purity enzymes). Reagents for the modified Fiske and Subbarow phosphate (Method A) were added: 8.0 ml acid molybdate solutions and 1.0 ml elon reducing solution (4.0 ml and 0.5 ml respectively for 0.5 ml phosphate solutions). Control or zero time assays were done by adding the 0.25 ml 10% TCA to the 0.75 ml reaction mixture before the enzyme was added.

P-Glycolate phosphatase (Phosphoglycolate phosphohydrolase E.C. 3.1.3.18): This enzyme was assayed by the method of Anderson and Tolbert (24). The reaction mixture contained 0.50 ml of 0.2 M sodium cacodylate buffer, pH 6.3, and 1 mM $MgSO_4$, 0.25 ml of 20 mM P-glycolate, and after equilibration to 30°, the assay was initiated with enzyme for a 10 minute reaction period and terminated with 0.25 ml of 10% trichloroacetic acid.

One unit of enzyme is defined as the amount which catalyzes the hydrolysis of 1 μmole substrate per minute under conditions of the standard 3-P-glycerate phosphatase assay. Specific activity is defined as units of enzyme per mg of protein.

Glycolate oxidase (E.C. 1.1.3.1), catalase (E.C.

1.11.1.6), and NADH-hydroxypyruvate reductase (E.C. 1.1.1.29) were assayed spectrophotometrically as described by Tolbert *et al.* (25).

Substrates

The 3-P-glycerate was obtained as the barium salt-dihydrate from C. F. Boehringer and Soehne, GmBH, Mannheim, West Germany. The barium salt was converted to free acid by passage through 200-400 mesh Dowex resin (BioRad AG-50W-X12) in the hydrogen form and then adjusted to pH 5.9 with base. Paper chromatography revealed no other organic phosphate esters or organic acids. About 1.6 percent of the phosphate present was as inorganic phosphate.

P-Glycolate was obtained as the tricyclohexylammonium salt from General Biochemicals and was converted to the free acid by passage through Dowex resin (BioRad AG-50W-12) in the hydrogen form and adjusted to pH 6.3 with base. Previously, Dr. D. E. Anderson (114) had established the purity of this substrate. In general, fresh solutions were prepared weekly and stored at -18° to minimize endogenous phosphate resulting from non-enzymatic hydrolysis.

All other substrates used were the most pure available and are listed in Chemicals section.

Isoelectric Focusing

The apparatus was an Electrofocusing Column type LKB 8100* having a capacity of 110 ml. A density gradient of

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*The author gratefully acknowledges the use of the electrofocusing equipment of John E. Wilson and his helpful advice.

sucrose, in which Ampholine, carrier ampholytes, in the pH range from 3 to 10 were dissolved, was made by successive dilution of twenty-four 4.6 ml aliquots of 47 percent (w/v) sucrose-water-ampholyte (1.25%) solution with a water-ampholyte (0.4%) solutition. These aliquots were layered on 22 ml of cathode solution containing 200 µl ethanolamine in 55 percent (w/v) sucrose. The gradient column was completed by layering 5 to 7 ml of anode solution (1.9% sulfuric acid) on the top of the gradient.

The enzyme was introduced into the column in one of the middle aliquots of the gradient in place of the non-sucrose containing portion. The column was operated at a potential of 300 V for 36-65 hours at 4°. The column was drained by gravity with a flow rate of 60 to 90 ml per hour. About 2 ml fractions were collected. The pH of the fractions was determined with a combination electrode on a Leeds and Northrup pH Meter.

Gel Filtration

Sephadex G-200 (40-120 µ) from Pharmacia was routinely prepared for chromatography as follows. The dry gel was suspended in water and swollen on a steambath for 5 hours. The suspended gel was allowed to settle for 9 minutes and the suspended material remaining in the supernatant was decanted. This fining process was repeated 10 times before the gel was equilibrated with 0.02 M sodium cacodylate buffer, pH 6.3 and 1 mM EDTA at 4°. Before pouring a column the gel

was degassed for 10 minutes using a water-aspirator system. The columns were poured and operated with the same head height of 10 cm. Most columns were 2.6 x 46 cm with a 2 cm pad of G-25 Sephadex (prepared in identical manner) on the top. The flow was descending and controlled by a mariotte flask. All molecular sieving gels used in this work were prepared in a similar manner.

Ion Exchange Chromatography

Phosphocellulose (cation exchange resin, 1.24 meq/g) from Serva (Gallard-Schleisinger) was suspended in H_2O, allowed to settle 20 minutes, and the remaining suspended material removed by decantation. This fining process was repeated 10 times. The resin was then washed with 0.1 N KOH and H_2O until the KOH wash was colorless. The resin was finally equilibrated with 0.05 M sodium acetate buffer, pH 4.5, at 4°. The 1.4 x 10 cm columns were poured and washed with 50 volumes of the 0.05 M sodium acetate buffer, pH 4.5.

DEAE-Cellulose DE-52 (Anion exchanger 1.0 meq/g) from Whatman was prepared in a manner similar to the P-cellulose ion exchanger.

Carboxymethyl-Sephadex (cation exchange gel, 4.5 meq/g), G-50 medium grade, from Pharmacia was suspended in H_2O and swollen 1 hour on a steam bath. The gel was allowed to settle for 8 minutes and then the remaining suspended material removed by decantation. This process of fining

was repeated 10 times before the gel was equilibrated with 10 volumes of sodium acetate buffer, 0.05 M, pH 5.0. After the 1.4 x 10 cm columns were poured, the column was washed with 50 volumes of the acetate buffer before application of enzyme.

Profiles of the gradients were determined by adding either DCPIP or Blue Dextran 2000 to the high-salt chamber of the gradient forming apparatus. Absorbance at 600 nm was determined on fractions of a calibration run, and all gradients used were found to be linear.

Dialysis tubing, a product of Union Carbide Co., was heated on a steam bath in 10 mM EDTA for 8 hours, with frequent changing of the EDTA solution. The tubing was then washed extensively with deionized, distilled water and stored at 4° in 20% ethanol.

Paper Chromatography

Paper chromatography on Whatman No. 1 filter paper was run on samples that were spotted on the origin and dried with warm air from a hair dryer. Chromatograms were pre-equilibrated with solvent and chromatographic development was done at room temperature. The solvent system of n-butanol-propionic acid-water (4) was prepared from equal volumes of solutions A and B (A: 1246 ml of n-butanol plus 84 ml H_2O; B: 620 ml propionic acid plus 790 ml H_2O). A basic solvent system used was iso-butanol-95% ethanol-water-diethyl amine (80:10:20:1) (116).

Phosphates on paper chromatograms were detected by the molybdate spray and UV light treatment method of Bandurski and Axelrod (124). Organic acids were detected by an indicator, 0.04% (400 mg/l 95% ethanol) bromocresol green, in the acid form for basic chromatograms and in the base form for acidic chromatograms.

Preparatory Methods

Chloroplast isolation from sugarcane leaves was by the method of Baldry et al. (125). Cacodylate or MES buffer was substituted for the pyrophosphate buffer in the grinding medium because of phosphate assays later. Subcellular particles were separated by differential centrifugation, and the isolation of peroxisomes from plant extracts followed procedures developed in this laboratory (25, 26).

Isolation of chloroplasts from spinach utilized a sorbitol medium containing 0.33 M sorbitol, 0.01 M $Na_4P_2O_7$ buffer, pH 6.5, 5 mM $MnCl_2$, 2 mM $MgCl_2$, and 2 mM isoascorbate. At 4°, washed and deribbed spinach leaves were homogenized at high speed for 8-10 seconds in a Waring Blendor. The homogenate was squeezed through 8 layers of cheese cloth and the resulting brei centrifuged at 4000 x g for 90 sec. The pelleted chloroplasts were resuspended in 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA and 50 mM Hepes buffer pH 7.6, and recentrifuged to pellet the chloroplasts. This process was repeated as many times as necessary to remove non-chloroplast enzymes. Glycolate oxidase was used as a marker enzyme for non-chloroplast enzymes.

Sucrose density centrifugations for sedimentation velocity determinations were run according to the procedure of Martin and Ames (126). Polyacrylamide gel electrophoresis was performed according to the method of Orstein (127), Davis (128), and Reisfeld et al. (129, 130). Running gels approximately 7 cm long were used with samples layered directly on the gels. A current of 5 milliamps per gel was applied. Electrophoresis at pH 4.3 was in β-alanine-glacial acetic acid buffer (130). Electrophoresis at pH 7.5 was in a barbitol-Tris buffer system and at pH 8.9 in Tris-glycine buffer (129).

SDS-Polyacrylamide electrophoresis was run according to the method of Shapiro et al. (131), with the help of J. C. Johnson and Dr. J. A. Boezi of this department. Samples of enzyme in a solution of 1% SDS, 1% 2-mercapoethanol and 0.1 M pH 7.1 phosphate buffer were incubated at 37° for 3 hours. Samples of the denatured and reduced enzyme were layered on 7 cm, 5% polyacrylamide gels and a current of 6-7 milliamps per gel was applied. Standard protein samples of known molecular weight were treated identically. The buffer system was 0.1 M, pH 7.1 phosphate with 1% SDS present.

RESULTS

PART A:

3-P-GLYCERATE PHOSPHATASE FROM SUGARCANE LEAVES

I. Purification of Sugarcane 3-P-Glycerate Phosphatase

The term enzyme as used in the subsequent paragraphs in synonymus with 3-P-glycerate phosphatase. Table II contains a summary of the purification procedure.

Extraction from Sugarcane Leaves

Leaves from sugarcane plants from the greenhouse were harvested in mid-afternoon only on days with full sunlight and, when possible, after a day with full sunlight. The chosen leaves were dark green, fully expanded, approximately 100 cm in length and 5 to 7 cm wide. The leaves were washed, deribbed, and diced into 4-5 cm pieces using a paper cutter. All subsequent steps were done at 0-4°. Sixty gram portions of the diced leaves were immersed in 5 volumes of extraction medium containing 20 mM sodium cacodylate buffer, pH 6.3 1 mM EDTA, 20 mM ascorbate and 2% (w/v) Polyclar AT (cross-linked, high molecular weight, polyvinyl pyrollidone). The tissue was allowed to soak for 15 minutes and then was homogenized by a Waring Blendor at high speed for two minutes. The homogenate was poured into a wine press lined with 6

Table II. Summary of the Purification of 3-P-Glycerate Phosphatase from 840 g Sugarcane Leaves*

Fraction	Units (μmoles/min)	Sp. Act.	% Yield	Enrichment	Total Protein (mg)
Crude extract	4,570	0.21	100	1.0	23,150
pH fractionation	4,220	0.50	93	2.4	9,250
First acetone fractionation	2,870	0.30	67	44.0	740
$(NH_4)_2SO_4$ fractionation	2,230	23.1	52	110.0	210
Second acetone fractionation	2,050	50.6	46	241.0	88
G-200 Sephadex chromatography	1,193	100.0	30	486.0	36
Phosphocellulose chromatography	1,420	413	13	1392	3.5
CM-Sephadex chromatography	646	489	5.7	1740	1.9
Concentrated enzyme	500	740	4.4	2530	0.8

*The results of the purification steps from the crude extract through G-200 Sephadex are the average of seven preparations of the enzyme. The results from the final 3 purification steps are the average of the last 2 preparations of the enzyme.

layers of cheesecloth. Extracts of 600-800 g of leaf tissue were pooled quickly in the wine press and the brei squeezed as dry as possible to yield the crude enzyme extract.

pH Fractionation

As rapidly as possible after the extraction, the pH of the crude extract was adjusted to 4.5 with 1 N HCl and centrifuged at 14,000 x g for 30 minutes. The precipitate was discarded and the supernatant was readjusted to pH 6.3 with 1 N NaOH. The pH stability of the crude extract and the specific activities of the crude extract and the specific activities of the crude enzyme after treatment at the various pH's are shown in Figure 8.

First Acetone Fractionation

To the acid fractionated enzyme, a volume of reagent grade acetone equal to 35% of the enzyme volume was added through 4 polyethylene tubes (1 mM I.D.) at approximately 5 ml per minute. The solution was stirred continuously and the acetone was kept at -5° by the use of a salt bath. After the acetone had been added, the mixture was equilibrated by slowly stirring for an additional 30 minutes and then centrifuged at 14,000 x g for 20 minutes. The precipitate was discarded and to the supernatant was added acetone, as before, equal to 35% of the starting enzyme volume. The resulting suspension was equilibrated for 30 minutes, centrifuged at 14,000 x g for 20 minutes and the supernatant was discarded. The precipitate was suspended in a solution

of 20 mM sodium cacodylate buffer, pH 6.3, and 1 mM EDTA equal to 20% of the acid fractionated enzyme volume. The use of a glass Potter-Elvejehm homogenizer was necessary to adequately resuspend the precipitate. The suspension was stirred for 2 hours to ensure that the enzyme was dissolved completely and then centrifuged at 14,000 x g for 30 minutes to remove the insoluble material. The supernatant was designated the first acetone fraction.

Ammonium Sulfate Fractionation

To the first acetone fraction was added 33 g solid $(NH_4)_2SO_4$ per 100 ml of first acetone fraction enzyme. The solution was equilibrated by slowly stirring for 20 minutes after the $(NH_4)_2SO_4$ had dissolved. The solution was centrifuged at 14,000 x g for 20 minutes and the precipitate discarded. To the supernatant, 22 g more solid $(NH_4)_2SO_4$ per 100 ml of the first acetone fraction was added, the solution equilibrated and centrifuged as for the first precipitation. The precipitate was dissolved in 20 mM sodium cacodylate buffer pH 6.3 and 1 mM EDTA equal to 10% of the volume of the first acetone fraction. This was termed the ammonium sulfate fraction.

Second Acetone Fractionation

In a manner similar to the first acetone fractionation, the second acetone fractionation was carried out by addition of acetone at -5° equal to 63% of the volume of the ammonium sulfate fraction. The system was equilibrated, centrifuged

at 14,000 x g for 15 minutes and the precipitate was discarded. To the supernatant, another volume of cold acetone equal to 47% of the volume of the ammonium sulfate fraction was added. After at least 30 minutes of equilibration the precipitate was collected by centrifugation at 10,000 x g for 10 minutes and the supernatant discarded. The precipitate was suspended in 5 ml of 20 mM cacodylate buffer, pH 6.3 and 1 mM EDTA. The suspension was centrifuged at 40,000 x g for 20 minutes to remove insoluble material, and the resulting supernatant was designated the second acetone fraction.

Sephadex G-200 Gel Filtration

Solid sucrose was added to the second acetone fraction to make it a 10% (w/v) sucrose solution. The enzyme-sucrose solution was layered under the top buffer of a 2.5 x 46 cm G-200 Sephadex Column operating in a descending manner and equilibrated with a solution of 20 mM cacodylate buffer, pH 6.3, and 1 mM EDTA. The column was eluted with the same buffer with a flow rate of 20 ml per hour and fractions of about 5.0 ml were collected. Figure 4 shows the 3-P-glycerate phosphatase activity and 280 nm absorbance profiles. The fractions with improved specific activity (hatched area) were pooled to yield the G-200 Sephadex enzyme preparation.

Chromatography on Phosphocellulose

The G-200 Sephadex fraction was dialyzed for 36 hours against 40 volumes of 0.05 M sodium acetate buffer, pH 4.5,

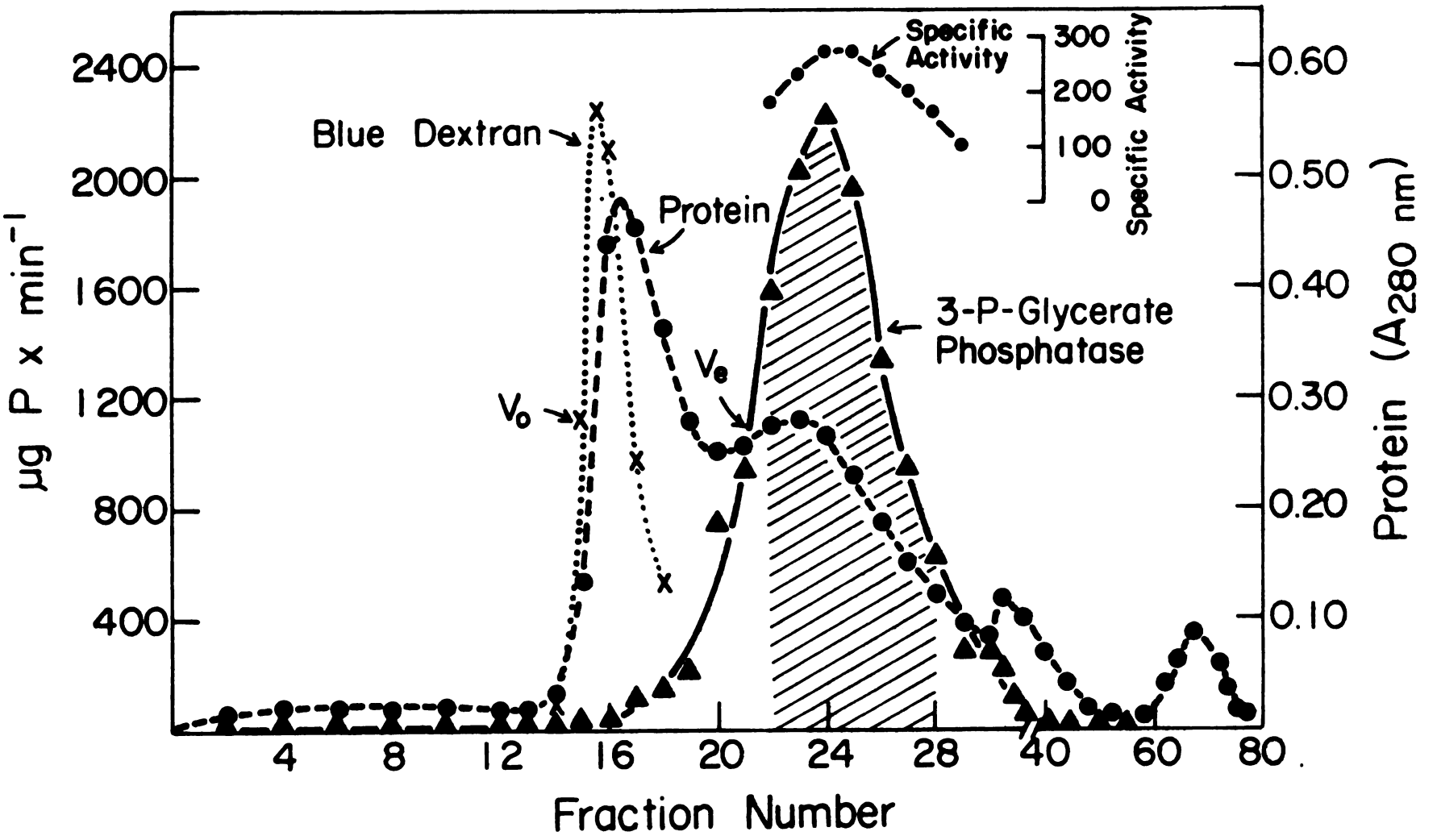
Figure 4. The Elution Pattern for Sephadex G-200 Gel Filtration

The 5.5 ml of a 3-P-glycerate phosphatase preparation from the second acetone fractionation, 10% (w/v) in sucrose, was applied to a 2.5 x 46 cm Sephadex G-200 column that had been equilibrated with 20 mM sodium cacodylate buffer, pH 6.3, and 1 mM EDTA. The column was eluted with the same buffer and 5 ml fractions were collected. Fractions of highest phosphatase specific activity, as shown in the hatched area, were pooled. The Blue Dextran 2000 was applied in a manner identical to the enzyme. The void volume (V_o) and elution volumes (V_e) were taken as the midpoint of the ascending side of the Blue Dextran peak and phosphatase peak respectively and the V_e/V_o was 1.39.

▲——▲ 3-P-glycerate phosphatase

●——● Absorbance 280 nm

X---X Blue Dextran 2000


2400
2000
1600
1200
800
400
μg P x min⁻¹
0.60
0.50
0.40
0.30
0.20
0.10
Protein (A280 nm)
300
200
100
0
Specific Activity
Specific Activity
Blue Dextran
Protein
3-P-Glycerate Phosphatase
Vo
Ve
4
8
12
16
20
24
28
40
60
80
Fraction Number

and 1 mM EDTA. The dialyzed enzyme was then adsorbed on 1.4 x 10 cm phosphocellulose ion exchange column which had been equilibrated with 0.05 M acetate buffer, pH 4.5. The flow rate of the column was about 1 ml per minute and 10 ml fractions were collected. Following enzyme adsorption, the column was washed with 240 ml of 0.05 M acetate buffer, and then the column was eluted with a 500 ml linear gradient from 0 to 0.5 M NaCl in 0.05 M sodium acetate buffer, pH 4.5 (Figure 5). The fractions of higher phosphatase specific activity were pooled (hatched area) to yield the phosphocellulose enzyme preparation.

Chromatography on Carboxymethyl Sephadex

The enzyme preparation from phosphocellulose chromatography was dialyzed for 18 hours against 40 volumes of 0.05 M acetate buffer, pH 5.0, and 1 mM EDTA. The dialyzed enzyme was adsorbed on a 1.4 x 10 cm CM-Sephadex column, previously equilibrated with 0.05 M acetate buffer, pH 5.0. The column was washed with 200 ml of buffer and eluted with a 500 ml linear gradient 0 to 0.5 M NaCl in 0.05 M acetate buffer, pH 5.0. The column was operated with a flow rate of 1 ml per minute and 10 ml fractions were collected. The phosphatase activity and 280 nm adsorbance profile are shown in Figure 6. The pooled fractions of highest specific activity were dialyzed against 40 volumes of 0.02 M sodium cacodylate buffer, pH 5.9, with 1 mM EDTA to yield the CM-Sephadex, 3-P-glycerate phosphatase preparation.

Figure 5. The Elution Pattern of the Phosphocellulose Ion Exchange Column

The enzyme after G-200 Sephadex step was dialyzed against 40 volumes of 0.05 M sodium acetate buffer pH 4.5 and 1 mM EDTA for 36 hours and applied to a 1.4 x 10 cm phosphocellulose column that had been equilibrated with 0.05 M acetate buffer, pH 4.5. The adsorbed enzyme and column were washed with 240 ml of buffer and then eluted with linear gradient of 0 to 0.5 M NaCl in the 0.05 M acetate buffer, pH 4.5. The fractions were collected in 10 ml volumes and the phosphatase assayed. Fractions in the hatched area were pooled.

▲——▲ 3-P-glycerate phosphatase

●——● Absorbance 280 nm

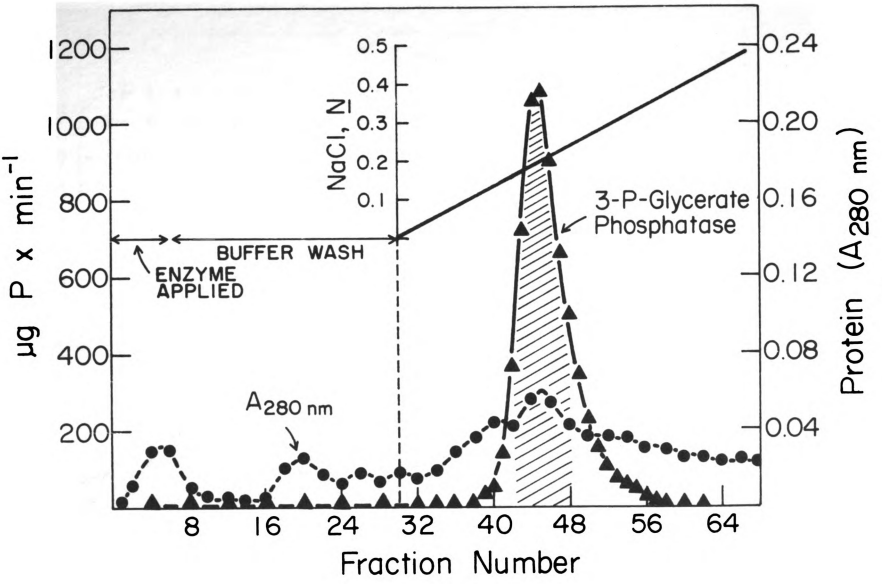
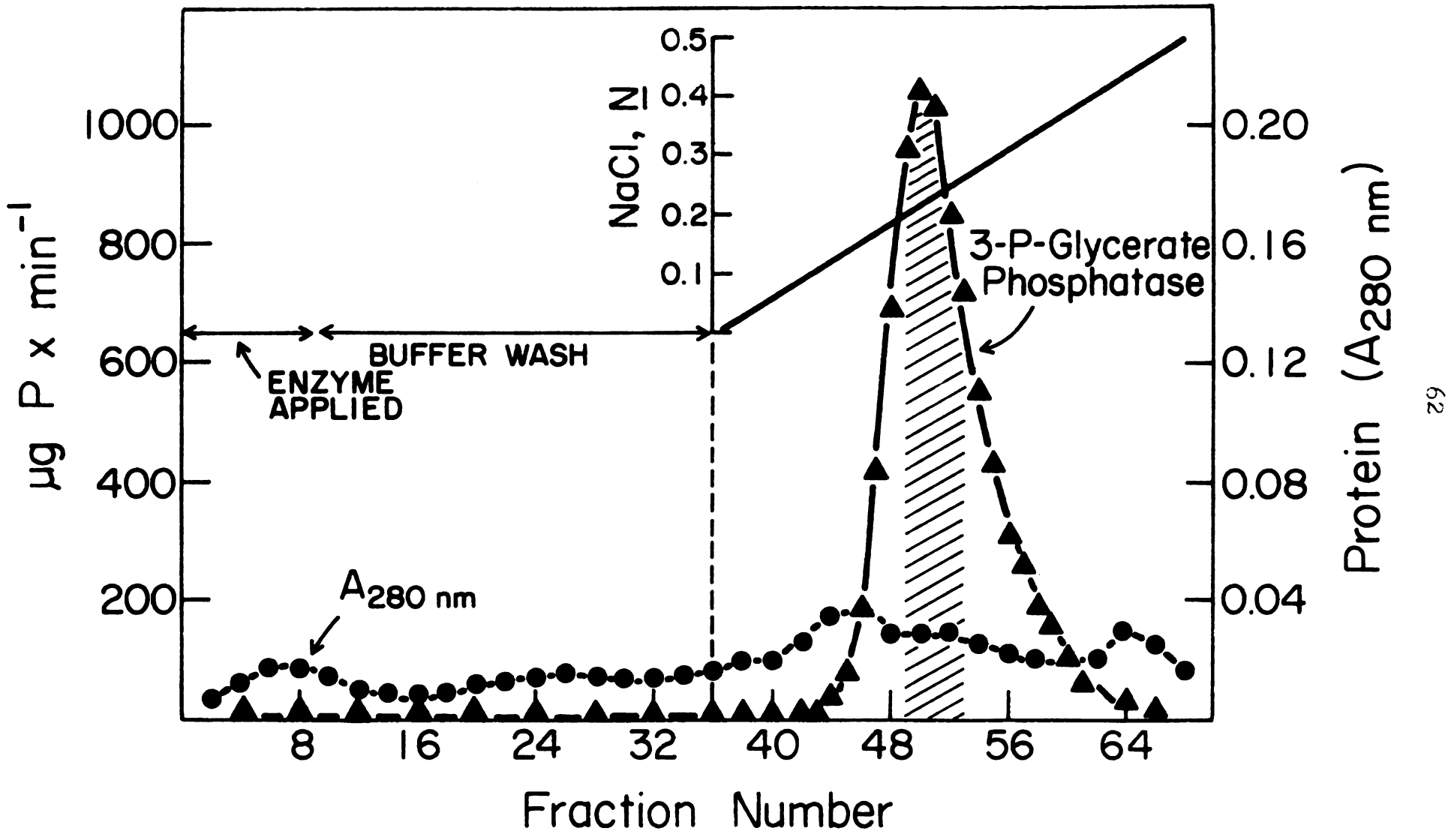

1200
1000
800
600
400
200
µg P x min⁻¹
0.5
0.4
0.3
0.2
0.1
NaCl, N
0.24
0.20
0.16
0.12
0.08
0.04
Protein (A280 nm)
3-P-Glycerate Phosphatase
BUFFER WASH
ENZYME APPLIED
A280 nm
8
16
24
32
40
48
56
64
Fraction Number

Figure 6. The Elution Pattern of the Carboxymethyl-Sephadex Ion Exchange Chromatography

The pooled 3-P-glycerate phosphatase fractions from the phosphocellulose column were dialyzed for 18 hours against 0.05 M acetate buffer, pH 5.0, and applied to a 1.4 x 10 cm CM-Sephadex column equilibrated with 0.05 M acetate buffer, pH 5.0. The column with the adsorbed enzyme was washed with 200 ml of the acetate buffer and eluted with a linear gradient of 0 to 0.5 M NaCl in 0.05 M acetate buffer, pH 5.0. Ten ml fractions were collected and the phosphatase assayed. The protein profile was obtained by 280 nm absorbance. The fractions shown by the hatched area were pooled and dialyzed against 0.02 M sodium cacodylate, pH 5.9, with 1 mM EDTA.

▲——▲ 3-P-glycerate phosphatase

●——● Absorbance 280 nm


μg P x min⁻¹
1000
800
600
400
200
NaCl, N
0.5
0.4
0.3
0.2
0.1
0.20
0.16
0.12
0.08
0.04
Protein (A280 nm)
3-P-Glycerate Phosphatase
BUFFER WASH
ENZYME APPLIED
A280 nm
8
16
24
32
40
48
56
64
Fraction Number

Concentration of Enzyme

The pooled enzyme fractions from CM-Sephadex column were concentrated by vacuum dialysis. A dialysis bag was attached to the stem of a 40 mm funnel by co-insertion through a rubber stopper that was the size of the neck of a 2 liter Buchner flask. The enzyme was continuously added to the dialysis bag through the funnel. The vacuum was drawn on the system by a Cenco Hyvac-2 pump until bubbles were observed in buffer that had been added to the bottom of the flask. The flask was closed by a stopcock connection between the pump and the flask and the system was held at 4°. Enzyme was added as the level receded in the system and the vacuum was renewed about every 3 to 4 hours. It was possible to concentrate 100 ml of enzyme to 5 ml in 24 hours or 5 to 10 ml in about 90 minutes. The inside surfaces of the dialysis tubing were rinsed with buffer to help reduce the loss of enzyme. The concentrated enzyme was removed from the sac by either pouring the enzyme out or using a long tip pipette.

Discussion of the Purification Procedure

Grinding Procedures: The age of sugarcane leaves was a factor to be considered only with regard to the breaking of the leaf tissue. Initial efforts to purify the enzyme utilized sugarcane leaves flown in from Canal Point, Florida. The leaves obtained in this manner were used 8-36 hours after harvesting, but still resulted in yields of enzyme on

a per gram fresh weight basis that were comparable to plants grown in the greenhouse. Older leaves, low on the stock, were so woody, stiff, and dry that they could not be properly homogenized by the equipment available. Even the young, relatively tender leaves were difficult to break completely, and it was necessary to grind small pieces of them for 2 minutes in the Waring Blendor at high speed to solubilize about 80-90 percent of the total activity (Figure 7). With shorter grinding periods very poor recoveries were obtained. This procedure extracted most of the P-glycerate phosphatase activity, which is mainly in the mesophyll cells, but not only about half of the P-glycolate phosphatase and glycolate oxidase activity of the bundle sheath cells, which could be completely broken only by further grinding in a roller mill (Figure 7). The significance of these results will be elaborated in Appendix A.

The most satisfactory grinding procedure started with 60 g of diced sugarcane leaves, which had been soaked in the grinding medium for 15 minutes. They were ground for 120 seconds in 350 ml of grinding medium (20 mM cacodylate, pH 6.3, and 1 mM EDTA, 20 mM ascorbate and 1% Polyclar AT) and the homogenate was squeezed through 6 layers of cheese cloth. In the large scale preparations this was repeated numerous times. However, this step plus the pH 4.5 fractionation had to be completed within one hour to prevent accumulation of excess phenolic oxidation products which could not be removed from subsequent enzyme fractions. Rapid performance of these

Figure 7. The Percentage of the Enzymes Solubilized as a Function of Homogenization Time or Use of the Roller Mill*

Triplicated samples (30 g) of leaves were extracted by the Waring Blendor at high speed in 150 ml of 20 mM cacodylate buffer, pH 6.3, and 1 mM EDTA. At the designated times an aliquot was removed and filtered through 6 layers of cheese cloth. After 120 sec the homogenate was squeezed through cheese cloth and the residue passed repeatedly through the roller mill while being mixed with the same buffer. Total enzyme activities, protein and chlorophyll were calculated from the sum of the two extraction procedures.

●——● 3-P-glycerate phosphatase

■——■ P-glycolate phosphatase

X---X Chlorophyll

O---O Protein

▲——▲ Glycolate oxidase

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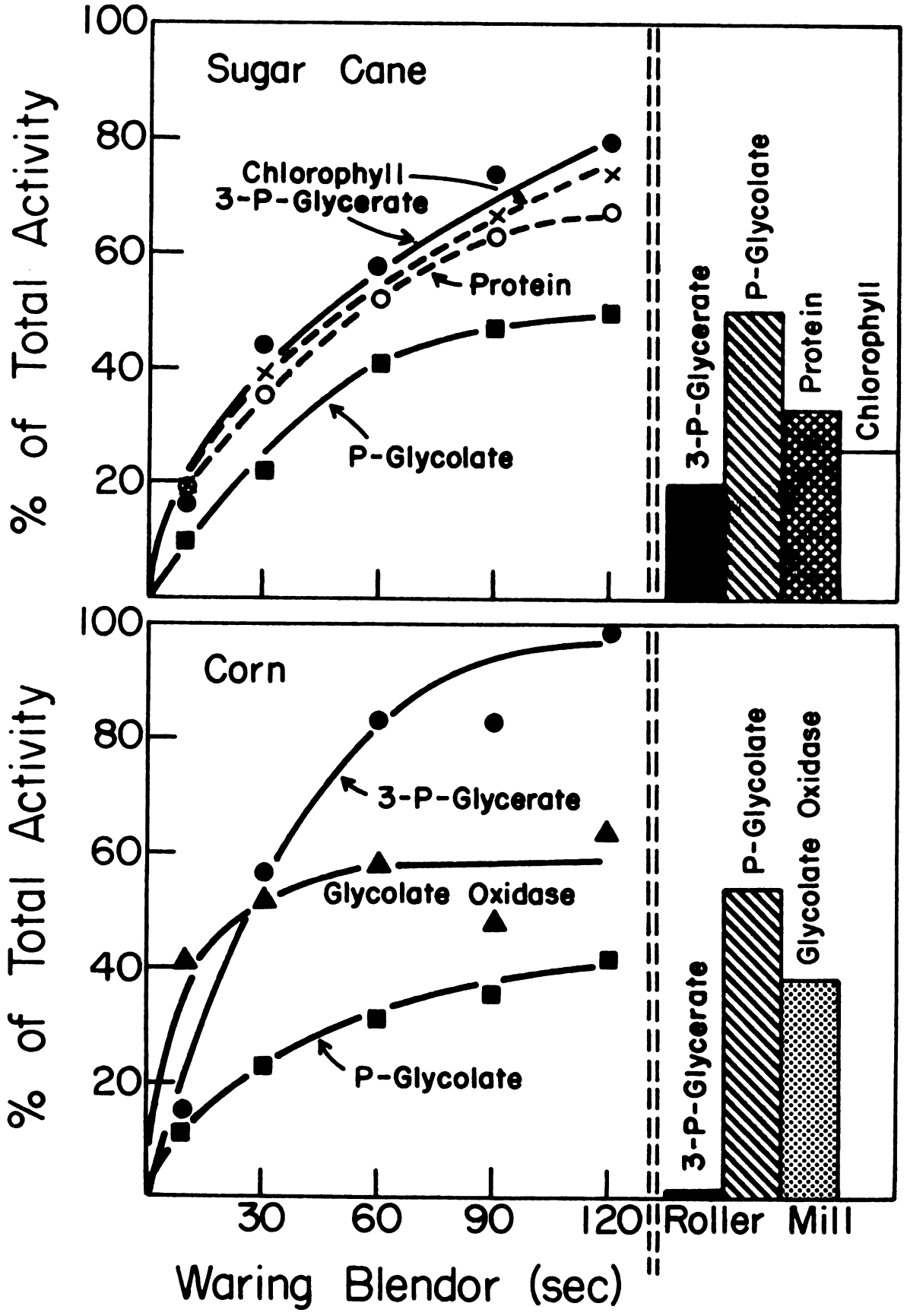
*A description and use of Roller Mill is found in Appendix A.


Sugar Cane
Chlorophyll
3-P-Glycerate
Protein
P-Glycolate
% of Total Activity
100
80
60
40
20
3-P-Glycerate
P-Glycolate
Protein
Chlorophyll
Corn
3-P-Glycerate
Glycolate Oxidase
P-Glycolate
% of Total Activity
100
80
60
40
20
3-P-Glycerate
P-Glycolate
Glycolate Oxidase
30
60
90
120
Roller Mill
Waring Blendor (sec)

initial steps yielded a clear, almost colorless supernatant devoid of chlorophyll after the pH fractionation, and subsequent precipitations were also almost colorless.

Other methods of breaking up the sugarcane leaves were tested and found unsatisfactory. The mortar and pestle was very limited in its capacity and exhaustingly slow. In the large commercial Waring Blendor inadequate breakage occurred as well as rapid heating of the extraction medium. The use of a large Hobart meat grinder, although efficient in macerating the leaf tissue, resulted in accelerated polyphenol oxidase activity, which interferred with subsequent purification of the phosphatase. When sugarcane leaves were ground in the Habart grinder without buffer and the macerated tissue then placed in extraction medium, the phenolic oxidation was severe. As described above, soaking the leaf tissue in the extraction medium before homogenization in the small Waring Blendor resulted in almost complete extraction of enzyme without heating the medium or allowing unnecessary oxidation.

Since the phenolic oxidation hindered subsequent purification of the phosphatase, 2% Polyclar AT was added to bind phenolic and phenolic like compounds so prevalent in plants (132). Likewise ascorbate was added to reduce unnecessary oxidation. Since in preliminary experiments the enzyme indicated no requirement for metal cations and was inhibited by various divalent cations, EDTA was routinely added. The EDTA also slowed the phenolic oxidations.

Acetone and ammonium sulfate fractionations: Complete equilibration during the precipitating procedures always resulted in improved yields. When considerable amounts of phenolic material were in the starting enzyme solution, the first acetone fractionation removed a large portion of them. Although P-glycerate phosphatase was soluble after acetone precipitation, the bulk of the insoluble sticky protein had to be dispersed by a glass homogenizer and prolonged stirring times were used to ensure complete suspension.

In scaling up to 800 to 1000 g of leaves for the final two large enzyme preparations, the yields from the first acetone fractionation were around 50% as compared to 80-90% in smaller runs. It is thought that a change in the precipitation pattern occurred and that more acetone should have been used to precipitate the enzyme.

Results from ammonium sulfate fractionation were often inconsistent for reasons unknown. The purification was either very good at this step or quite low with a parallel poor recovery. The rate of addition of the $(NH_4)_2SO_4$ was not critical, but complete equilibration after addition of the salt always improved the results and yield by at least a 2-fold purification.

The second acetone fractionation resulted in a 2 to 5 fold purification with good recovery and served to concentrate the enzyme for the G-200 Sephadex gel filtration. The precipitated enzyme was collected in one centrifugation and then dissolved in a small volume of 20 mM cacodylate buffer,

pH 6.3, and 1 mM EDTA. Good recovery required careful and complete resuspension of the precipitate. The insoluble material was removed by centrifuging at 40,000 x g.

Solid sucrose up to 10% (w/v) was added to the second acetone fraction for efficient sample application to the Sephadex gel column. The enzyme-sucrose solution was layered on a G-25 pad on top of the G-200 Sephadex column in order to prevent disruption of the G-200 gel by the heavy solution. A longer column should have improved the results, but ascending column equipment was unavailable at the time. A Blue Dextran sample through the G-200 Sephadex column permitted estimation of the void volume for an approximate estimate of molecular weights. The enzyme activity was slightly retarded by the gel giving a ratio of the elution volume to void volume (V_e/V_o) of 1.36 (Figure 4). The fractions were pooled, as indicated, on the criteria of a higher specific activity than the applied fraction. The phosphatase was completely excluded from G-100 Sephadex.

Ion exchange chromatography: It was at this point in the purification that difficulties arose. Since the enzyme from the gel filtration step was diluted, it was felt that adsorption on an ion exchange column would be desirable. DEAE-Cellulose or DEAE-Sephadex was picked as the most likely exchanger, but this was a most unfortunate choice. The 3-P-glycerate phosphatase activity gave a different pattern for every DEAE column attempted. Sometimes it was adsorbed entirely and other times only partially.

The enzyme activity was eluted in 2-7 peaks depending on pH, type of gradient, or concentration of applied enzyme. Rechromatography of that enzyme not adsorbed gave a pattern similar to the original attempt--some unadsorbed and the remaining eluting in several peaks. Rechromatography of individual, separated peaks gave the original peak and any that followed in the original column, but none preceeding it. Because of the potential of DEAE exchanger, considerable effort was expended in trying to perfect the process and determine the causes of multiple peaking and adsorbing properties.

In the pH range of 5.6 to 7.0, it was not possible to elute the enzyme from DEAE-cellulose in a reproducible manner. Below pH 5.6 increasing amounts of the enzyme came through without sufficient purification. Above pH 7.0 one peak was obtained but only 10-20% of the activity was recovered with equally poor purification.

The multiplicity of the enzyme peaks from DEAE columns suggested the possibility of a number of phosphatases or isoenzymes being present. Since specificity data was inconclusive, an isoelectric focusing experiment was performed in a pH range of 3 to 10. The isoelectric focusing resulted in only one peak of enzyme activity (see Figure 14, Section II). Since the isoelectric point (pI) of the phosphatase was about 6, a plausible explanation for the multiple peaking may be the running of the DEAE columns too close to the pI. Any slight differences in extraneous material or

other proteins with the enzyme or bound to it could cause a "different" enzyme to elute just by slight charge differences. At the higher pH's (>7.0) the differences would become negligible but the enzyme stability then became a problem. At pH's less than 6.0, the enzyme became positively charged and more of it came through the column in the void volume. Therefore, further purification attempts were directed towards cation exchangers.

Phosphocellulose chromatography: The phosphocellulose cation exchanger was tried with the idea that the phosphate group on the cellulose might bind the enzyme quite preferentially. A 3-fold purification was obtained (Figure 5 and Table I). The amount of buffer used to wash the column had no effect on the elution pattern nor did the type of gradient. For either a linear and concave gradient the enzyme eluted at about 0.2 M NaCl.

CM-Sephadex chromatography: The protein profile from the phosphocellulose elution suggested that a second cation exchange column could give further purification. The CM-Sephadex cation exchange column proved to be most useful at pH 5.0 from which the enzyme eluted at about 0.25 M NaCl (Figure 6). The fact that very little 280 nm absorbance was found anywhere on the column made protein determinations very difficult, and probably greater in error. For more accurate determination of protein and the fact that enzymes are frequently unstable when very dilute, the enzyme was concentrated by vacuum dialysis before characterization. Concen-

tration of the enzyme also resulted in increased specific activity (Table II).

Some other purification procedures which were tried and proved unsuccessful in addition to the DEAE-cellulose were: TEAE-cellulose; positive or negative absorption on alumina Cγ gel; heat fractionation; ethanol precipitation; and calcium phosphate gel.

Stability: In a crude extract of sugarcane leaves 3-P-glycerate phosphatase was stable at 4° for about a week before microbial growth destroyed the preparation. The activity in crude leaf extracts was completely stable to -18° for at least 6 months. A reason for not storing the enzyme in the crude extract was the slow continuous oxidation of phenolic compounds which interferred with subsequent purification of the enzyme.

The pH stability curve for the crude extraction of 3-P-glycerate phosphatase and the G-200 (Figure 8-A) indicates that it was unstable to the high pH and quite stable to low pH. The stability of the enzyme at low pH was utilized for the acid fractionation step in purification. The specific activity of the enzyme at the various pH's after removal of precipitated protein is also shown in Figure 8-A. The pH stability was unaffected by increasing ionic strength. However, enzyme from G-200 Sephadex fractionation was rapidly inactivated by $(NH_4)_2SO_4$ and $(NH_4)_2SO_4$ precipitation around pH 6.0.

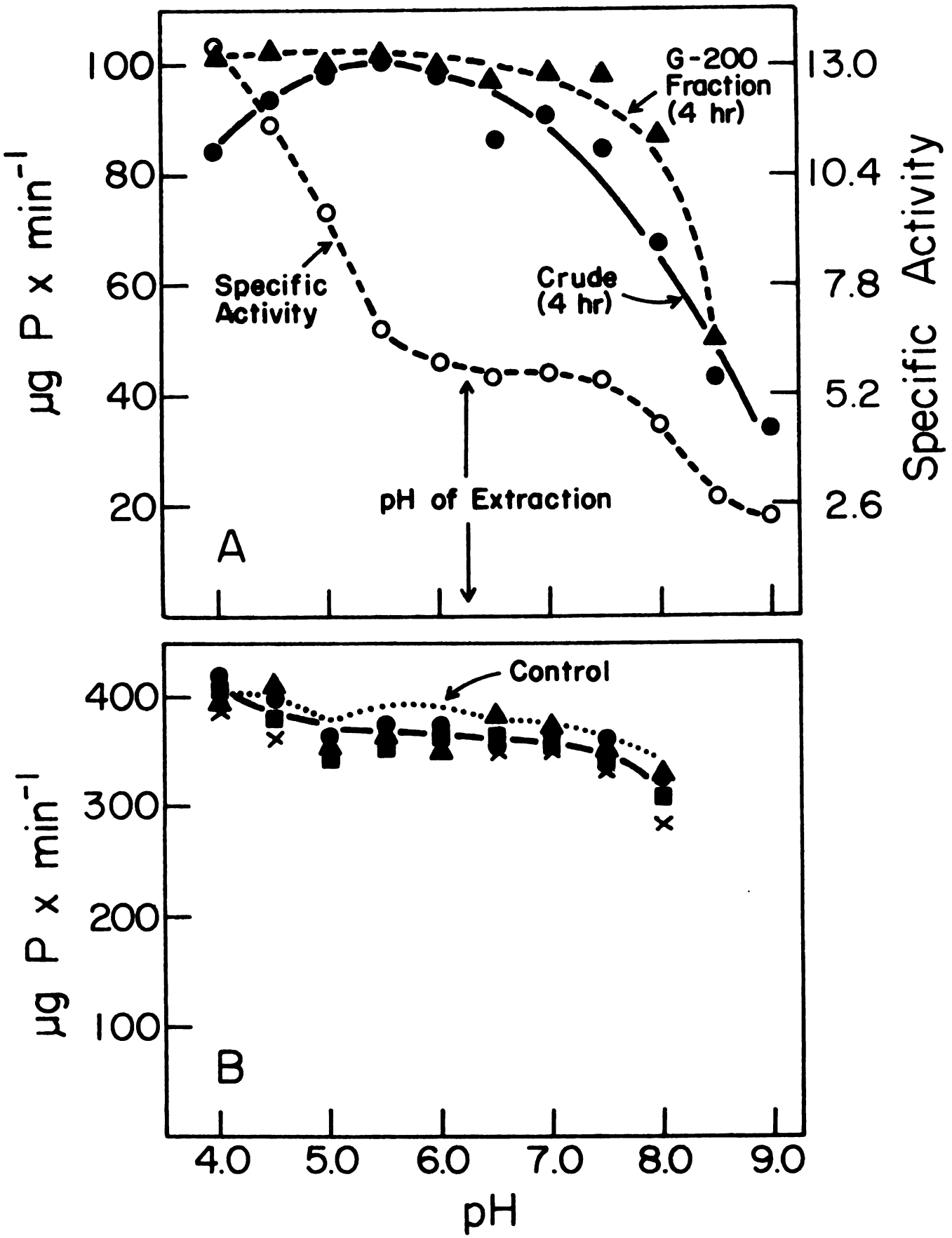
Partially purified enzyme was completely stable at

Figure 8. The Stability of 3-P-Glycerate Phosphatase as Function of pH and Ionic Strength

A. Aliquots of 20 ml of crude enzyme were adjusted to indicated pH with 1 N NaOH or 1 N HCl. The volumes were then adjusted to 25 ml and incubated at 4° for 4 hours. Aliquots of the samples were assayed and compared with controls (●——●). The crude enzyme at the various pH's was then centrifuged and adjusted to pH 6.3 with 1 N HCl or 1 N NaOH and their volumes equalized by adding 20 mM cacodylate buffer, pH 6.3 and 1 mM EDTA.

Aliquots of 100 µl of the Sephadex G-200 fraction of 3-P-glycerate phosphatase were diluted with 0.9 ml of 0.1 M cacodylate plus 0.1 M acetate buffer or 0.1 M cacodylate plus 0.1 M glycylglycine buffers at indicated pH. After 4 hours incubation at 4° the samples were assayed and relative 3-P-glycerate phosphatase activities were determined (▲——▲).

B. Aliquots of 50 µl of G-200 Sephadex fraction at the various pH's were diluted with NaCl or sodium acetate solutions of ionic strengths of 0.4 M (▲), 0.6 M (●), 0.8 M (■), and 1.0 M (X) and assayed after 4 hours.


100
80
60
40
20
μg P x min⁻¹
G-200
Fraction
(4 hr)
Specific
Activity
Crude
(4 hr)
pH of Extraction
A
13.0
10.4
7.8
5.2
2.6
Specific Activity
Control
400
300
200
100
μg P x min⁻¹
B
4.0
5.0
6.0
7.0
8.0
9.0
pH

pH 5.9 and 4° for several weeks or as long as it was kept sterile. Less than 5% of the activity was lost after storing at -18° for up to 3 months. The most purified enzyme preparations were stable for at least 2 months at 4° at concentrations so dilute the 280 nm absorbance readings were negligible.

II. Biochemical Properties of 3-P-Glycerate Phosphatase

The Enzyme Assay

Enzymatic hydrolysis of 3-P-glycerate was linear over the time period used and was also a linear function of the enzyme concentration (Figure 9). The amount of enzyme used in the enzyme assays was selected so that the phosphate released in a 10 minute period was between 0.2 μmole and 1.2 μmole. These conditions of linearity over the assay time and enzyme concentration were checked frequently and at each purification step of the enzyme isolation.

pH Optimum

The pH activity curves for 3-P-glycerate phosphatase are in Figure 10. These curves were determined using the highly purified enzyme after CM-Sephadex fractionation. The pH optimum in the 5 buffering systems was between 5.7 and 6.0. When the activity values from the different buffer systems were normalized to their highest value and then averaged, the optimum pH was 5.9. The addition of the divalent cation, Mg^{++} did not change the pH optimum in any

Figure 9. 3-P-Glycerate Hydrolysis by the Phosphatase as a Function of Time and Enzyme Concentration

A. Reaction mixtures (0.75 ml) containing 10 μmoles 3-P-glycerate, 100 μmoles sodium cacodylate buffer, pH 5.9, were equilibrated to 30°. The reactions were initiated by addition of 20 μl 3-P-glycerate phosphatase with thorough mixing and maintained at 30° until terminated by addition of 0.25 ml 10% trichloroacetic acid. The amount of phosphate released was determined by method A.

B. Reaction mixtures (0.50 ml) containing 100 μmoles sodium cacodylate, pH 5.9, and the indicated amount of 3-P-glycerate phosphatase were incubated for 5 minutes at 30°. The enzyme reaction was initiated by additions of 10 μmoles 3-P-glycerate at 30°. After 10 minutes the reaction was terminated by addition of 0.25 ml 10% trichloroacetic acid. The amount of phosphate released was determined by method A.

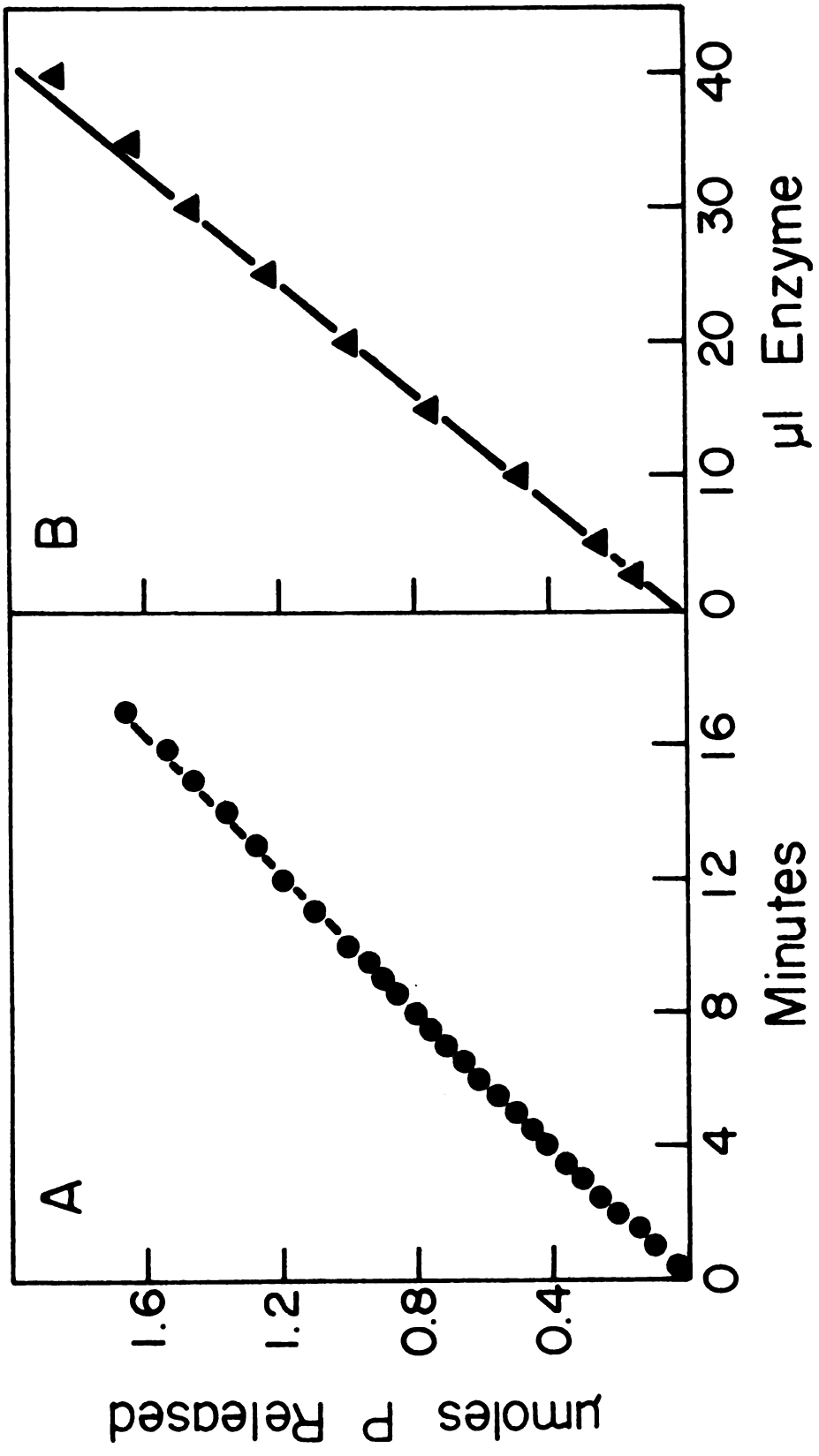
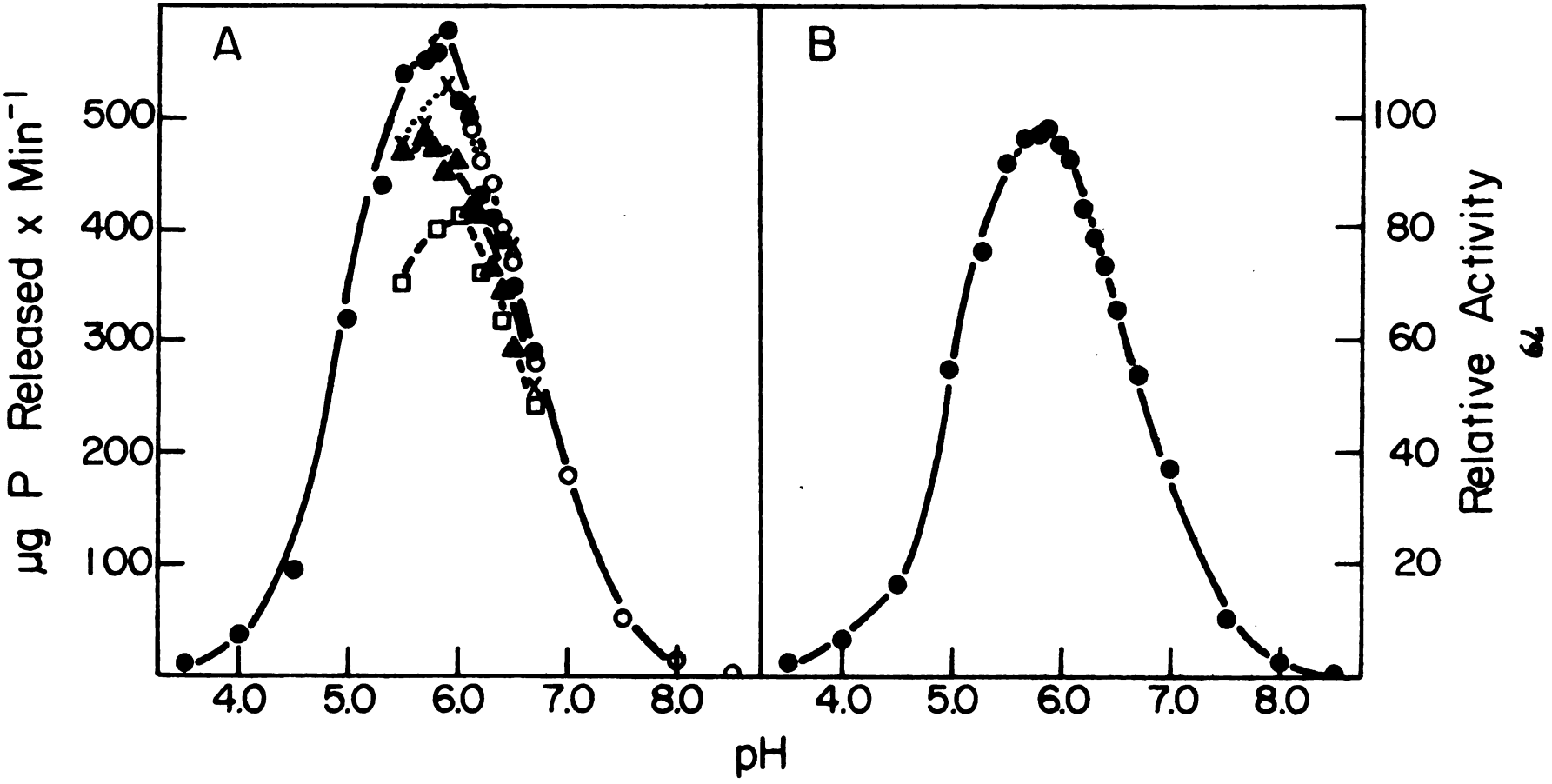

A
B
μmoles P Released
1.6
1.2
0.8
0.4
0
4
8
12
16
Minutes
0
10
20
30
40
μl Enzyme

Figure 10. The pH Activity Curves for 3-P-Glycerate Phosphatase

All points of part A are averages of triplicated assays of the enzyme after CM-Sephadex fractionation. The 5 buffer systems were: (●——●) for 0.1 M acetate + 0.1 M cacodylate; (O——O) for 0.1 M glycylglycine + 0.1 M cacodylate; (□——□) for 0.2 M malate; (▲——▲) for 0.2 M cacodylate; and (X——X) for 0.2 M MES. Part B is an average plot obtained by normalizing the points of each buffer system to their highest value.


A
B
µg P Released x Min⁻¹
500
400
300
200
100
4.0
5.0
6.0
7.0
8.0
pH
100
80
60
40
20
Relative Activity

way. The pH curves in the different buffers were quite narrow and did not show distinct characteristics of either acid or alkaline phosphatases. The pH optimum is not in the range of most chloroplast enzymes (pH 7 to 8.5).

The pH activity curves for less pure enzyme were shifted slightly to higher pH values. The enzyme had a pH optimum of 6.3 in early stages of purification (Figure 11) through the second acetone fractionation. Consequently pH 6.3 was used for assays of less pure preparations and is possibly closer to the optimum *in vivo*.

Kinetic Characteristics

The apparent K_m of 3-P-glycerate phosphatase from sugarcane leaves purified through CM-Sephadex fractionation for substrate ranged from 2 to 5 x 10^{-4} M from several different enzyme preparations (Figure 12). The average apparent K_m was 2.85 x 10^{-4} M. Considering the somewhat non-specific nature of the enzyme, it should be emphasized that there was no indication in the Lineweaver-Burk plots of two enzymes acting on the substrate.

Effect of Temperature

The enzyme in sugarcane leaf homogenates was 50% inactivated by heating to 50° for 3 minutes and about 80% inactivated by 60° (Figure 13-A). The effect of temperature on the reaction rate with the purified 3-P-glycerate phosphatase is shown in Figure 13-B. In a parallel experiment aliquots (5 µl) of the enzyme at 4° was added to assay

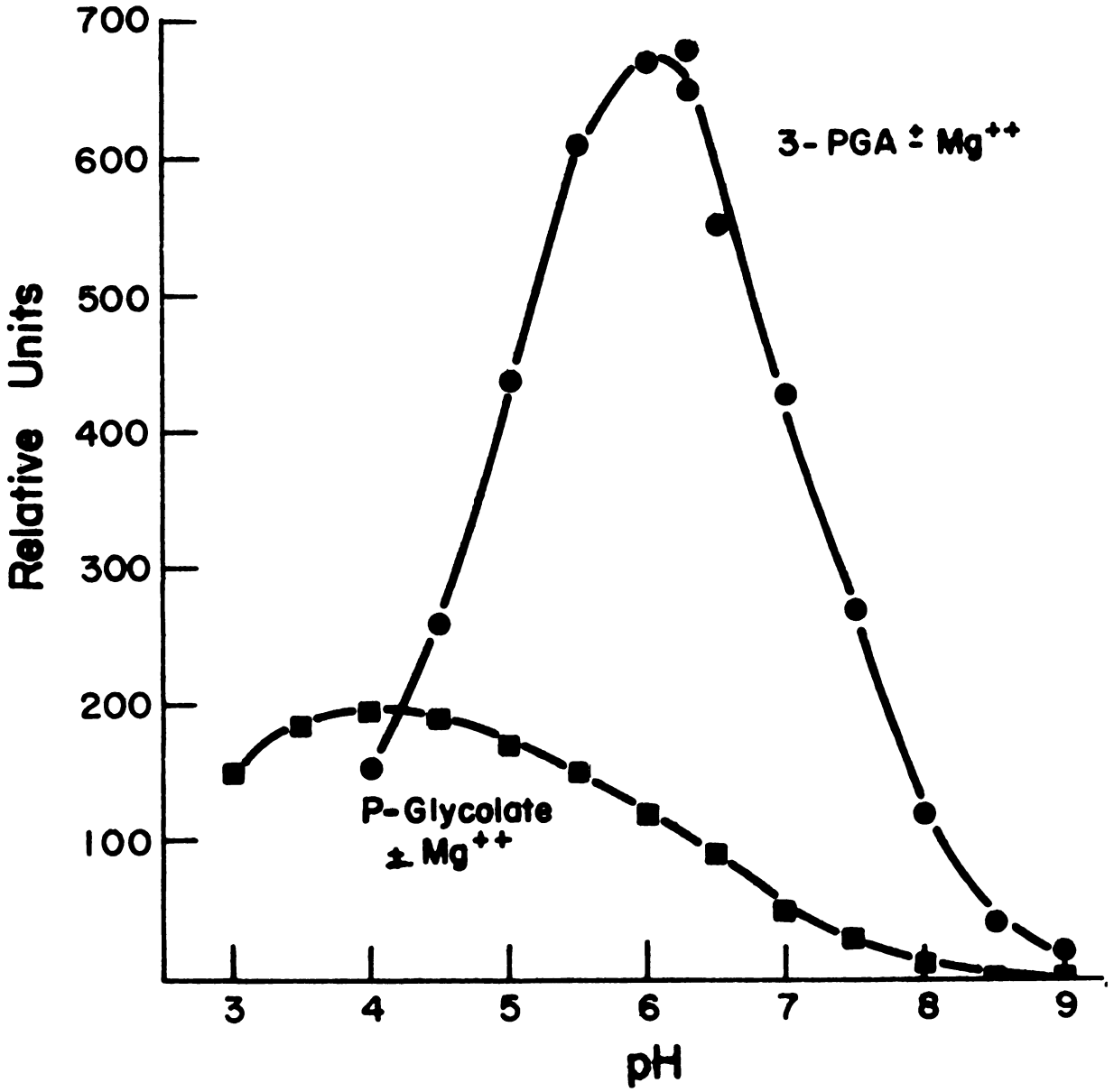


Figure 11. The pH Activity Curve for 3-P-Glycerate Phosphatase and P-Glycolate Phosphatase Using First Acetone Fractions

Phosphatase activity on 3-P-glycerate and P-glycolate was determined at indicated pH's using buffer systems of 0.1 M cacodylate plus 0.1 M acetate and 0.1 M cacodylate plus 0.1 M glycylglycine. Phosphatase activity was determined in presence and absence of 10^{-3} M $MgCl_2$.

Figure 12. The Relationship of Substrate Concentration to Reaction Velocity and a Lineweaver-Burk Plot for 3-P-Glycerate Phosphatase

The 3-P-glycerate phosphatase was assayed in the standard manner but with a reaction time of 1 minute. The amount of phosphate released was determined by the method B. Aliquots of 100 µl were used for phosphate determination. Each point is the average of triplicated assays. The two Lineweaver-Burk plots were obtained with the purified enzyme after CM-Sephadex fractionation (A) and after concentration of the CM-Sephadex fraction (B) (Table I).

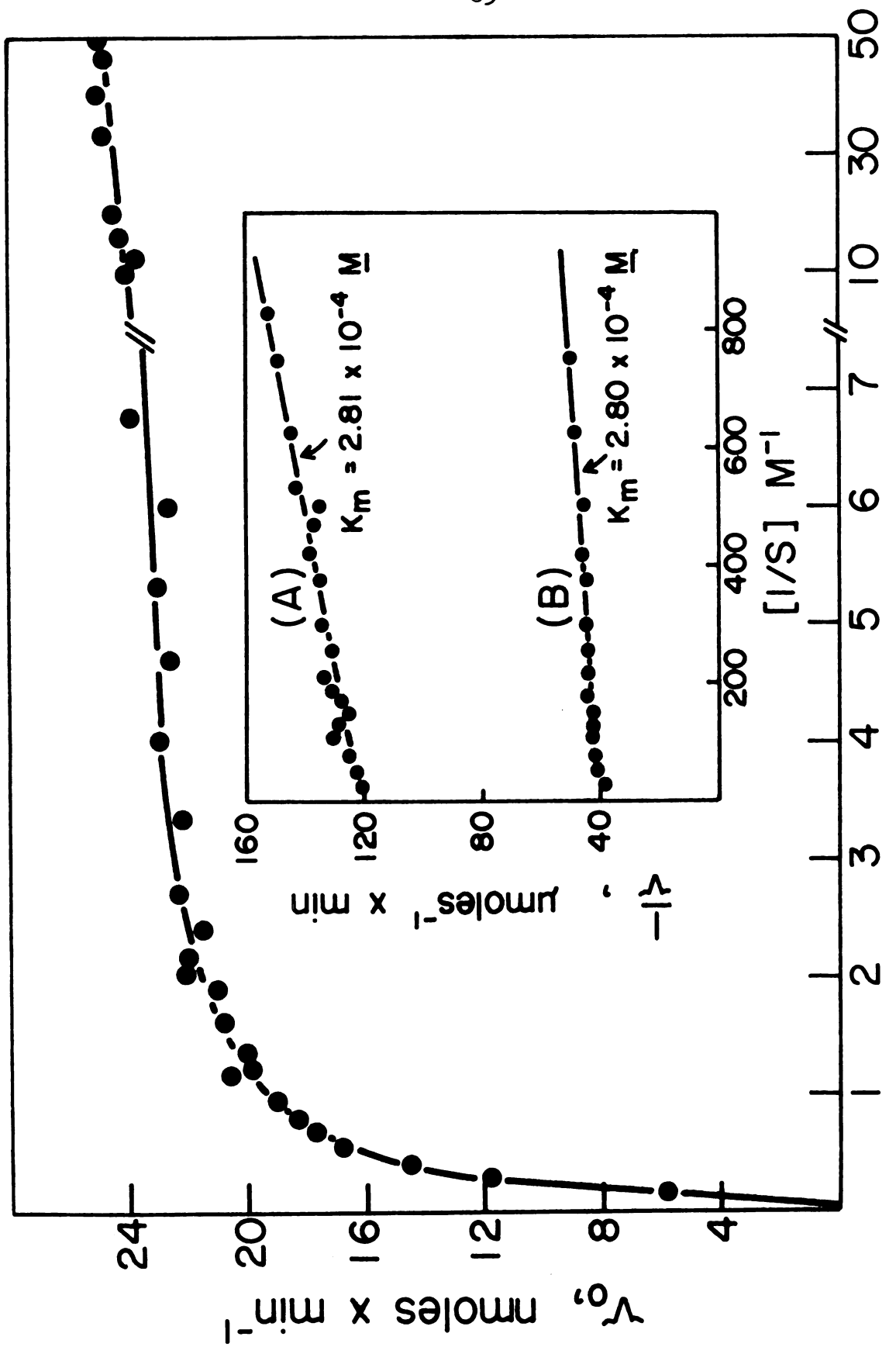
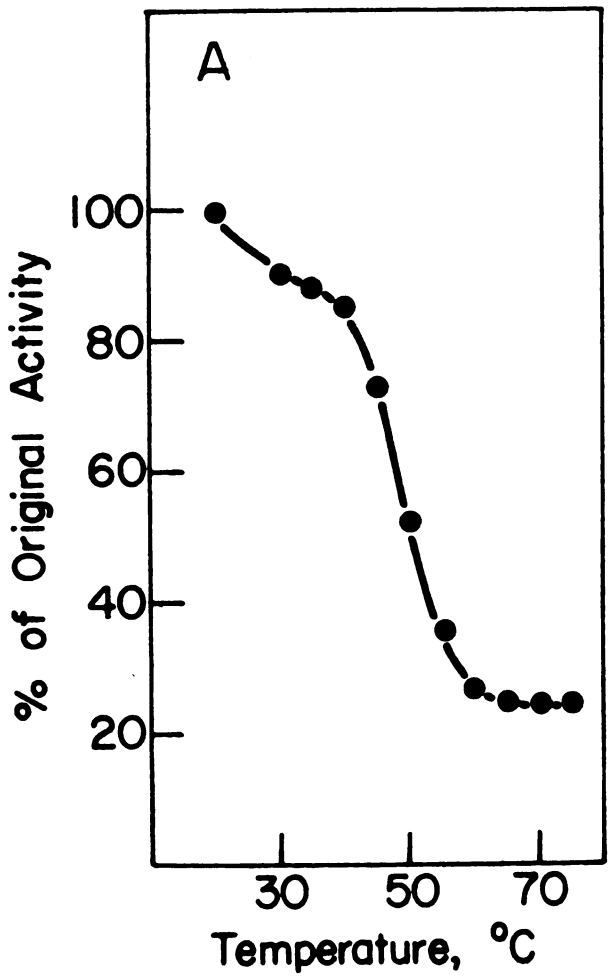
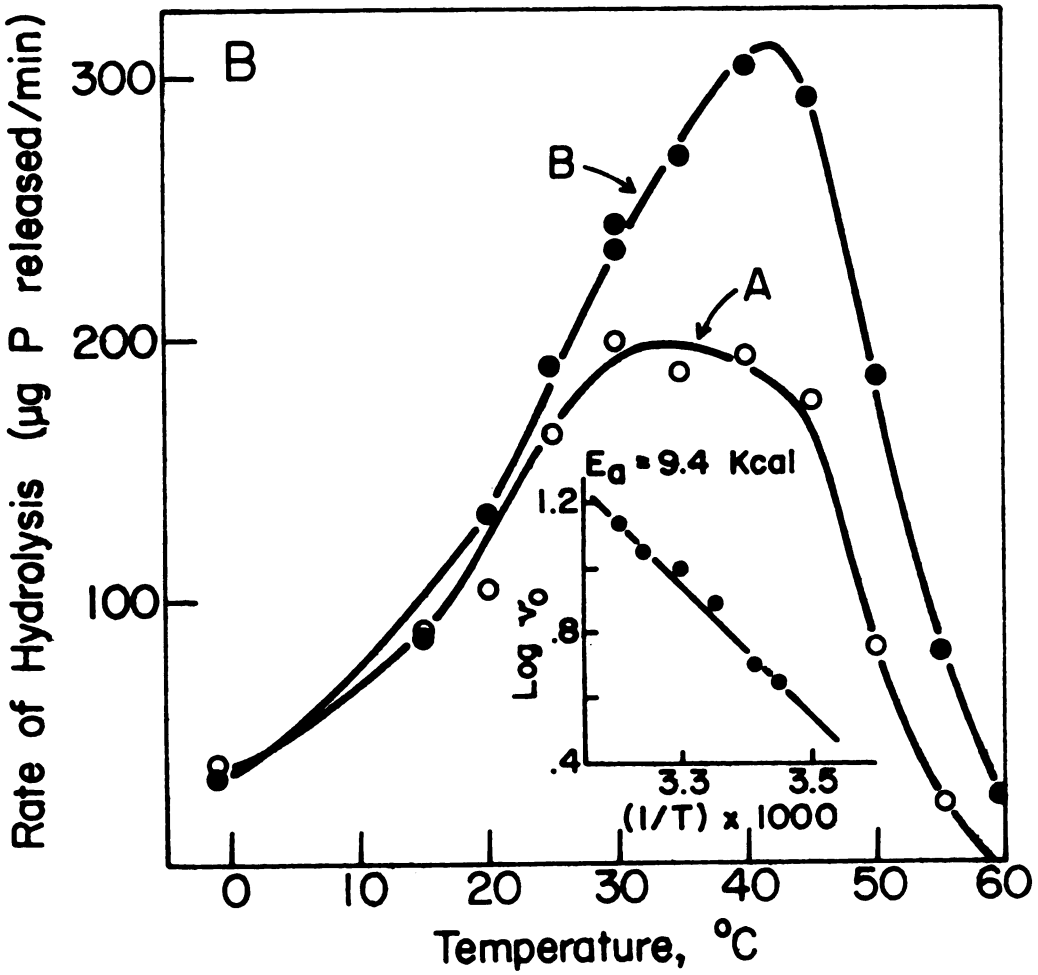

V_o, nmoles x min^{-1}
[S], mM
$\frac{1}{v}$, μmoles^{-1} x min
[1/S] M^{-1}
(A)
K_m = 2.81 x 10^{-4} M
(B)
K_m = 2.80 x 10^{-4} M

Figure 13. Effect of Temperature of 3-P-Glycerate Phosphatase Stability and Rate of Hydrolysis of 3-P-Glycerate

A. Triplicated samples (10 ml) of crude enzyme in conical test tubes were heated to indicated temperatures and held there with continuous stirring for 3 minutes. The samples were then immediately cooled in an ice bath to 4^{o} and assayed.

B. Samples of crude enzyme from leaf homogenates were incubated for 15 minutes in 0.5 ml of 0.1 M sodium cacodylate buffer, pH 5.9, at indicated temperature. The enzyme reaction was initiated by addition of 7.5 µmoles of 3-P-glycerate at the same temperature. Reaction time was 1 minute and the reaction was terminated by addition of 0.25 ml 10% TCA O——O. Samples of 0.75 ml of reaction mixture, containing 10 mM 3-P-glycerate, 0.1 M sodium cacodylate buffer at pH 5.9, were incubated at the desired temperatures and the reaction initiated by addition of 5 µl of enzyme from the CM-Sephadex fraction. After 1 minute the reaction was terminated by addition of 0.25 ml 10% TCA. The phosphate was determined by method B ●——●.


A
% of Original Activity
100
80
60
40
20
30
50
70
Temperature, °C


B
Rate of Hydrolysis (µg P released/min)
300
200
100
B
A
E_a = 9.4 Kcal
1.2
.8
.4
Log V_0
3.3
3.5
(1/T) x 1000
0
10
20
30
40
50
60
Temperature, °C

buffer and substrate at the desired temperature and the reaction run for 1 minute. The rate of 3-P-glycerate hydrolysis was maximal about 42°. The enzyme stability declines rapidly after 50° but is quite stable at 30° where the enzyme was assayed in the standard procedure. From the Arrhenius plot (insert Figure 13) the activation energy was found to be about 9.8 Kcal. The Q_{10} value for the enzyme activity in the 15° to 35° range was about 1.7.

Isoelectric Point

The isoelectric point (pI) of 3-P-glycerate phosphatase after the CM-Sephadex step of purification was about pH 6.8 (Figure 14). Nearly an identical profile was obtained with the enzyme obtained from the G-200 gel filtration step, except that the pI was near to pH 6.5 (data not shown). About 60% of either enzyme preparation that was applied to the column was found in the peak, and 20-25% of the activity trailed the peak in the fractions towards the acidic side in the elution profile. The reason or significance for the long trailing pattern is not known. It is possible that some of the enzyme adhered to the sides of the column or that the manner of draining the column did not ensure even removal. The results are consistent with the probability that only one 3-P-glycerate phosphatase was present.

Effect of Ionic Strength

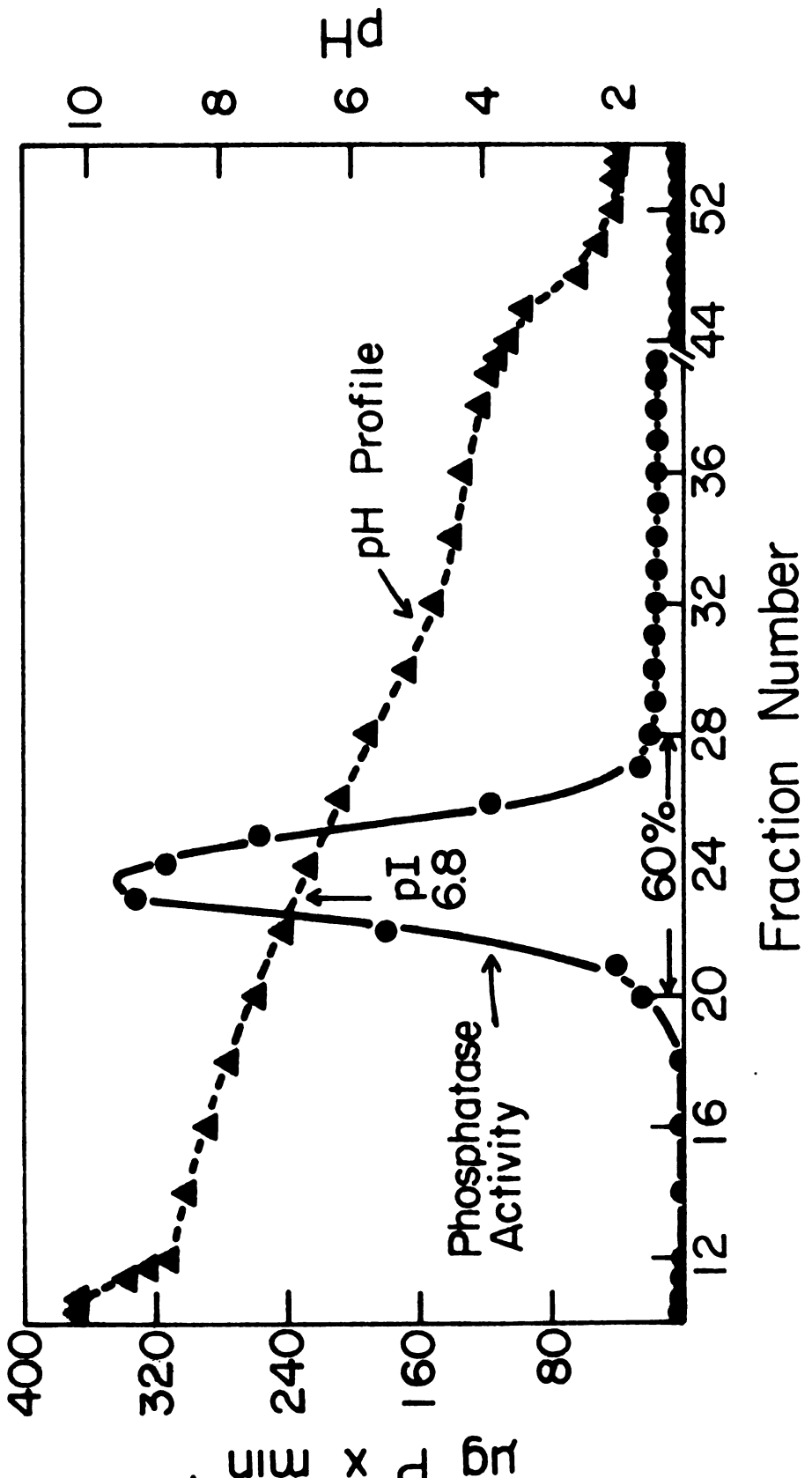
When the purified enzyme from the CM-Sephadex fractionation was assayed in the presence of NaCl or sodium

Figure 14. The Isoelectric Focusing Profile of 3-P-Glycerate Phosphatase

A sample of 3-P-glycerate phosphatase purified through the CM-Sephadex step and concentrated by vacuum dialysis was run on the LKB-Isoelectric Focusing Column. The column was prepared from the Ampholine-sucrose media with pH range of 3 to 10 as described in Materials and Methods. After 48 hours of equilibration at 300 volts and 4°, 2 ml fractions were drained from the bottom of the column by gravity at a rate of 1 ml per minute. The isoelectric point was taken as the midpoint of the 3-P-glycerate phosphatase peak.

●——● 3-P-glycerate phosphatase

▲---▲ pH


pH
10
8
6
4
2
pH Profile
pI
6.8
60%
Phosphatase
Activity
μg P x min⁻¹
400
320
240
160
80
12
16
20
24
28
32
36
44
52
Fraction Number

acetate there was a slight (12-15%) loss of activity above 0.3 M salt (Figure 15). When the enzyme was diluted 10-fold at low ionic strengths (< 0.05 M) in buffer at pH 5.9 and held at room temperature for 5 hours before assaying about 30% of the activity was lost. However the enzyme was perfectly stable to dilution and room temperature at an ionic strength of 0.2 to 0.5 M (Figure 15). Prolonged stability of the enzyme at 4° with various ionic strengths was excellent with essentially no loss of activity until the pH was increased above 7.5 (Figure 8-A).

Substrate Specificity

The substrate specificity for 3-P-glycerate phosphatase was examined using 44 substrates of which very few (phosphoenol pyruvate and p-nitrophenylphosphate) showed activity greater than 50% of that for 3-P-glycerate hydrolysis (Table III). For physiological reasons (Literature Review) the functions of 3-P-glycerate phosphatase and P-glycolate phosphatase may be related. P-Glycolate phosphatatase was specific, but no absolute substrate specificity was found for 3-P-glycerate phosphatase. Since the purity or homogenity of the enzyme has not yet been established, one cannot state that the non-specificity is due to only one phosphatase. However, the single peak in isoelectric focusing would support only one enzyme. Also the Lineweaver-Burk plots indicate only one enzyme acting on 3-P-glycerate.

During purification of the enzyme, the relative activity for 3-P-glycerate hydrolysis increased through the

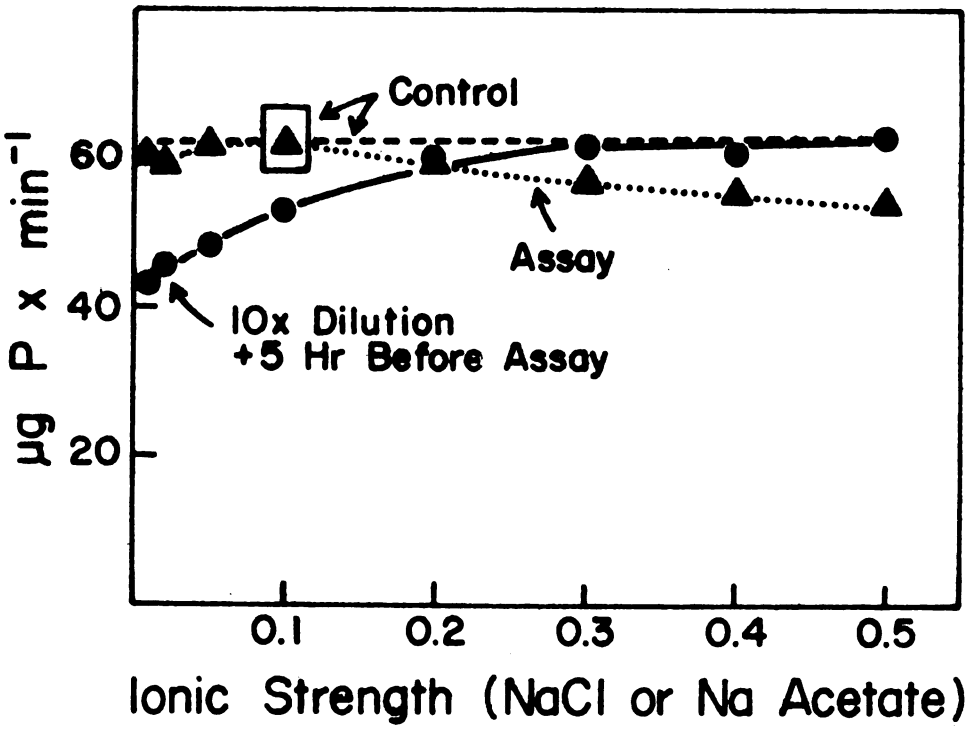


Figure 15. The Effect of Ionic Strength on 3-P-Glycerate Phosphatase Activity

The CM-Sephadex fraction was assayed in the presence of either NaCl or sodium acetate of indicated ionic strength. This standard assay was initiated by addition of a small aliquot of enzyme (▲——▲). Also the activity in the CM-Sephadex fraction was diluted 10 fold into 0.01 M sodium cacodylate buffer, pH 5.9, containing NaCl or sodium acetate of indicated ionic strengths, and allowed to stand 5 hours at room temperature before assaying at standard considions (●——●).

Table III. Specificity of 3-P-Glycerate Phosphatase

All substrates were present in 10 μmole amounts in the standard assay described in Materials and Methods. Enzyme purified through CM-Sephadex and concentrated was used (1 μl). Activities are shown relative to 3-P-glycerate at 100.

Substrates	Rel. Act.	Substrates	Rel. Act.
3-P-glycerate	100	Nicotinamide adenine dinucleotide-P	7
DL-glyceraldehyde-3-P	41	Nicotinamide adenine dinucleotide-P reduced	11
2-P-glycerate	24	α-Phenylphosphate	48
P-glycolate	11	bis-p-Nitrophenylphosphate	0
P-glycoaldehyde	13	p-Nitrophenylphosphate	66
Phosphoenolpyruvate	64	Phenophthalein diphosphate	48
Dihydroxyacetone phosphate	24	Pyridoxal-5-phosphate	11
α-Glycerol phosphate	14	Phosphocholine	1
β-Glycerol phosphate	16	Glucose-6-phosphate	7
Phospholactate	11	Glucose-1-phosphate	0
Propanediol phosphate	37	Fructose-6-phosphate	6
Phosphoserine	10	Fructose-1,6-diphosphate	31
Phosphohydroxypyruvate	17	Ribose-5-phosphate	7
Phosphoethanolamine	2	Ribulose-1,5-diphosphate	11
Adenosine triphosphate	37	6-P-Gluconate	17
Adenosine diphosphate	18	Carbamyl phosphate	0
3'-Deoxyadenosine monophosphate	0	Creatine phosphate	0
3'-Adenosine monophosphate	9	Pentaphosphate	0
5'-Adenosine monophosphate	2	Decaphosphate	0
5'-Uridine monophosphate	14	Pyrophosphate	9
5'-Guanosine triphosphate	26	Phytic acid	5
5'-Cytosine triphosphate	28		
5'-Cytosine monophosphate	5		

ammonium sulfate fractionation step to 110-fold purification (Table IV). However further purification of the phosphatase to 2530-fold did not significantly alter the ratio of activity toward the various substrates. Consequently it is probable that one phosphatase is present which catalyzes the hydrolysis of these various substrates. After the initial steps of the purification, rates of hydrolysis of P-glycolate, p-nitrophenylphosphate, and pyrophosphate decreased and rates of hydrolysis of ATP and fructose-1,6-diphosphate increased relative to 3-P-glycerate. During one purification attempt the specificity of 3-P-glycerate phosphatase was checked immediately after the second acetone fractionation and then rechecked after the enzyme had been stored in the coldroom for 2 weeks and then for another 2 weeks at -18°. The rate of hydrolysis of 3-P-glycerate remained unchanged, but the rate of hydrolysis of several other substrates had increased for the enzyme stored at 4° and -18°. Thus there appeared to be a loss of specificity with aging.

EDTA (1 mM) was used in the isolation procedure and should inactivate divalent cation requiring phosphatases such as P-glycolate phosphatase. The results in both Tables III and IV were from assays without added Mg^{++}, and thus the activity of other phosphatases would be minimal. Addition of $MgCl_2$ to the assays with purified P-glycerate phosphatase did not alter the activity ratios for P-glycerate and the other substrates, suggesting that the purified enzyme did not contain other divalent cation requiring phosphatases.

Table IV. Relative Specificity of 3-P-Glycerate Phosphatase at Various Stages of Purifications

The various substances were assayed using the standard 3-P-glycerate phosphatase assay with substrates at 10 mM concentrations.

Substrate	Crude	pH Fractionation	1st Acetone	$(NH_4)_2SO_4$	2nd Acetone	G-200 Sephadex	CM Sephadex	pI*
3-P-glycerate	100	100	100	100	100	100	100	100
P-glycolate	29	67	20	15	13	11	11	11
2-P-glycerate	11	11	1	14	12	17	24	27
p-Nitrophenyl phosphate	156	156	72	76	74	76	66	66
Phenolphathalein diphosphate	71	86	56	60	54	-	55	48
Glucose-6-phosphate	9	5	0	5	2	9	-	-
Pyrophosphate	39	43	16	16	15	22	-	-
Adenosine diphosphate	29	29	25	22	21	-	-	-
Adenosine triphosphate	32	29	62	61	52	59	52	48
Phosphoserine	28	28	22	29	34	-	10	10
Fructose diphosphate	15	15	54	54	54	44	48	48
β-glycerol phosphate	-	-	-	-	-	17	-	-
α-glycerol phosphate	-	-	-	-	-	15	-	-
Phosphoenol pyruvate	-	-	-	-	-	-	64	61

*Enzyme pooled from isoelectric focusing experiments.

According to Thorn (133) and Dixon and Webb (134) one means of determining whether the activity toward two substrates is due to one or two enzymes is to compare the activity with such substrate separately against the substrates together. If two enzymes were present, the activities should be additive, and if one enzyme was present, the activity would be the average of the two separately. When this was done (Table V) the activity with 3-P-glycerate alone was always greater than with 3-P-glycerate plus a second substrate. Thus the activities were not additive, but neither were they the average of the two alone. Since the rates were not additive for 3-P-glycerate with 11 other substrates, one enzyme is suggested. A basic assumption for this type of experiment is that the apparent K_m's for the two substrates should be about the same (134). Although the apparent K_m's for the other phosphate esters were not determined, different values might well be expected and probably could account for the varying results.

In other phases of this investigation we have been concerned with activity ratio of P-glycolate phosphatase to 3-P-glycerate phosphatase and with the physiological function of these two phosphates. Consequently, the P-glycolate phosphatase activity in the first acetone fractionation of the 3-P-glycerate phosphatase was examined. This activity did not have the properties of the specific P-glycolate phosphatase (23, 24), but rather it appeared to be a non-specific Mg^{++}-dependent acid phosphatase. The pH optimum

Table V. Activity of 3-P-Glycerate Phosphatase with Two Substrates Persent

Phosphatase activity was determined using the substrate alone (10 μmoles) and for 3-P-glycerate (10 μmoles) plus a second phosphate ester (10 μmoles).

Phosphate Ester	Activity (nmoles P released/10 min)		
	Alone	+ PGA	Calculated*
Control, 3-P-glycerate	700	700	700
P-Glycolate	88	476	394
2-P-Glycerate	155	583	424
α-Glycerol phosphate	78	661	389
Phospholactate	58	593	379
Phosphoserine	78	661	389
Phosphoethanolamine	15	662	354
Propanediol phosphate	252	680	476
Glucose-6-phosphate	10	661	355
Phosphocholine	78	642	389
Ribose-5-phosphate	97	718	399
Fructose-1,6-diphosphate	330	447	515

*The calculated value is based on the average of the activities of the substrates alone.

for P-glycolate hydrolysis was around pH 4 (Figure 12), where as the specific P-glycolate phosphatase has a pH optimum of 6.3. The difference in rate of hydrolysis by the first acetone preparation of the two substrates and the pH optimum are emphasized in Figure 12.

Effect of Metal Complexing Agents

Dialysis of 3-P-glycerate phosphatase at each stage of purification against 40 volumes 10^{-3} M EDTA at 4° for 48 hours did not cause any loss of activity. It was initially concluded that the enzyme had no easily removable metal cation, and EDTA was employed in the isolation procedure for stability of the protein and for inactivating other phosphatases. In order to determine whether or not the enzyme had a metal requirement, a number of metal complexing or chelating agents were incubated with the enzyme for 15 minutes under assay conditions and then the enzyme reaction was initiated with substrate (Table VI). None of the complexing agents, even at 10^{-2} and 10^{-3} M showed significant inhibition of 3-P-glycerate phosphatase that had been purified 1740-fold through the CM-Sephadex step of the fractionation procedure.

Effect of Cations

The effects of various metal cations on 3-P-glycerate phosphatase activity are shown in Table VII. Enzyme (5 µl) with a specific activity of 562 purified through the

Table VI. The Effect of Metal Complexing Agents on 3-P-Glycerate Phosphatase Activity

The enzyme was from CM-Sephadex fractionation. The activities were calculated relative to the control (no additives) at 100.

Complexing Agent	Final Concentration of Complexing Agent During Assay		
	10^{-2} M	10^{-3} M	10^{-4} M
Dithiothreitol	109	101	102
EDTA	107	102	102
o-Phenanthroline	*	93	104
m-Phenanthroline	*	100	100
8-Hydroxyquinoline	100	102	104
8-Hydroxyquinoline-5-sulfonic acid	102	100	100
αα-Dipyridyl	*	96	106
Diethyldithiocarbamate	92	98	100
Sodium azide	99	101	100

*Formed a precipitate with reagents for determining phosphate.

Table VII. Effect of Metal Ions on 3-P-Glycerate Phosphatase

Reactions were carried out as described in Materials and Methods except that the enzyme reaction was initiated with substrate after a 15 minute incubation of the enzyme with the cation in the assay buffer. The activities from various salts of each cation were averaged. Each salt was evaluated in triplicate. Aliquots (5 µl) of enzyme purified through CM-Sephadex were used for the study.

Cation		Final Concentration of Cations			Cation		Final Concentration of Cations		
		10^{-2} M	10^{-3} M	10^{-4} M			10^{-2} M	10^{-3} M	10^{-4} M
Control		100	100	100	Cu^{++}	$SO_4^=$	4	10	37
Mg^{++}	SO_4^-, Cl^-, $Acetate^-$	97	95	94	Hg^{++}	Cl^-, $Acetate^-$	1	18	58
					Sn^{++}	Cl^-	9	75	85
Mn^{++}	$Acetate^-$, Cl^-	88	98	90	Sr^{++}	Cl^-	103	93	84
Ca^{++}	Cl^-, NO_3^-	91	97	89	Zn^{++}	$Acetate^-$, $SO_4^=$	4	26	63
Co^{++}	$SO_4^=$, Cl^-	73	98	90	K^+	Cl^-, $SO_4^=$	98	100	100
Pb^{++}	NO_3^-, $Acetate^-$	8	57	88	Na^+	Cl^-, $Acetate^-$	100	101	100
Ni^{++}	$SO_4^=$	50	71	87	NH_4^+	Cl^-	99	101	101
Fe^{++}	$SO_4^=$	39	72	85	Cr^{+++}	Cl^-	86	97	84
Fe^{+++}	Cl^-, NO_3^-, $NH_4(SO_4^=)_2$	37	76	80					

CM-Sephadex was used. Each of the salt forms of each divalent cation was added to a reaction buffer to give final concentrations of 10^{-2} M, 10^{-3} M and 10^{-4} M. The enzyme was added and the solutions were incubated for 15 minutes at 30°, before the reaction was initiated by the addition of substrate.

None of the cations resulted in any significant stimulation of the 3-P-glycerate phosphatase activity. Most of the cations caused at least some inhibition at the highest concentration except for the monovalent cations and 10^{-2} M strontium. Hg^{++}, Cu^{++}, Pb^{++}, Fe^{++}, Fe^{+++}, Zn^{++} and Sn^{++} were inhibitory at 10^{-2} M and in some cases at 10^{-3} M, but no significance is attached to these facts, because of the high concentrations. Cu^{++} and Zn^{++} ions were inhibitory at 10^{-4} M. Zn^{++} ion was of particular interest because P-glycolate phosphatase had previously been found to be the most active in the presence of Zn^{++} at 10^{-2} and 10^{-3} M, as are other phosphatases.

Effect of Related Compounds and Other Inhibitors

The effect of a series of compounds which are either inhibitors of other phosphatases, structural analogs of 3-P-glyceric acid, inhibitors of the glycolate pathway, compounds of the glycolate pathway, possible glycolate metabolites or amino acid modifying reagents was determined on 3-P-glycerate phosphatase of high specific activity from the CM-Sephadex fractionation step. An aliquot of the

enzyme (5 µl) in 0.10 M sodium cacodylate buffer, pH 5.9 was treated as in the standard assay (0.75 ml final volume) with the indicated compound (Table VIII) at three final concentrations of 10^{-2} M, 10^{-3} M and 10^{-4} M for 15 min at 30°. Then substrate (5 µmoles) was added to initiate the reaction. Of the amino acid modifiers only p-chloromercuribenzoate showed inhibition (24%) at 10^{-2} M.

Sodium fluoride and molybdate exhibited the typical phosphatase inhibition but inhibition by L(+)tartrate was less than for other phosphatases and was only 29% at 10^{-2} M. Lineweaver-Burk plots of L(+)tartrate inhibition, as shown in Figure 16, indicated that it exerted a typical competitive inhibition.

Two inhibitors of the glycolate pathway, hydroxypyridinemethane sulfonate and isonicotinyl hydrazide, were ineffective in reducing 3-P-glycerate phosphatase activity. None of the metabolites of glycerate or the glycolate pathway affected the enzyme.

Of the structurally similar compounds, only arsenate, bicarbonate, carbonate, borate, dihydroxy tartrate, and amino-(oxy)acetic acid showed some inhibition. Isocitrate, cis-aconitate and citrate, which stabilizes the P-glycolate phosphatase (114) and which are also competitive inhibitors of P-glycolate phosphatase, were only slightly inhibitory at 10^{-2} M to 3-P-glycerate phosphatase. It seems characteristic of 3-P-glycerate phosphatase to be stable and insensitive to most of the usual inhibitors.

Table VIII. Effects of Other Inhibitors and Related Compounds

A 5 µl aliquot of enzyme (S.A. = 562) in a final volume of 0.5 ml of 0.1 M pH 5.9 sodium cacodylate and 10^{-2} M, 10^{-3} M, and 10^{-4} M in effector reagent were incubated 15 min at 30°. Phosphatase activity was determined by adding 5 µmoles 3-P-glycerate (6.7 mM final conc) and incubating 10 minutes more at 30°. The reaction was terminated by the addition of 0.25 ml 10% TCA. The activities were stated as percent of control.

Effector	Concentration of Effector		
	10^{-2} M	10^{-3} M	10^{-4} M
Control	100	100	100
Amino Acid Modifiers			
p-Chloromercurobenzoate	76	86	98
Iodoacetate	83	102	102
N-Ethyl maleimide	100	98	95
Phosphatase Inhibitors			
KF	7	55	93
L(+)Tartrate	71	94	98
Oxalate	95	93	86
Ascorbate	100	97	93
Cysteine	98	100	100
Glutathione	99	96	93
$NaMoO_4$	2	28	45
Sodium arsenite	87	96	98
Inhibitors of Glycolate Pathway			
Hydroxy-pyridine methane sulfonate	100	100	98
Isonicotinyl hydrazide	91	98	100

Table VIII. Continued

Effector	10^{-2} M	10^{-3} M	10^{-4} M
Structurally Similar or Related Compounds*			
Sodium arsenate	66	90	96
Glycidol	91	101	100
Glycerol	100	104	100
Pyruvic acid	103	100	98
β-Chloropropionate	100	97	96
L(+)Lactic acid	94	100	95
D(-)Lactic acid	92	102	94
L-α-Alanine	110	107	109
β-Alanine	113	101	104
Di-Hydroxymalic acid	100	95	92
Di-Hydroxytartaric acid	54	63	73
meso-Tartric acid	81	99	101
(Amino)oxy-acetic acid	54	100	100
Malonic acid	83	92	96
$NaBO_3$	26	78	90
Products and Related Metabolites			
DL-Glyceric acid	97	97	97
DL-Serine	94	95	97
Glycine	91	97	97
Glycolic acid	100	102	100
Glucose	104	102	97
Sucrose	111	105	101
$NaHCO_3$	79	89	96

Table VIII. Continued

Effector	10^{-2} M	10^{-3} M	10^{-4} M
Na_2CO_3	76	86	98
Maleic acid	90	100	100
Malic acid	98	104	101
Aspartate	106	110	104
Other Possible Effectors			
Succinic acid	103	98	99
Phlorizin	89	100	100
Thioglycolate	112	107	102
DL-Hydroxybutyric acid	100	96	96
Isocitric acid	94	98	102
Citric acid	85	103	104
cis-Aconitic acid	72	95	98
Citraconitic acid	96	96	102

*See mixed substrate assays for phosphorylated compounds.

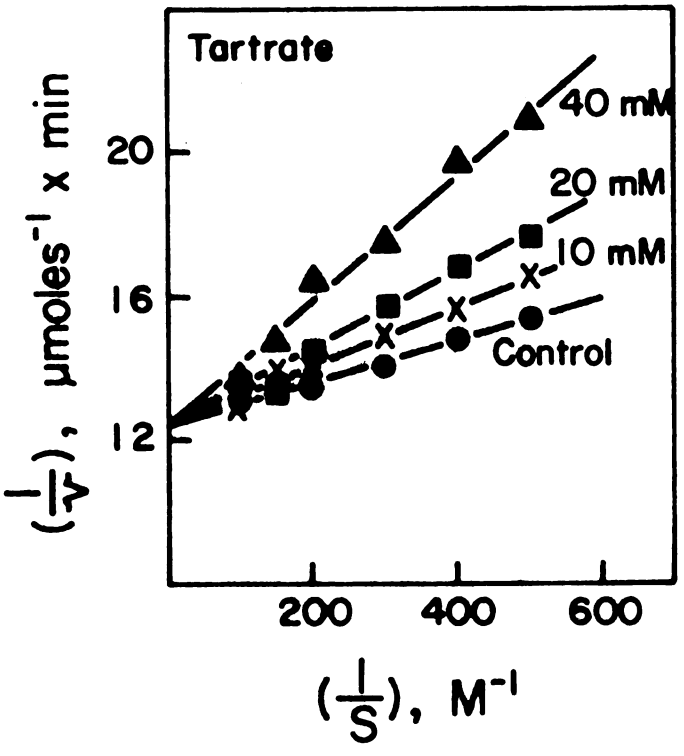


Figure 16. Lineweaver-Burk Plot of L (+) Tartrate Inhibition

The 3-P-glycerate phosphatase used had a specific activity of 562 after CM-Sephadex fractionation. The standard assay was used except that the reaction time was 1 minute, and L (+) tartrate at 10, 20, and 40 mM final concentration was added as indicated. The phosphate released was determined by method B, using 100 µl aliquots of the reaction mixture.

Inhibition by Glycidol-P

Glycidol-P (1,2, epoxipropanediol-P*) has been reported to be a potent inhibitor of rabbit muscle triose phosphate isomerase and enolase (135). When glycidol-P and P-glycerate phosphatase were incubated together at 23°, the enzymatic activity was inhibited about 50% in 15 minutes and 75% in 1 hour (Figure 17). At 4° this inactivation was much slower, but after 5 hours the enzyme was 75% inhibited. This loss of phosphatase activity was not reversible by an 18 hour dialysis of the inhibited enzyme. This slow but irreversible inhibition probably was due to binding of the inhibitor at or near the active site. This idea is consistent with the kinetics of inhibition by glycidol phosphate, which appear to be competitive (Figure 18).

Effect of Phosphonic Acid Derivatives

Three derivatives of phosphonic acids** were selected on the basis of structural similarity to 3-P-glycerate. The phosphonic acid derivatives were examined with the idea that one of two effects might result. First, we looked for inhibition of the phosphatase since the C-P bond is supposed to be resistant to normal phosphatase action (136). And secondly, we looked for an effect on the enzyme by Compound III that would be analogous to any effect produced by the structural

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*The sample of glycidol-P was the gracious gift of I. A. Rose.

**The phosphonic acid derivatives were the gracious gift of I. F. Isbell, Texas A & M University.

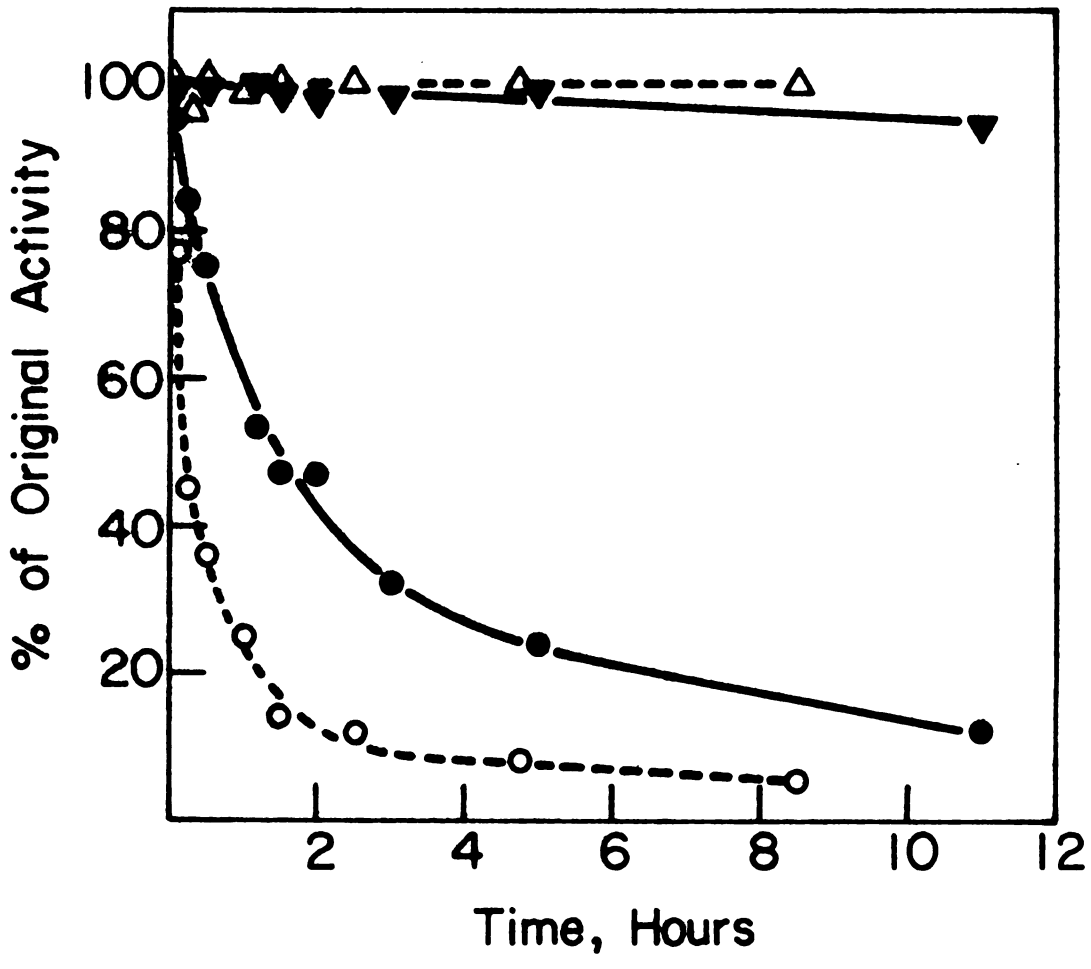


Figure 17. Time Dependence of Enzyme Inactivation by Glycidol-P

3-P-Glycerate phosphatase (3 units) was incubated with 4 mM P-glycidol in 10 mM sodium cacodylate buffer at pH 5.9 and 1 mM EDTA at 4° and 23°. Aliquots from the enzyme-inhibitor incubation were removed at indicated times, assayed, and phosphate determined by method A.

O——O Incubated with glycidol-P at 4°

O——O Incubated with glycidol-P at 23°

△——△ Incubated without glycidol-P at 4°

▼——▼ Incubated without glycidol-P at 23°

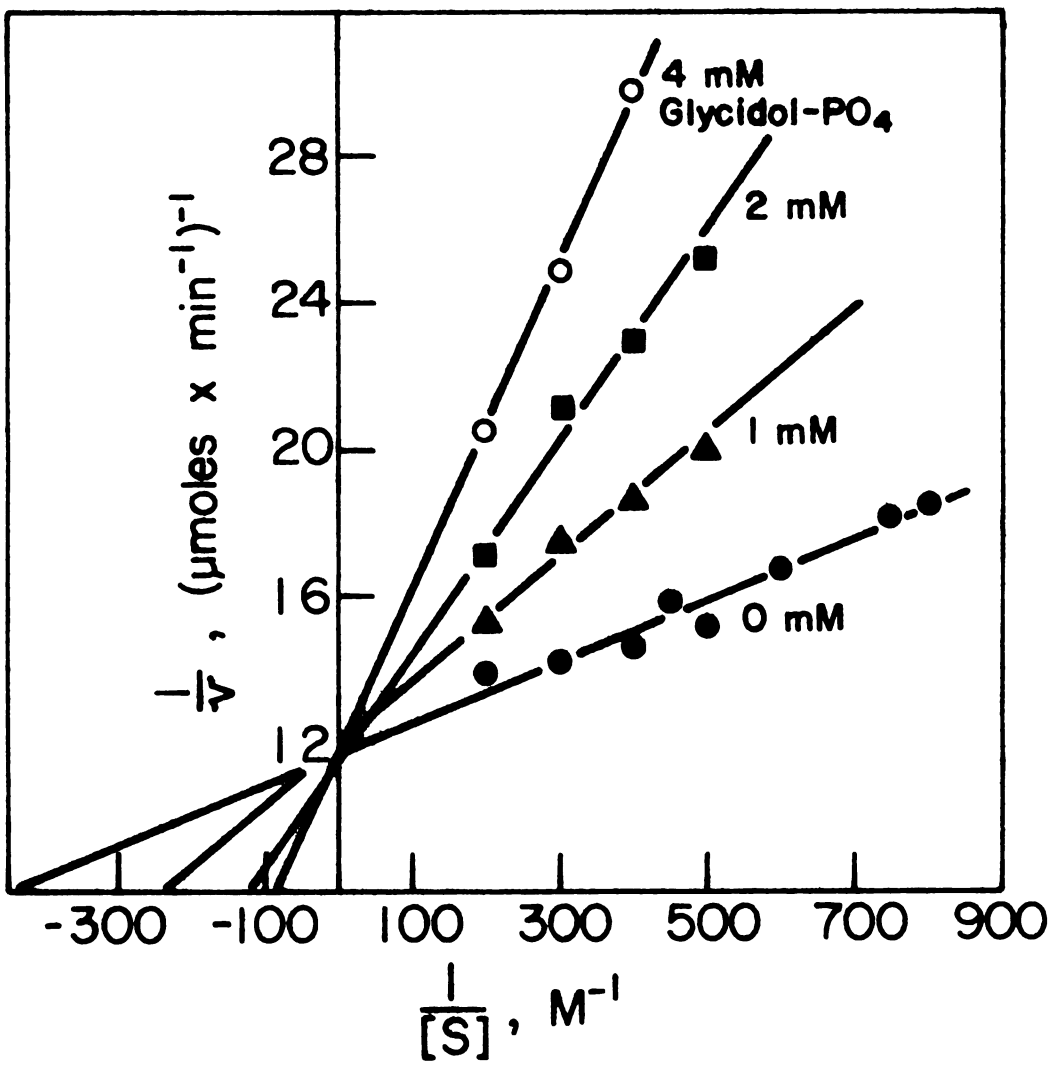
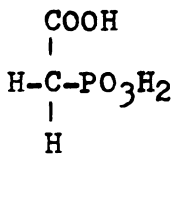


Figure 18. Lineweaver-Burk Plots of Glycidol-P Inhibition

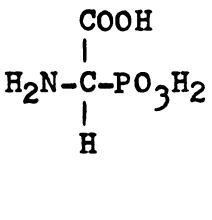
The enzyme (purified through the CM-Sephadex step) was assayed in 0.3 ml reaction volumes containing the indicated concentrations of 3-P-glycerate, 0.1 M sodium cacodylate buffer, pH 5.9, and 0, 1, 2 or 4 mM glycidol-P. The reaction time was 1 minute, terminated by the addition of 0.3 ml of 10% TCA. The amount of phosphate released was determined by method B.

analog aspartate, which is a major product of the photosynthetic C_4-dicarboxylic pathway in the same mesophyll cell with the phosphatase.



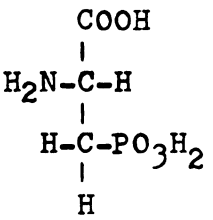
I

Phosphono-acetic acid



II

2-Amino phosphono-acetic acid



III

2-Amino, 3-phosphono-propionic acid

The enzyme did not hydrolyze the phosphonic acids to orthophosphate (Table IX). The phosphonates did not inhibit 3-P-glycerate phosphatase from sugarcane leaves. However the phosphonates at 1 mM final concentration stimulated enzymic hydrolysis of 3-P-glycerate, which was at a 10 mM concentration. In a more detailed experiment (Figure 19), the phosphonic acid stimulation became maximum as the amount of the 3-P-glycerate substrate approached a saturating concentration for the enzymatic hydrolysis. At low concentrations of 3-P-glycerate, stimulation did not occur. The significance of this stimulation is not known. The stimulation was reproducible but not great. Similarly aspartate, also at 1 mM concentration, stimulated (10%) the phosphatase (Table VIII).

Table IX. Hydrolysis of Phosphonic Acid Derivatives and Their Effect on 3-P-Glycerate Hydrolysis by 3-P-Glycerate Phosphatase

The phosphono compounds were added to the standard enzyme assay. The enzyme was a CM-Sephadex fraction whose specific activity was 489. Final concentrations are cited.

Substrates	Phosphonic acid derivatives					
	Phosphonoacetate		2-Amino Phosphonoacetate		2-Amino 3-Phosphonopropionate	
	μgP/min	% of control	μgP/min	% of control	μgP/min	% of control
Control (10 mM 3-PGA only)	204		204		204	
Phosphonic acid only (10 mM)	0		0		0	
Phosphonic acid, 10 mM + 10 mM 3-PGA	180	(88)	207	(101)	214	(105)
Phosphonic acid, 1 mM + 10 mM 3-PGA	248	(121)	234	(115)	230	(113)
Phosphonic acid, 0.1 mM + 10 mM 3-PGA	214	(105)	210	(103)	225	(110)

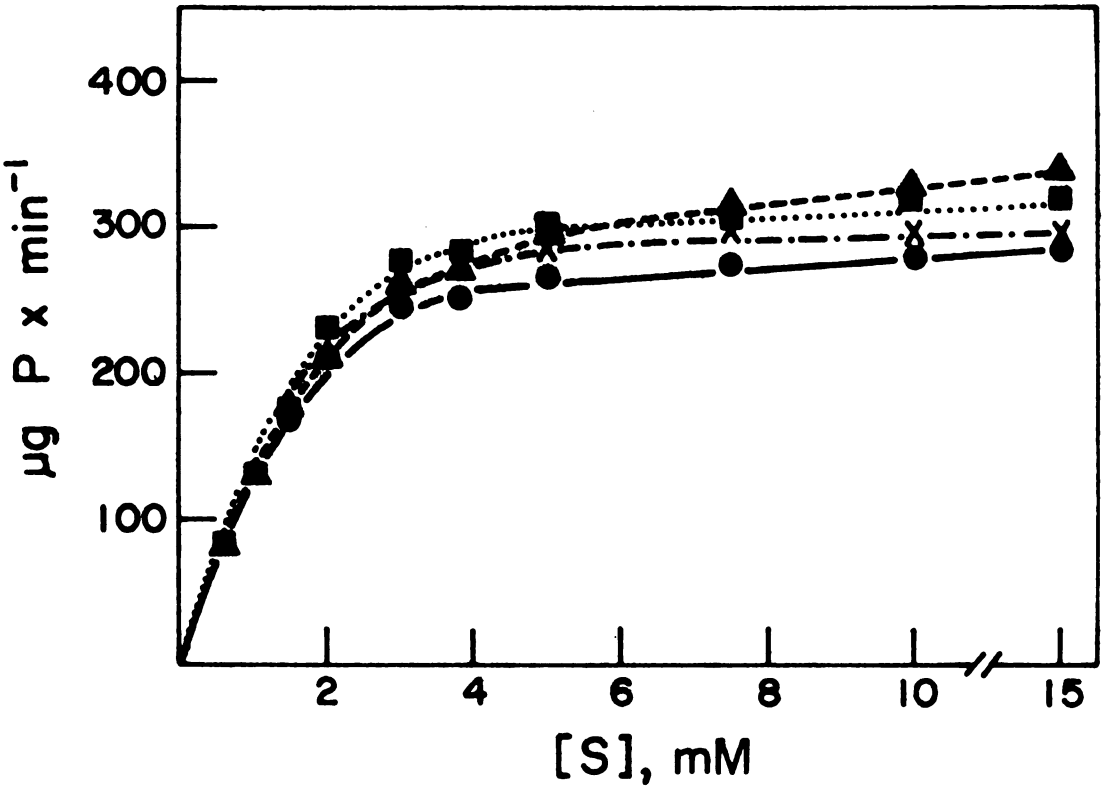


Figure 19. The Michaelis Curve for 3-P-Glycerate Phosphatase in the Presence of Phosphonic Acid Derivatives

The activity of 3-P-glycerate as a function of substrate concentration was determined using 0.3 ml reaction volumes containing 0.1 M sodium cacodylate buffer at pH 5.9, 2 mM of phosphonic acid derivative and indicated concentration of substrate. The amount of phosphate released was determined by method B.

●——● Control

▲---▲ Phosphonoacetate

■•••■ 2-amino-phosphonoacetate

X-·-X 2-amino,3-phosphonopropionate

Products of the Enzyme Reaction

Enzymatic hydrolysis of 3-P-glyceric acid with the 2530-fold purified phosphatase was complete and yielded stoichiometric amounts of glycerate and inorganic phosphate under the conditions described in Table X.

Table X. Stoichiometry of 3-P-Glycerate Phosphatase

The reaction mixture contained in 2 ml: 0.52 units of 3-P-glycerate phosphatase, 100 μmoles of sodium cacodylate buffer at pH 5.9, and 3-P-glycerate as indicated

Substrate	Hydrolysis Products			
3-P-Glycerate (μmoles)	Phosphate (μmoles)			Glycerate (μmoles)
	Method A	Method B	Method C	
1.0	0.80	0.95	1.05	1.30
2.0	1.85	1.80	2.02	2.15
3.0	3.20	2.90	3.21	3.30
4.0	4.17	3.85	3.84	4.10
6.0	6.30	5.95	6.50	6.00
8.0	7.85	8.00	7.82	7.90
10.0	10.00	9.45	10.40	10.10

In another experiment reaction mixtures of 2 ml, containing 20 μmoles 3-P-glycerate at pH 5.9, and 1.06 units of enzyme, were allowed to react for 0, 2, 4, 6, 8, and 10 minutes. The reactions were stopped by placing the tubes in a boiling water bath. The cooled samples were converted to

acid form by passage through 0.3 mm x 20 mm Dowex 50 (H^+) columns and aliquots were spotted on Whatman No. I paper. The chromatograms were developed and compounds located as described in Materials and Methods. The chromatograms showed that the hydrolysis products of 3-P-glycerate chromatographed with the R_f's of glycerate and orthophosphate, and that the amount of these products increased with increasing hydrolysis time.

III. Physical Characteristics

Sucrose Density Gradient Centrifugation*

To estimate the size of 3-P-glycerate phosphatase, sedimentation velocity experiments were run with sucrose density gradients. A sample of the purest enzyme (CM-Sephadex preparation) was layered on a 5-20% sucrose gradient prepared in 20 mM cacodylate buffer, pH 6.3, and 1 mM EDTA. Crystalline bovine catalase (Worthington) was used as a marker enzyme with a known $s_{20,w}$ value of 11.3 S for a molecular weight of 250,000 (155). Phosphatase and catalase were placed in separate tubes (Figure 20-A) and in the same tube (Figure 20-B). When run separately 3-P-glycerate phosphatase was distributed in a very broad peak with a maximum around 13.6 S. A significant portion of the enzyme had been pelleted to the bottom of the tube. About

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*The author would like to thank Dr. John Boezi for his assistance and counsel on this part of the investigation.

Figure 20. Sucrose Density Gradient Profiles

A. Samples (0.10 ml) of 3-P-glycerate phosphatase from CM-Sephadex fractionation in 0.02 M, sodium cacodylate, pH 6.3, buffer and 1 mM EDTA and crystalline bovine catalase in an identical buffer were layered on separate 4.8 ml, 5-20% sucrose gradients prepared in 0.02 M sodium cacodylate buffer, pH 6.3, and 1 mM EDTA. The gradients were centrifuged for 10 hours at 4° in a SW-39L rotor at 38,000 rpm in a Spinco Model L-2 centrifuge. Fractions of six drops were collected and the enzymes assayed by standard procedure.

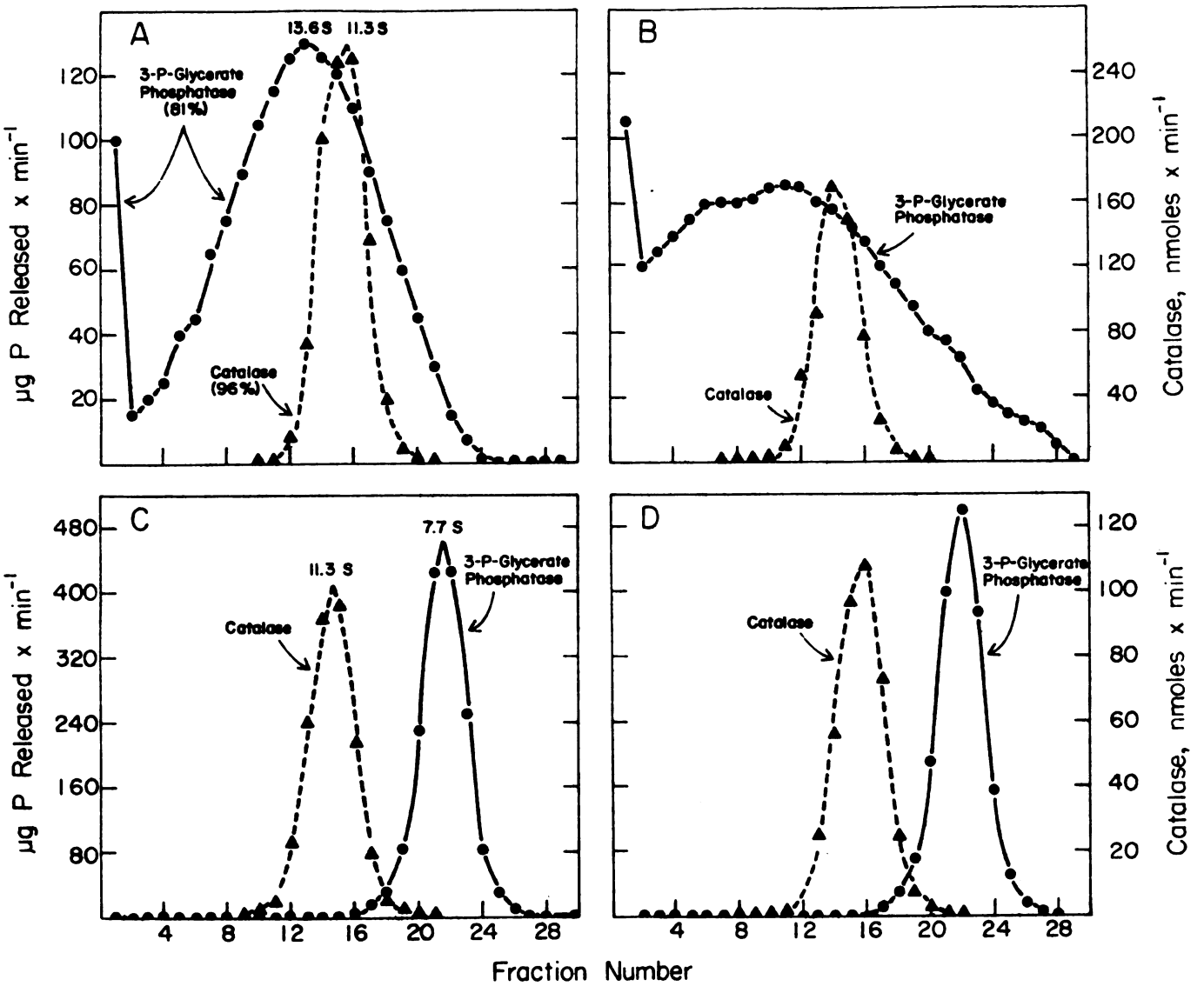
▲——▲ Catalase

●——● 3-P-Glycerate phosphatase

B. Same as A, only both phosphatase and catalase were placed on the same gradient.

C. A sample (0.10 ml) of the same 3-P-glycerate phosphatase in a solution of 0.1 M, sodium cacodylate buffer, pH 6.3, 1 mM EDTA and 0.1 M KCl and a sample (0.10 ml) of crystallize bovine catalase in identical buffer and salt were layered on 4.8 ml 5-20% sucrose gradients prepared in 0.02 M, sodium cacodylate buffer, pH 6.3, 1 mM EDTA and 0.25 M KCl. Centrifugation, sample collection and assay were similar to part A.

D. Same as C, only both enzymes were in the same sample and were placed on one gradient.


A
13.6 S
11.3 S
3-P-Glycerate Phosphatase (81%)
Catalase (96%)
B
3-P-Glycerate Phosphatase
Catalase
C
11.3 S
7.7 S
Catalase
3-P-Glycerate Phosphatase
D
Catalase
3-P-Glycerate Phosphatase
μg P Released x min⁻¹
Catalase, nmoles x min⁻¹
Fraction Number

81 percent of the phosphatase that had been applied was recovered in the gradient, and it is assumed that the rest of the activity (19%) was centrifuged to the bottom of the tube. When the phosphatase and catalase were on the same gradient, the phosphatase profile was smeared. It appears that something in the catalase preparation caused some manner of heterogeneity in the phosphatase. The catalase peak with the phosphatase was comparable to the catalase peak when run separately, and therefore no interaction of the phosphatase with the catalase was apparent. About 96 percent of the catalase activity was accounted for in both gradients.

The gradient profiles of the 3-P-glycerate phosphatase appeared characteristic of an aggregating system with a multiplicity of active forms. This suggested some sort of ionic interaction could be taking place and it was felt that high salt concentrations might be instrumental in overcoming the protein-protein interaction. Enzyme aliquots of the most pure CM-Sephadex fraction were placed in a solution of 0.1 M sodium cacodylate buffer, pH 6.3, 1 mM EDTA and 0.1 M in KCl and layered on a 5-20% sucrose gradient prepared in 20 mM sodium cacodylate buffer, pH 6.3, 1 mM EDTA and 0.25 M in KCl. The phosphatase and catalase were run on separate gradients (Figure 20-C) and they were run together on one gradient (Figure 20-D). Greater than 90 percent of the activity of both enzymes in both experiments was recovered in the peak fractions. In the presence of the

high salt condition, 3-P-glycerate phosphatase exhibited an $s_{20,w}$ value of about 8.0 S. This S value is characteristic of a molecular weight in the area of 160,000. This molecular weight is in agreement with the enzyme behavior on the G-200 gel filtration, where it had an elution volume to void volume ratio of 1.4:1 (137). However no aggregation was observed on the Sephadex G-200 column, suggesting that aggregation phenomenon is not just a function of slow ionic strength. The significance of the multiple forms, as suggested by the enzyme distribution in sucrose gradients, is not known but is of interest to note that all enzymes so far that are involved in regulation behave in this manner (138).

SDS-Polyacrylamide Electrophoresis

Thirty-five microgram samples of concentrated 3-P-glycerate phosphatase from the CM-Sephadex fraction were denatured and reduced by incubation at 37° for 3 hours in a solution of 1% sodium dodecylsulfate, 1% 2-mercaptoethanol and 0.1 M potassium phosphate buffer, pH 7.1. Samples of the denatured and reduced enzyme and appropriate standards treated identically were layered directly on 5% polyacrylamide gels and electrophoresed at 7.5 milliamps per gel for 3 hours and 55 min. The gels were fixed in 12% TCA and stained in 0.125% Coomassie brilliant blue (R-250) in 12% TCA. After the proteins were stained for 5 hours, the gels were destained in several changes of 12% TCA. A 550 nm scan

of the gel with 3-P-glycerate phosphatase revealed one major and one minor band of protein (Figure 21). The movement of the two phosphatase bands are shown in Figure 22 relative to the standard proteins of known molecular weight. The major polypeptide band of the phosphatase had an estimated molecular weight of 51,000 and the minor polypeptide band a molecular weight of 62,000. The relative amounts of the major and minor bands were calculated to be 176 to 49 by determining the areas under the peaks of the gel scan shown in Figure 21. The molar ratios were 4.3 to 1. It is unknown whether the two bands are both subunits of the phosphatase or whether one band is an impurity.

Polyacrylamide Gel Electrophoresis

Attempts were unsuccessful to electrophores concentrated 3-P-glycerate phosphatase (70 μg and 105 μg) from CM-Sephadex fractionation on 5% polyacrylamide gels using three buffer systems, β-alanine-glacial acetic acid, pH 4.3, Tris-glycine, pH 8.9 and Tris-barbitol pH 7.5. Coomassie brilliant blue was used to stain for protein but no bands were seen in the gels. Standard proteins were electrophoresed simultaneously with 3-P-glycerate phosphatase. These proteins migrated and were stained by the Coomassie blue procedure, confirming that the system was operational.

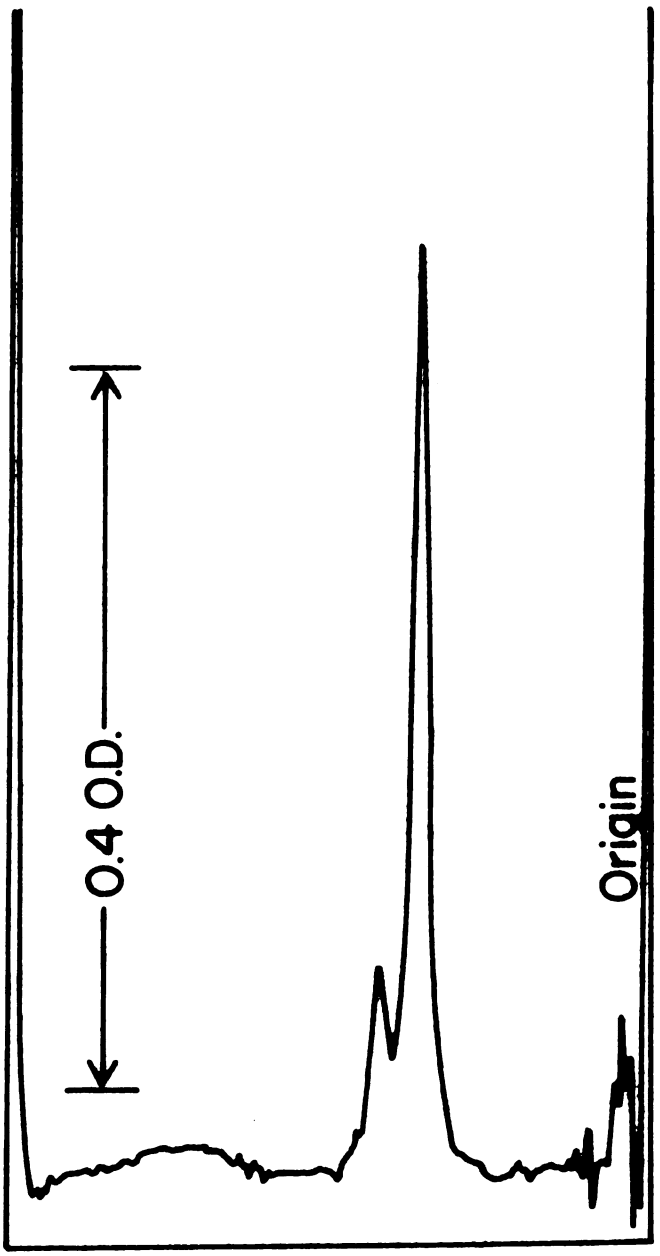


Figure 21. Gel Scan of SDS-Polyacrylamide Gel Electrophoresis of 3-P-Glycerate Phosphatase

Polypeptide chains were stained with Coomassie brilliant blue (R-250) and the gel scanned at 550 nm.

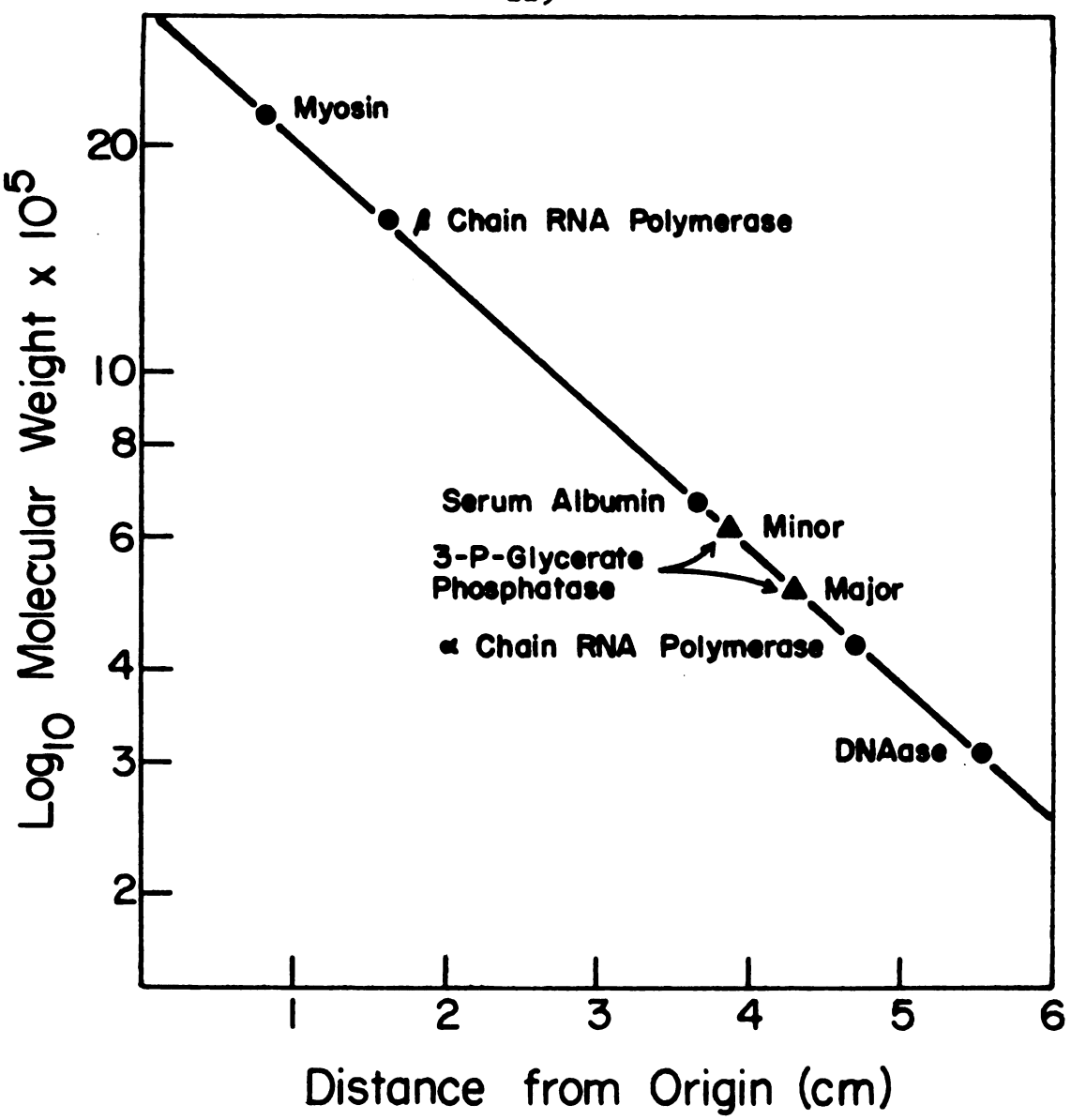


Figure 22. Molecular Weight of Polypeptide Chains of 3-P-Glycerate Phosphatase

The semi-log plot of molecular weight of standard proteins versus distance of migration from origin is shown. Standards of known molecular weight were myosin 220,000 (139); β-chain RNA polymerase 16,000 (140); bovine serum albumin 68,000 (141); α-chain RNA polymerase 42,000 (140); and DNase 31,000 (142). 3-P-Glycerate phosphatase sample was purified through CM-Sephadex and concentrated to give a specific activity of 740.

IV. Physiological Considerations of 3-Phosphoglycerate Phosphatase in Sugarcane Leaves

Location in the Sugarcane Plant

Activity for 3-P-glycerate phosphatase was located almost entirely in the leaves of 6 month old, greenhouse-grown sugarcane plants (Table XI). The tissue was extracted by the standard method for preparing crude extracts with a Waring Blendor and assayed immediately. Small amounts of activity in stems and roots may be from non-specific phosphatases. These results are consistent with the fact that P-glycolate phosphatase was also found only in green leaf tissue (23, 76).

Table XI. Distribution of 3-P-Glycerate Phosphatase in Sugarcane Plant

Samples of root, stem and leaf tissue (50 g) were extracted for 2 minutes at high speed in Waring Blendor with 200 ml 20 mM sodium cacodylate buffer, pH 6.3, 1 mM EDTA, 20 mM ascorbate and 2% Polyclar AT. The extract was filtered through 6 layers of cheese cloth before assaying by Method A.

Tissue	μmoles Phosphate Released x gm^{-1} Fresh Wt x Min^{-1}	Relative Distribution
Leaves	11.30	1000
Stem	0.50	44
Roots	0.15	13

Localization of the Sugarcane 3-P-Glycerate Phosphatase in Leaf Tissue

Sugarcane is an example of those plants with well developed parenchyma sheath cells containing chloroplasts similar to plastids in the mesophyll of a C_3-plant. In addition, in a sugarcane leaf, green mesophyll cells surround the bundle sheath cells, and in the mesophyll cells are located the key enzymes of the C_4-pathway of photosynthesis, and chloroplasts different from those in parenchyma sheath cells (16). In an investigation on the distribution of enzymes between the bundle sheath and mesophyll cells, 3-P-glycerate phosphatase was found to be predominately located in mesophyll cells and P-glycolate phosphatase in the bundle sheath cells. These results as well as the distribution of other related enzymes have been published (143) and a reprint is attached as Appendix A. In addition to this published work, additional experiments were done to attempt to determine if the phosphatase was in a subcellular organelle or in the cytoplasm.

In the plants such as sugarcane, the distribution of 3-P-glycerate phosphatase among the cell types and subcellular organelles is difficult to determine, because the grinding procedures necessary to crush these hard leaves also break most cells and particles indiscriminately. Methods for isolating whole, intact chloroplasts from these types of plants are only now under development. Baldry *et al.* (125) claim to have separated heavy, starch contain-

ing, chloroplasts of the bundle sheath cells (C_3-type of chloroplasts) from the lighter, (non-starch containing) chloroplasts of the mesophyll cells by density centrifugation of crude sugarcane extracts on 50% sucrose solution. Since these chloroplasts did not catalyze complete photosynthesis as $^{14}CO_2$ fixation in the light, retention of all the chloroplastic enzymes is in doubt. Using their method (Baldry et al., 125) sugarcane leaves, which had been exposed to 6 hours light to ensure adequate starch formation, were ground in a sorbitol medium and the chloroplasts isolated by fractional centrifugation and density centrifugation on 50% sucrose (Table XII).

Nearly all of the 3-P-glycerate phosphatase and P-glycolate phosphatase activities were in the soluble fraction. Further, when the chloroplast pellet was fractionated by density centrifugation on 50% sucrose, only 5% of the 3-P-glycerate phosphatase activity was found with non-starchy chloroplasts (interface) as compared to 18% of the P-glycolate phosphatase. The starchy chloroplasts (mainly bundle sheath chloroplasts) found in the density centrifugation pellet had less than 3% of the 3-P-glycerate phosphatase activity as compared to 31% of the P-glycolate phosphatase activity. These results exemplify the difficulties inherent in isolating subcellular particles from C_4-type plants. However the data strongly suggested that the 3-P-glycerate was a soluble or cytoplasmic enzyme. The retention of some P-glycolate activity by these chloroplasts is remarkable

Table XII. Distribution of 3-P-Glycerate and P-Glycolate Phosphatase in Isolated Sugarcane Chloroplasts

Triplicated samples (20 g each) of sugarcane leaves harvested after 6 hours of sunlight were ground with 300 ml of 0.33 M sorbitol, 0.02 M sodium cacodylate buffer, pH 6.5, and 1% $MgCl_2$ for 8 seconds at high speed in the Waring Blendor. The chloroplasts were pelleted by a 4000 x g, 1 minute centrifugation. The chloroplast pellets were resuspended in 10 ml of 0.33 M sorbitol, 4 mM EDTA, 2 mM $MnCl_2$, 2 mM $MgCl_2$, 50 mM cacodylate buffer, pH 6.5, 5 mM isoascorbate, 1 mM thioglycolate and 1% carbowax 4000. They were layered on 30 ml of 50% (w/w) sucrose in a glass centrifuge tube and centrifuged for 20 minutes at 1000 x g. The layers were separated and the chloroplasts diluted with suspension media and centrifuged at 4000 x g for 5 minutes. These pellets were resuspended in suspension medium. The phosphatase activities were determined by standard assay.

	3-P-Glycerate Phosphatase			P-Glycolate Phosphatase		
	Units x 10^3	S.A.	%	Units x 10^3	S.A.	%
Original extract	198	91	100	113.5	20	100
Supernatant	185	172	93	38.6	30	89
4000 x g pellet	4	34	2	.66	40.5	1.6
Density Centrifugation						
Supernatant	3.7	60	92.0*	.53	0.9	51*
Interface	.23	0.8	5.4	.19	0.6	18
Pellet	.11	40	2.6	.32	12.0	31

*Percent of activity recovered in sucrose density centrifugation process.

considering that this phosphatase is probably in the very tough bundle sheath cells (Appendix A), and this supports the conclusions that P-glycolate phosphatase is in or on the C_3-type chloroplasts, as observed with spinach leaves (Literature Review).

A second method for examining the localization of the 3-P-glycerate activity was by fractional centrifugation (Table XIII). The 3-P-glycerate phosphatase was found almost entirely in the soluble fraction and this distribution strongly supports the conclusion that the 3-P-glycerate phosphatase is a soluble, cytoplasmic enzyme. Only 4% of the P-glycolate phosphatase was in the chloroplast fraction isolated by these grinding and centrifugation procedures. This is consistent with observations that this phosphatase is readily solubilized from the chloroplasts (76).

Similar results were obtained when sugarcane leaves were ground in a medium used for the isolation of peroxisomes from leaf tissue by Tolbert *et al.* (25, 26). No significant amount of 3-P-glycerate phosphatase activity was found in any particulate fraction (Table XIV). Only 6.3% of the 3-P-glycerate activity was found in the fraction which contained 26% of the glycolate oxidase as a peroxisome marker. The percentage of the cytochrome *c* oxidase activity, which was a marker enzyme for mitochondria, was 3.6 times greater than the 3-P-glycerate activity in the mitochondrial fraction. Again there was no evidence to suggest that the sugarcane 3-P-glycerate phosphatase is particulate.

Table XIII. Fractional Centrifugation of an Isotonic Extraction of Sugarcane Leaf Tissue

Triplicated leaf samples (15 g) were passed through the serrated roller mill* to break both mesophyll and bundle sheath cells. The macerated tissue was extracted with a chloroplast isolation medium of Baldry et al. (125) containing 0.33 M sorbitol, 0.05 M sodium cacodylate buffer, pH 6.5 and 1% $MgCl_2$. The homogenates were filtered through 6 layers of cheese cloth and centrifuged as indicated. The phosphatases were assayed by standard procedure.

Fraction	3-P-Glycerate			P-Glycolate		
	Units x 10^3	S.A.	%	Units x 10^3	S.A.	%
Original extract	229.00	149	100	45.3	29	100
100 x g pellet, 10 min (nuclei)	1.13	26	0.5	0	--	0
11,000 x g pellet, 1 min (chloroplasts)	.66	1.2	0.3	1.8	31	4
25,000 x g pellet, 20 min (mitochondria)	3.02	13.9	1.3	0	--	0
120,000 x g, 2 hr (pellet)	.5	6.1	0.2	0	--	0
120,000 Supernatant	225.00	488	98.4	43.0	93	95

*For description of roller mill see Appendix A.

Table XIV. Distribution of 3-P-Glycerate Phosphatase after Fractional Centrifugation for Isolation of Peroxisomes

An 80 g sample of sugarcane leaves was ground in 200 ml 0.5 M sucrose in 0.02 M glycylglycine, pH 7.5, for 45 seconds at high speed in the Waring Blendor. The extract was squeezed through 8 layers of cheese cloth and centrifuged as described.

Fraction	3-P-Glycerate Phosphatase	Glycolate Oxidase	Cytochrome c Oxidase
	% of total	% of total	% of total
Original	100	100	100
80-100 x g (nuclei, whole chloroplasts)	1.2	0	-
6000 x g (broken chloroplasts, peroxisomes)	6.3	25.7	7.8
39,000 x g (mitochondria)	2.9	4.4	10.4
Supernatant	89.6	70.0	81.8

Localization of 3-P-Glycerate Phosphatase by Non-Aqueous Density Fractionation

Samples of destarched maize leaves which had been freeze-dried and fractionated non-aqueously by the method of Smillie (40) were obtained from Dr. J. J. Andrews. These dried, non-aqueous fractions were reconstituted in 5 ml of 20 mM sodium cacodylate buffer, pH 6.3, and 1 mM EDTA and the 3-P-glycerate phosphatase and P-glycolate phosphatase activities determined. The results of this experiment, along with some of the results from Dr. Andrew's investigations on these same samples are presented in Table XIV.

The fraction of density <1.30 is supposed to represent the chloroplasts with some also in the 1.30 to 1.33 fraction. The other 3 fractions are considered to be non-chloroplastic or cytoplasmic.

The 3-P-glycerate phosphatase activity does not correlate with chlorophyll or chloroplast marker enzymes. It correlates very well with the acid phosphatase distribution reported by Slack et al. who located an acid phosphatase in the cytoplasm (41). If the fractions of <1.30 to 1.33 are chloroplasts and 1.33 to >1.40 are cytoplasmic the cytoplasm has 89% of 3-P-glycerate phosphatase while 87% of P-glycolate phosphatase is with the chloroplasts. Our investigations (Appendix A) have shown that, in corn and sugarcane, 3-P-glycerate phosphatase and P-glycolate phosphatase are present in the mesophyll and bundle sheath cells respectively.

The P-glycolate phosphatase distribution (Table XV) again confirmed the conclusions that the enzyme was attached to or in the chloroplasts with the Calvin pathway.

The conclusion that 3-P-glycerate is a soluble, cytoplasmic enzyme was strongly supported by all four approaches to particle isolation. This conclusion also is in agreement with the fact that the pH optimum is below 7.0 as are most non-chloroplastic enzymes.

Development of the 3-P-Glycerate Phosphatase in Etiolated Sugarcane after Illumination

Nodes of sugarcane stalks were held for 21 days in the dark at 23° and in vermiculite moistened with Hoagland's

Table XV. Distribution of Chlorophyll and Enzymes after Non-Aqueous Isolation and Density Fractionation of Destarched Corn Leaves

Non-aqueous density fractions were reconstituted and assayed for enzyme activity. The total activity of each enzyme and the total chlorophyll contact are the sum of the individual fractions.

Fraction Density	Chlorophyll** (% of Total)	3-P-Glycerate Phosphatase*	P-Glycolate Phosphatase*	Malic Enzyme**[a]	NADP Malate Dehydrogenase**[b]
$\leq$ 1.30	63	3	53	55	50
1.30-1.33	6	8	34	16	14
1.33-1.36	6	9	10	12	9
1.36-1.40	16	30	0	12	22
> 1.40	9	50	4	6	6
Total activity µmoles/min	47 µg	0.08	0.06	0.18	0.22

*The amount of phosphate released was determined by Method B.

**Results of Dr. T. John Andrews.

[a]An enzyme associated with parenchyma sheath chloroplasts (Slack et al., 41).

[b]An enzyme associated with mesophyll chloroplasts (Slack et al., 41).

nutrient solution. Etiolated sugarcane "leaf" tissue grew from these nodes, and at the time of harvest the etiolated tissue of the poorly developed leaves was approximately 40 cm in height. Tissue samples from the top of the tissue spikes (3 g) were ground in a mortar and pestle with washed Ottawa sand, squeezed through 6 layers of cheese cloth and centrifuged at 14,000 x g for 10 minutes. 3-P-Glycerate phosphatase activity was determined over a 2 day period during exposure to 1800 foot candles of incandescent white light in the plant growth chamber (Figure 23). Some P-glycerate phosphatase activity was present in the etiolated tissue. This amounted to about 65 units per g fresh weight, whereas a green leaf of the same age contained about 620 units per g fresh weight. It is not known whether this activity of 11 or 12% of the level in normal green tissue represents non-specific phosphatases or is the same 3-P-glycerate phosphatase of the green leaf. After 24 hours of light a small amount of chlorophyll could be seen in the tissue, but it was too small to be measured after extraction. Between 12 and 24 hours of light, 3-P-glycerate phosphatase activity about doubled. After 47 hours of exposure to light, 3-P-glycerate phosphatase activity had increased at least 4-fold, and on a gram fresh weight basis it was about 46% of that in greenhouse tissue of the same age. The tissue was still very light green in color. During greening, the protein in the leaf tissue also increased in parallel with the phosphatase activity, and after 47 hours of light

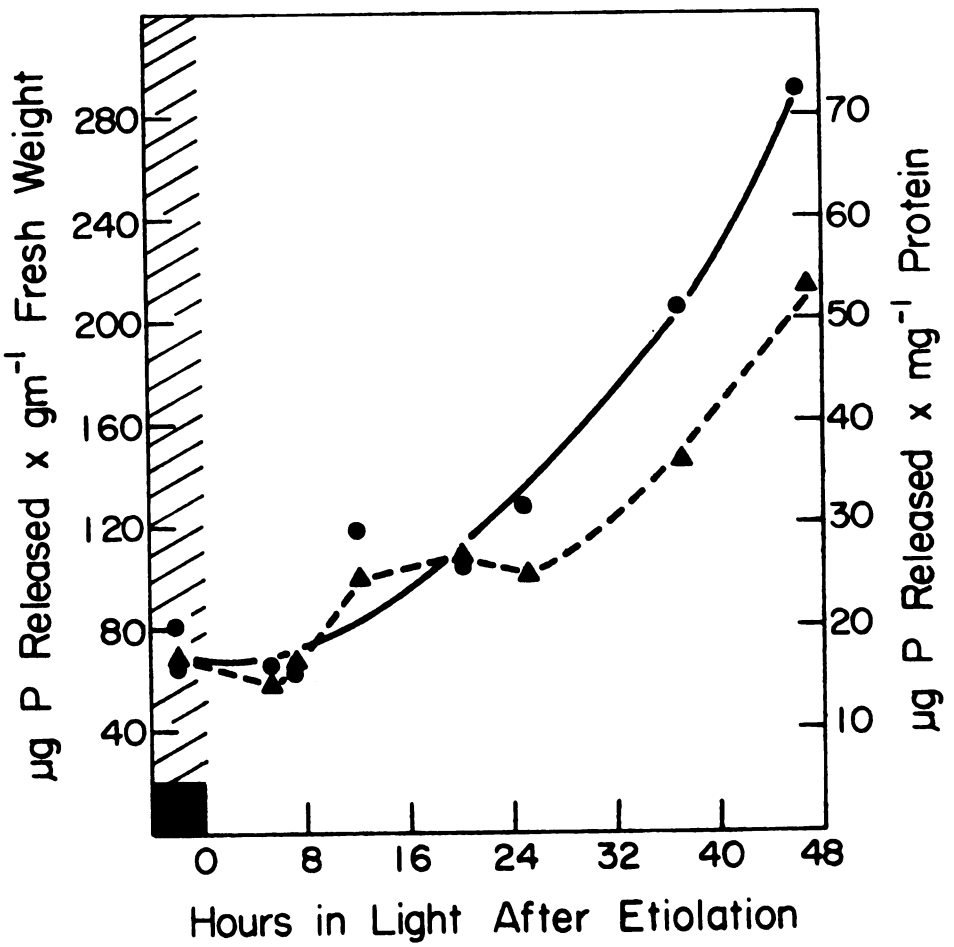


Figure 23. 3-P-Glycerate Phosphatase Development from Etiolated Sugarcane Tissue Upon Illumination

Samples (3 g) from etiolated sugarcane leaf tissue (21 days old) from stem nodes were extracted before and after exposure to 1800 foot candles of light. The 3-P-glycerate phosphatase activity is expressed on the basis of g fresh weight (●——●) and mg protein (▲---▲). Enzyme activity of greenhouse grown sugarcane of same age from same source of stem nodes was 620 units x g^{-1} fresh weight or 63 units x mg^{-1} protein.

there was 87% as much protein as in the comparable green leaf. It can be concluded that P-glycerate phosphatase develops in light and, therefore, possibly functions in a manner related to photosynthesis. This result is similar to the light catalyzed development of P-glycolate phosphatase in etiolated wheat leaves, which does not have 3-P-glycerate phosphatase activity (76), or the development of glycolate oxidase which is at a level of about 10% of normal in etiolated cereal leaves (75, 77).

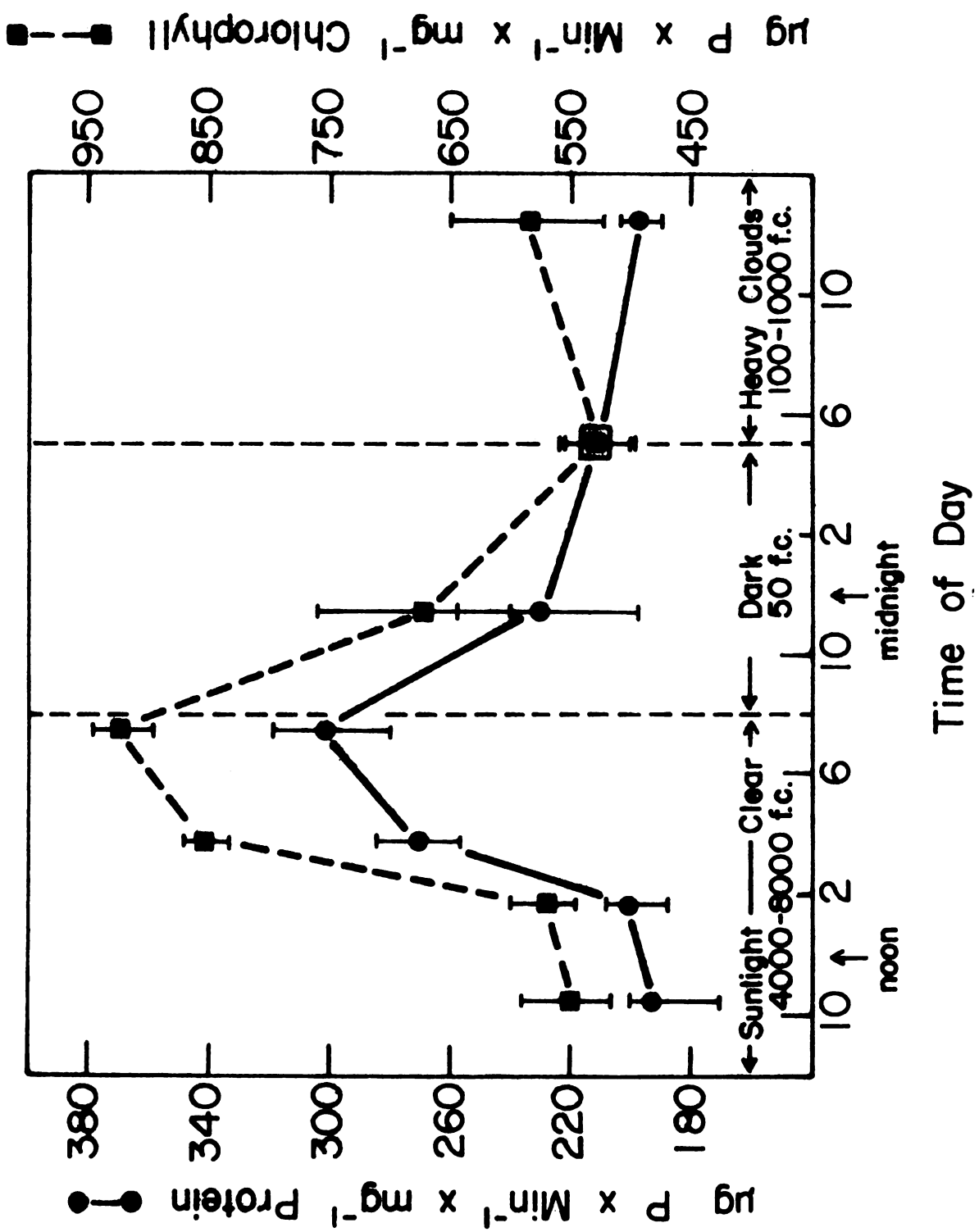
Diurnal Variation of the Enzyme Activity

A distinct diurnal variation in 3-P-glycerate phosphatase activity in sugarcane leaves was found when total activity was expressed either on a protein or chlorophyll basis (Figure 24). High sunlight intensity was required for this phenomenon, and when light intensities were reduced by heavy cloud cover, as on the second day of the experiment shown in Figure 24, 3-P-glycerate phosphatase activity did not increase. In these experiments the day preceding also had at least 8 hours of strong sunlight. 3-P-Glycerate phosphatase activity rose in the late afternoon, reaching a maximum in early darkness and then decreased by midnight. The maximum increase in late afternoon was about 50% on a protein basis and 60% on a chlorophyll basis.

Interpretations of these results will require more physiological experiments. Careful attention was taken to assure that the plants had sufficient water to prevent

Figure 24. Diurnal Variation of 3-P-Glycerate Phosphatase Activity

Triplicated leaf samples (5 g each) from greenhouse plants were extracted with 10-12 ml of 20 mM sodium cacodylate buffer, pH 6.3, and 1 mM EDTA by grinding in mortar and pestle with washed Ottawa sand. Results are plotted as μg P released x mg^{-1} chlorophyll x min^{-1} (■ --- ■) or μg P released x mg^{-1} protein x min^{-1} (●——●).


μg P x Min⁻¹ x mg⁻¹ Protein
μg P x Min⁻¹ x mg⁻¹ Chlorophyll
380
340
300
260
220
180
950
850
750
650
550
450
Sunlight — Clear → 4000-8000 f.c.
Dark 50 f.c.
Heavy Clouds → 100-1000 f.c.
10
noon
2
6
10
midnight
2
6
10
Time of Day

water stress, which would have occurred during middle of the day rather than at the end of the day. The diurnal cycle for photosynthesis would have risen in the morning, peaked before noon, decreased around noon to 1:00 pm and peaked a second time in early afternoon (144). P-Glycerate phosphatase activity began to increase when the photosynthetic rate was decreasing and in the evening when the least photosynthetic activity was expected the most phosphatase activity was found. The results suggest that the phosphatase may not be directly associated with CO_2 fixation, but possibly is involved in regulating starch formation (or breakdown). It is late in the day that starch formation may be occurring in mesophyll chloroplasts (62). Perhaps the phosphatase is an enzymatic mechanism for reducing the rate of CO_2 fixation or making it more efficient (no carbon through glycolate). There may also be a need based on permeability for the glycerate to be in a non-phosphorylated form. Certainly the hydrolysis of 3-P-glycerate, the first product of photosynthesis would drastically curtail the C_3-photosynthetic carbon cycle. However neither of these hypotheses is supported by the fact that the phosphatase is located in the mesophyll cells and the C_3-carbon cycle and the starch containing chloroplasts are located in the bundle sheath cells.

The diurnal variation in 3-P-glycerate phosphatase activity occurs in spite of the excellent stability of this enzyme in homogenates or when partially purified. These facts suggest that the regulation of the phosphatase activity

in vivo is probably by some effector rather than by protein turnover; but protein turnover cannot be ruled out with the present information.

Because of the diurnal variation in 3-P-glycerate phosphatase activity, plants, whenever possible, were harvested in the afternoon for enzyme preparations. My major professor was not sure that this was a valid excuse for not coming to work until noon, but he was unavailable for comment after midnight.

PART B:

THE DISTRIBUTION OF 3-P-GLYCERATE AND P-GLYCOLATE PHOSPHATASE IN VARIOUS PLANTS

In the Literature Review of this thesis is cited some of the evidence for similarities between the glycolate pathway, peroxisomes and photorespiration. Investigations to establish the extent of this correlation are underway in numerous laboratories. One of the approaches initiated by us in 1966 was to determine the relative amounts of P-glycolate phosphatase and 3-P-glycerate phosphatase activities in different leaf tissues. Since P-glycolate seems to be the precursor of glycolate (22), then P-glycolate phosphatase should be present in those cells or C_3-plants with the glycolate pathway, peroxisomes and CO_2-photorespiration. Likewise non-CO_2-photorespiring C_4-plants or cells would be expected to have less P-glycolate phosphatase and perhaps more 3-P-glycerate phosphatase, if it were substituting for the other phosphatase. This hypothesis was supported by our initial investigations with sugarcane (145). Sugarcane leaves had low levels of peroxisomal enzymes (Appendix A) and also relative low levels of P-glycolate phosphatase (Table XV). However we did find in sugarcane leaves a large amount of phosphatase activity towards 3-P-glycerate. These findings prompted the isolation, partial purification and characterization of 3-P-glycerate phosphatase from sugarcane as presented in Part A.

A survey of plants for the two phosphatases was based upon the hypothesis that other CO_2-photorespiring plants should have high levels of P-glycolate phosphatase activity relative to the levels of 3-P-glycerate phosphatase. Non-CO_2-photorespiring plants it was postulated would have high levels of 3-P-glycerate phosphatase and low levels of the P-glycolate phosphatase activity.* Another purpose of the survey was to determine the feasibility of using the relative levels of the two phosphatases as one criterion for distinguishing between CO_2-photorespiring and non-CO_2-photorespiring plants. We have been intrigued by the fact that glycerate is a major product of the glycolate pathway, but it can also be formed from enzymatic hydrolysis of 3-P-glycerate, a major photosynthesis product, essentially bypassing the glycolate pathway (Figure 2).

I. Special Materials and Methods

The plants used for the survey were all obtained locally in the greenhouse or from field plots. They were harvested during the middle part of the day and assayed within an hour of harvest. The washed, deribbed leaf tissue was diced into 2 x 2 cm pieces and homogenized at high speed in a Waring Blendor for 2 minutes in 4 to 5 volumes of

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*Initially the survey was aided by the diligent efforts of Dennis Gremel, an Honors College student at Michigan State University, who is now in graduate school in Biochemistry.

grinding medium at 4°. The grinding medium was 20 mM sodium cacodylate buffer, pH 6.3, and 1 mM EDTA. For some plants (where noted) 2% Polyclar AT and 20 mM ascorbate were added to protect against the oxidation of the phenolic compounds. The homogenate was filtered through 6 layers of cheese cloth and the phosphatase activities determined in the crude plant extract by Method A. For data in Experiment II, Table XVI, the residue remaining in the cheese cloth after the Waring Blendor homogenization was re-extracted by putting it repeatedly through a roller mill (described in Appendix A) while washing the residue as it was squeezed on the rollers until the chlorophyll was visually all extracted from the tissue. All enzyme assays were at least duplicated and most plants were examined using triplicated samples and extractions.

II. Results and Discussion

During part of the survey for the two phosphatases (Experiment I, Table XVI) only the extract from Waring Blendor homogenate of whole leaves were assayed. Later, from our own work (Appendix A) and from Björkman's (58), it was realized that in the C_4-plant the bundle sheath cells required vigorous grinding by mortar and pestle or roller mill for complete breakage. A differential grinding procedure was developed (Appendix A) employing first the Waring Blendor to break mainly mesophyll cells and then a roller mill extraction to break the remaining bundle sheath

Table XVI. The Levels of Activity and the Distribution of 3-P-Glycerate Phosphatase and P-glycolate Phosphatase in Various Higher Plants, Algae, and Liverwort.

The activities are presented as μg P released per minute per mg chlorophyll (S.A.). Experiment I uses only 2 minute Waring Blendor extraction. Experiment II is 2 minute Waring Blendor extraction followed by roller mill extraction of the cheese cloth residue of the Waring Blendor extraction.

Plants[+]	Experiment I			Experiment II									
	Waring Blendor Extraction			Waring Blendor Extraction					Roller Mill Extraction				
	3-PGA S.A.	P-glyc S.A.	3-PGA:P-glyc Ratio	3-PGA S.A.	3-PGA %	P-glyc S.A.	P-glyc %	3-PGA:P-glyc Ratio	3-PGA S.A.	3-PGA %	P-glyc S.A.	P-glyc %	3-PGA:P-glyc Ratio
WITH PHOTORESPIRATION													
Cotton	101	390	1/4	-	-	-	-	-	-	-	-	-	-
Tobacco[a]	24	160	1/6.7	72	(100)	324	100	1/4.5	0	(0)	0	(0)	-
Spinach	58	296	1/5	49	(96)	101	(89)	1/2	15	(4)	85	(11)	1/5.7
Sunflower	49	338	1/7	-	-	-	-	-	-	-	-	-	-
Wheat	36	304	1/8.4	36	(100)	415	(94)	1/11.5	0	(0)	40	(6)	0
Sugar beet	130	714	1/5 5	-	-	-	-	-	-	-	-	-	-
Alfalfa	62	545	1/8.8	-	-	-	-	-	-	-	-	-	-
Tomato	33	78	1/2.4	-	-	-	-	-	-	-	-	-	-
Soybean	157	293	1/1.9	-	-	-	-	-	-	-	-	-	-
Bean[a] (Sanalac)	292	115	2.5/1	300	100	300	100	1/1	0	(0)	0	(0)	-
Atriplex patula hastata	100	119	1/1.2	47	(88)	74	(87)	1/1.6	9	(12)	20	(13)	1/2.2

WITHOUT CO_2 PHOTORESPIRATION													
Sudan Grass	91	33	2.8/1	135	(86)	40	(77)	3.4/1	5	(14)	19	(23)	1/3.8
Sorghum	162	42	3.9/1	176	(85)	48	(73)	3.7/1	8	(15)	28	(27)	1/3.5
Sudum	38	15	2.5/1	86	(84)	30	(66)	2.9/1	7	(16)	12	(34)	1/1.7
Pigweed	169	95	1.8/1	-	-	-	-	-	-	-	-	-	-
Sugarcane	159	24	6.6/1	173	(80)	44	(50)	4.1/1	45	(20)	45	(50)	1/1
Corn	22	63	1/2.9	38	(81)	85	(60)	1/2.2	29	(19)	185	(40)	1/6.4
Atriplex rosea	160	36	2.9/1	132	(92)	56	(64)	2.4/1	21	(8)	42	(36)	1/2
Crab Grass	62	8	7.7/1	75	(62)	10	(27)	7.5/1	94	(38)	55	(73)	1.7/1
Bermuda grass	341	94		438	(82)	98	(66)	4.5/1	200	(18)	100	(34)	2/1
Poa grass	56	258	1/4.6										
Bent grass	600	123	4.9/1										
Marion blue grass	56	125	1/2.2										

[a] P-glycolate phosphatase is inactivated by long extraction time, see text.

* Percent of total activity (Waring Blendor extraction and roller mill extractions).

+ Scientific names of plants are in Appendix B.

Table XVI. Continued

Plants	Waring Blendor Extraction -- Experiment I		
	3-PGA S.A.	P-glyc S.A.	3-PGA/P-glyc Ratio
Aquatic Plants			
Elodea densa	46	142	1/3.1
Sagittaria	14	46	1/3.3
Algae			
Chlamydomonas reinhardtii	14	114	1/8.1
Ankistrodesmus braunii	15	34	1/2.2
Chlorella pyrenoidosa	7	117	1/16.7
Scenedesmus	12	207	1/17.2
Crassulacean Plants			
Kalanchoe vulcan[b]	15	26	1/1.7
Bryophyllum[b]	5	9	1/1.8
Sedum spectabile[b]	98	180	1/1.8
Liverwort			
Marchantia[b]	126	42	3/1
Trees			
White oak[b]	13	130	1/10
Red maple[b]	5	23	1/4.6
Mountain ash[b]	240	187	1.3/1
Cottonwood[b]	11	176	1/16
American elm[b]	32	87	1/2.7
Red spruce[b]	9	31	1/3.4

[b] 20 mM ascorbate and 2% (w/v) polyclar AT added to grinding medium.

cells (Experiment II, Table XVI).

The phosphatase specific activity is reported on a per mg chlorophyll basis, since we felt the plant's photosynthetic ability is more closely related to its chlorophyll content than protein. The data was summarized by grouping the plants in Table XVI according to their ability to photorespire or by their general type. The data is also summarized by the relative ratio of 3-P-glycerate phosphatase activity to P-glycolate phosphatase. In general C_3-plants known to have CO_2-photorespiration had very high P-glycolate phosphatase activities, 3 to 23 μmoles phosphate released per mg chlorophyll per min. Rates of CO_2 fixation probably and photophosphorylation rates range between 2 to 6 μmoles per mg chlorophyll per min. Thus in general the P-glycolate phosphatase activity considerably exceeds maximum photosynthetic rates. In these C_3-plants 3-P-glycerate phosphatase activity was 1/2 to 1/8 as much as the P-glycolate phosphatase. In a group of known C_4-plants without CO_2-photorespiration there was more 3-P-glycerate phosphatase and much less P-glycolate phosphatase, so that the 3-P-glycerate:P-glycolate phosphatase ratios in these plants were nearer 2/1 to 4/1. This approximate correlation was valid for the two well-studied Atriplex species (58). For A. patula with CO_2-photorespiration the 3-P-glycerate:P-glycolate ratio was 1/1.2 while for A. rosea without CO_2-photorespiration the ratio was 2.9/1. However the phosphatase activities in the C_3-bundle sheath cells of

A. rosea had a ratio of 1/2 favoring P-glycolate phosphatase as predicted by the peroxisomal activity in these C_3-cells within this C_4-plant.

Spinach and sunflower are good sources of stable peroxisomes (26) and contain high levels of P-glycolate phosphatase in comparison to the 3-P-glycerate phosphatase. Likewise wheat, tobacco and soybean contain high levels of glycolate pathway enzymes and high specific activity of P-glycolate phosphatase. In all of these C_3-plants the Waring Blendor adequately broke all the cells (Experiment II, in Table XVI) and no further release of enzyme occurred with the roller mill. In contrast, sugarcane, sorghum, sudan grass and pigweed which are low in peroxisomes and glycolate pathway enzymes (26), had low levels of P-glycolate phosphatase and relatively high levels of 3-P-glycerate phosphatase. In addition it was necessary to use the roller mill to break the bundle sheath cells to release much of the P-glycolate phosphatase activity.

From leaves of 15 soybean varieties, C_3-plants, the P-glycerate phosphatase to P-glycolate phosphatase ratio also varied as predicted from 1:2 to 1:5 (Figure 26). Bush beans (Phaseolus vulgaris, v. Sanalac) are characterized by having large amounts of both phosphatases. Beans appear to be an exception (Table XVI) to this hypothesis. In this bean leaf both phosphatases were equally as active, and 3-P-glycerate phosphatase activity was almost double the activity in sugarcane. The P-glycolate phosphatase was

almost double the activity in sugarcane. The P-glycolate phosphatase was partially inactivated by the routine long extraction time (Figure 25-A) which was employed to solubilize the phosphatase from corn or cane (Figure 25-C). After 30 seconds of grinding of Sanilac bean leaves the amount of both enzymes was about equal, and in Experiment II (Table XVI) both phosphatases had the same activity after 2 minutes. P-Glycolate phosphatase from tobacco leaves undergoes similar inactivation, but not to such a degree that the ratio is greatly affected. The extraction time versus percent activity plots (Figure 25) were done on a large portion of the plants examined, especially those that did not seem to follow the predicted pattern. Figure 25-B and C, were typical of the plots for other plants examined.

In extracts of bundle sheath cells of C_4-plants obtained by the roller mill, there was more P-glycolate phosphatase activity than 3-P-glycerate phosphatase. This distribution of the phosphatases is in complete agreement with the concept that enzymes for the glycolate pathway and the C_3-photosynthetic carbon cycle are located in the bundle sheath cells and the C_4-photosynthetic carbon cycle in the mesophyll cells (Table 1). It is from such results that it is postulated that plants which utilize the C_4-acid pathway will have higher levels of 3-P-glycerate phosphatase. Plants or cells utilizing the C_3- or Calvin cycle pathway will have higher levels of P-glycolate phosphatase activity.

Some plants have not been characterized by the plant

Figure 25. Extraction Time Versus Percent of the Phosphatase Activity Solubilized for 3 Types of Plants*

A. Bean (phaseolus vulgaris, V. Sanalac) showing inactivation of P-glycolate phosphatase (▲——▲) after 30 seconds of homogenization and 3-P-glycerate phosphatase (●——●) which was stable.

B. Typical extraction time versus percent activity solubilized from leaves of C_3-type plants (e.g., spinach), showing no inactivation of the phosphatase.

C. Typical extraction time versus percent activity solubilized from leaves of a C_4-plant (e.g., corn), showing no inactivation of enzyme but incomplete extraction.**

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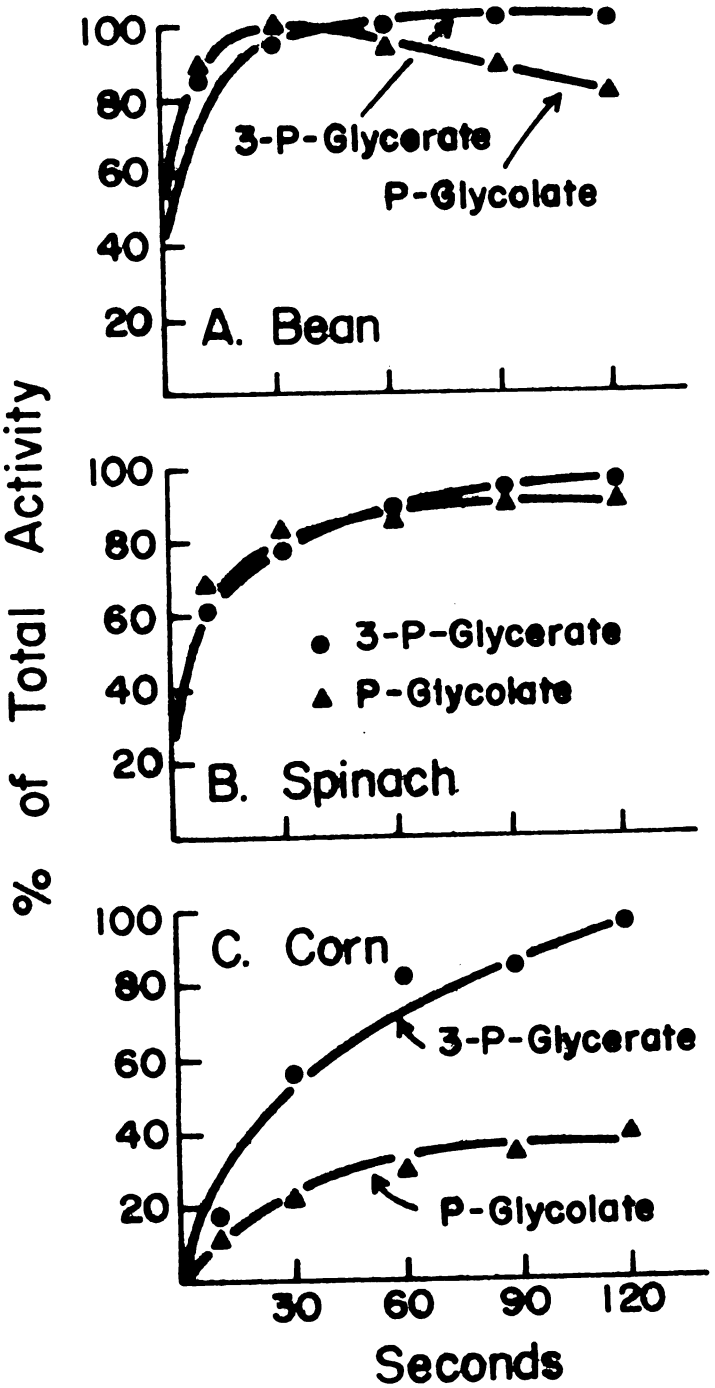
*All points are obtained by averaging duplicate assays of triplicated samples.

**Figure 7-B shows results of roller mill extraction after incomplete Waring Blendor extraction.


100
80
60
40
20
3-P-Glycerate
P-Glycolate
A. Bean
% of Total Activity
100
80
60
40
20
3-P-Glycerate
P-Glycolate
B. Spinach
100
80
60
40
20
C. Corn
3-P-Glycerate
P-Glycolate
30
60
90
120
Seconds

physiologists according to their manifestation of CO_2-photorespiration. Some of these plants are in a third group in Table XVI. According to their phosphatase activities Bermuda grass and Bent grass should not have CO_2-photorespiration. Indeed they are in sub-familier of Chlorideae and Agrostideae of which there are many examples of the C_4-pathway (57). On the other hand Poa and Merion bluegrasses with high P-glycolate phosphatase are of sub-families of the Festuceae which is generally considered to be comprised of C_3-pathway plants (57). The two aquatic plants examined had phosphatase activities of typical C_3-pathway plants.

The unicellular algae examined all actively biosynthesize glycolate and have high levels of P-glycolate phosphatase. These algae are considered non-photorespiring however, since they either excrete glycolate or metabolize it slowly by a glycolate dehydrogenase (146). The primitive, non-vascular liverwort (Marchantia) however had higher levels of 3-P-glycerate phosphatase. Other investigators in Tolbert's laboratory also find low levels of glycolate oxidase (no glycolate dehydrogenase) in this tissue (S. Wardell and N. E. Tolbert, unpublished). The crassulacean plants with their β-carboxylation system for acid accumulation might be thought of as C_4-plants, yet they appear to be C_3-pathway plants according to their relative phosphatase activities.

Among the C_4-plants without photorespiration, corn leaves was an exception to the above hypothesis. It had

relatively low levels of 3-P-glycerate phosphatase activity, and even the amount of extractable P-glycolate phosphatase was low. However the 3-P-glycerate phosphatase was located primarily in the C_4-mesophyll cells and the P-glycolate phosphatase in the C_3-bundle sheath cells (Appendix A). This distribution is consistent with the hypothesis. The reason for the lower levels of total 3-P-glycerate phosphatase activity is not understood.

Leaves of trees are grouped separately. The CO_2 compensation point of these leaves is as high as 150 ppm, and, as such, tree leaves (North American, temperate zone trees) are in general the most actively photorespiring leaf tissue known. As predicted, they have high levels of P-glycolate phosphatase and low levels of P-glycerate phosphatase. These values must be qualified by the difficulty in obtaining adequate amounts of active enzymes from homogenate of tree leaves. The tree leaves were ground in the presence of Polyclar AT in order to reduce the tanning action. These homogenates were still so full of tannic material that all particles and most of the protein were coagulated and enzyme assays could only be run on the small portion of the protein which is not precipitated. Because of this problem with tree leaves many enzymes, including the peroxisomal enzymes, cannot be detected in tree leaf extracts (Tolbert, unpublished). Likewise unknown and different amounts of the phosphatases may have been precipitated by the tannins and the levels reported in Table XVI can only

be considered as exploratory values.

The fact that high levels of P-glycolate phosphatase was present in plants which have high peroxisomal respiration (oxidation of glycolate in the light = photorespiration) supports the conclusion that P-glycolate is the immediate precursor of glycolate. The fact also that 3-P-glycerate phosphatase was present at relatively high levels (except corn) whenever the C_4-dicarboxylic acid pathway is present supports the conclusion that its presence is related to the C_4-pathway.

III. Relative Levels of 3-P-Glycerate and P-Glycolate Phosphatases as a Function of the Rate of Photosynthesis in Soybeans

The initial results of the survey of plants for the relative levels of 3-P-glycerate and P-glycolate phosphatases activities supported the conclusion that 3-P-glycerate phosphatase was the more active in C_4-plants (e.g., sugarcane, sorghum) which are considered more efficient or have a lower CO_2 compensation point than C_3-plants (Literature Review). In an attempt to apply this hypothesis it was postulated that the relative levels of the two phosphatases might be a criterion of photosynthetic efficiency or growth. Professor R. H. Hageman suggested to us that varieties of soybeans which had a range of known CO_2 fixation rates (147), would fit the requirements for such an experiment. Through the cooperation of Dr. R. H. Hageman of the Agronomy

Department and W. L. Ogren of the U.S.D.A. Regional Soybean Research Laboratory at the University of Illinois, Urbana, the activities of 3-P-glycerate phosphatase and P-glycolate phosphatase in 15 varieties of soybeans (Glycine max. L. Merrill) were determined. The soybean varieties had rates of photosynthesis from 14 to 24 μmoles CO_2 fixed per minute per square decimeter of leaf tissue (147). All of the varieties were planted at the same time in the same field plots. Three, 20 g leaf (7-9 leaves) samples of each of the varieties and 2 complete replications were used for each experiment. The activities of the phosphatases were determined in June on small plants (5-10 cm in height) and in July on large plants, just before the onset of flowering. The results of the July experiment are presented in Figure 26 in which the phosphatase activities are expressed on a per mg protein or chlorophyll basis. Although there is considerable scatter in the points, a regression analysis (Table XVII) of the data showed that they were significant at the 0.05 level.

Soybean varieties with increasing rates of CO_2 fixation had higher levels of P-glycolate phosphatase and lower levels of 3-P-glycerate phosphatase activities. The initial hypothesis was that plants with higher rates of CO_2 fixation would have relatively more 3-P-glycerate phosphatase as do sugarcane and sorghum while those plants with low CO_2 fixation rates would have more P-glycolate phosphatase, because considerable amounts of the CO_2 fixed would be respired by

Figure 26. The Activities of 3-P-Glycerate and P-Glycolate Phosphatases Versus Rate of Photosynthesis in Soybean Varieties

The averages are from 2 replications with triplicated samples for each of 15 varieties harvested in July experiment. The μmoles of CO_2 fixed per minute per square decimeter of leaf tissue are values from Curtis et al. (147). The data is significant at the 0.05 level. The soybean varieties are:

1. Seneca
2. Hawkeye 63
3. Lee
4. Kanrich
5. Wayne
6. Corsoy
7. Clark 63
8. Scott
9. Kent
10. Hark
11. Chippewa 64
12. Harosoy 63
13. Grant
14. Amsoy
15. Richland

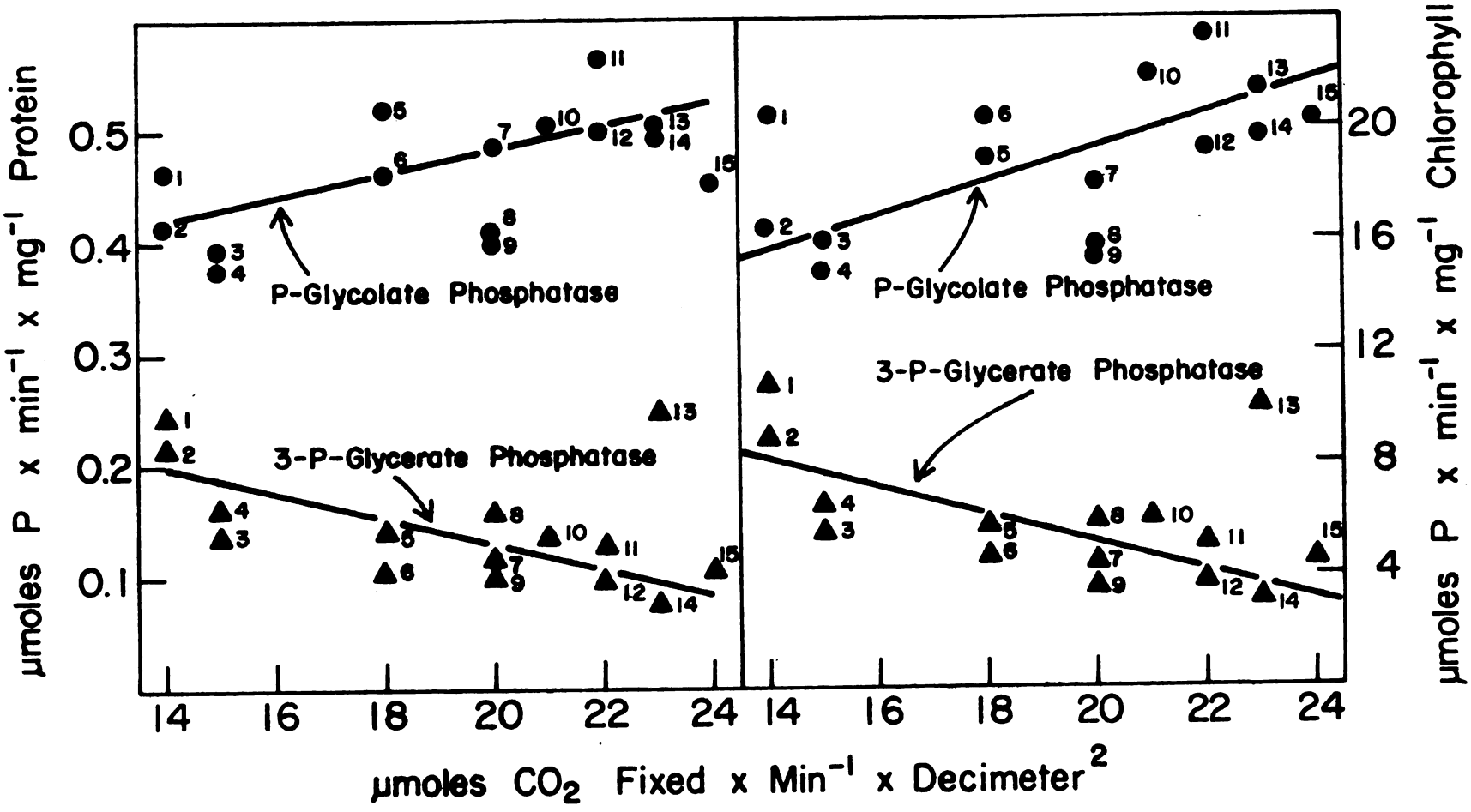

µmoles P x min⁻¹ x mg⁻¹ Protein
µmoles P x min⁻¹ x mg⁻¹ Chlorophyll
P-Glycolate Phosphatase
3-P-Glycerate Phosphatase
P-Glycolate Phosphatase
3-P-Glycerate Phosphatase
0.5
0.4
0.3
0.2
0.1
20
16
12
8
4
14
16
18
20
22
24
µmoles CO2 Fixed x Min⁻¹ x Decimeter²

Table XVII. Correlation of 3-P-Glycerate Phosphatase and P-Glycolate Phosphatase Activities with Photosynthesis in 15 Soybean Varieties[+]

	3-P-Glycerate Phosphatase				P-Glycolate Phosphatase			
	June		July		June		July	
	I	II	I	II	I	II	I	II
Prot. S.A.	-.733**	-.625*	-.483	-.363	.346	.203	.506*	.572*
Chl. S.A.	-.681**	-.522*	-.413	-.370	.383	.194	.538*	.413

	June Avg.	July Avg.	June Avg.	July Avg.
Prot. S.A.	-.702**	-.441	.285	.596*
Chl. S.A.	-.660**	-.399	.296	.494

	Overall Avg.	Overall Avg.
Prot. S.A.	-.580*	.532*
Chl. S. A.	-.512	.477

[+]Replications I and II were run with 3 samples each time in both June and July.

*Significant at .05 level (.514).

**Significant at .01 level (.641).

the flow of carbon through the glycolate pathway and peroxisomal respiration. The results were just the opposite.

There are some qualifications to the results which could have a considerable influence on the validity of the relationship indicated. First of all, the data of Curtis et al. (147) for the photosynthesis rates were considered to be absolute. However investigators at the University of Minnesota and Iowa State disagree with the rates of some of the varieties (R. H. Hageman, personal communication). Secondly, the rates that Curtis reported were from seedlings grown in a growth chamber whereas field grown plants were used for the phosphatase assays. Third, the genotypes used are quite variable, with regard to maturity types, growth requirements and grain composition. Certainly the metabolism must also vary with genotype. Considering these factors it is amazing that any relationship or correlation was found at all.

The initial hypothesis for this experiment had assumed that the higher the net rate of CO_2 fixation the more efficient would be the net photosynthetic growth and peroxisomal or glycolate metabolism would be less. The assumption was based upon the fact that glycerate, glycine, serine and C_1-moieties are all products of the glycolate pathway which starts with the hydrolysis of two P-glycolate (Figure 2). During this conversion of two P-glycolates molecules to one molecule of glycerate, considerable energy is lost and one CO_2 is respired. To make glycerate

by the hydrolysis of one 3-P-glycerate molecule is much more efficient, both from the standpoint of a carbon conservation and of phosphorylation. From the data in Figure 26 this assumption appears to be in error. The original hypothesis would have been reversed to accomodate this data to state that soybean plants with more P-glycolate phosphatase activity and presumably peroxisomal respiration are photosynthetically more active. If peroxisomal metabolism is essential for photosynthesis and polysaccharide synthesis, then increasing capacity for glycolate metabolism should coincide with increase rate of photosynthesis.

PART C:

A PARTICULATE 3-P-GLYCERATE PHOSPHATASE FROM SPINACH LEAVES

During my thesis research, isolation and characterization of leaf peroxisomes has been in progress by others in the laboratory (25, 26, 82). The metabolic sequence of the glycolate pathway associated with the peroxisomes begins with glycolate and ends with glycerate (Figure 2). Consequently the cellular location of P-glycolate and 3-P-glycerate phosphatase activities have been of particular concern to the peroxisomal investigations. Neither phosphatase was found in the peroxisomes, but rather P-glycolate phosphatase is located in the chloroplast and part of the 3-P-glycerate phosphatase in an apparent starch grain from spinach leaf extracts.

P-Glycolate Phosphatase in Chloroplasts

P-Glycolate phosphatase was first reported to be associated with the chloroplasts of spinach by Yu *et al.* (76). Thompson and Whittingham (70) confirmed by non-aqueous density gradient techniques the location of the P-glycolate phosphatase with the chloroplasts (89-97%). Using an aqueous sorbitol medium we also concluded that the enzyme is with chloroplasts (Table XVIII) but that the 3-P-glycerate phosphatase did not appear to be with the chloroplasts. The recovery of only 12% of the P-glycolate phosphatase in the chloroplast fraction indicated that the

enzyme was loosely associated with the plastids. However the advantage of sorbitol medium over NaCl or sucrose isolation medium was that most of the P-glycolate phosphatase activity with the chloroplast fraction remained with the chloroplasts upon repeated washings which completely removed other glycolate metabolizing enzymes.

Table XVIII. Phosphatase Activity of Isolated Spinach Chloroplasts Prepared in Sorbitol Medium as Described in Materials and Methods

Fraction	P-Glycolate Phosphatase		3-P-Glycerate Phosphatase	
	Units	%	Units	%
Original extraction	11,350	100	2,480	100
Supernatant	8,580	77	1,080	43
Chloroplasts	1,400	12 (100)*	45	2
1x Washed chloroplasts	1,300	9.1 (74)	Trace	
2x Washed chloroplasts	950	8.4 (68)	Trace	
3x Washed chloroplasts	916**	8.1 (66)	Trace	

*Values in parenthesis are percent of enzyme in chloroplast fraction after the first centrifugation.

**Equivalent to 39.6 μmoles of phosphate released x $minute^{-1}$ x mg^{-1} chlorophyll.

3-P-Glycerate Phosphatase in Starch Pellet

During the isolation and characterization of the spinach leaf peroxisomes from the 6000 x g fraction by Tolbert et al. (25), the various fractions from the isopycnic sucrose density gradients were assayed to determine the activities of the 3-P-glycerate and P-glycolate phosphatases. Neither phosphatase was found in the peroxisomal, mitochondrial, or chloroplast fractions under these conditions. However as much as 38% of the 3-P-glycerate phosphatase activity was in the 6000 x g pellet (Table XIX). About half of this activity was apparently soluble enzyme and remained on the top of the discontinuous sucrose gradient. The other half of the activity pelletted to the bottom of the sucrose gradient through 2.3 to 2.5 M sucrose. Microscopic examination of the small pellet indicated that the major constituent was small starch-like grains, although the pellet was somewhat gray in contrast to the usual white expected from starch. The fact that it gave a blue-purple stain with iodine and KI agrees with the idea that the only logical particle which could pass through such high density sucrose would be starch or a similar polysaccharide. Thus the particle has been termed the starch particle and the pellet, the starch pellet. The few whole cells, cell fragments and broken chloroplasts that were present in the pellet should not have been able to sediment through the 2.5 M sucrose and probably moved down the side of the centrifuge tube. The nature and composition of the starch

pellet and the 3-P-glycerate phosphatase with it were subjected to further investigation because the phosphatase activity in the pellet appeared to be a new discovery.

Table XIX. Distribution of 3-P-Glycerate Phosphatase in Peroxisome Preparation

The peroxisomes were prepared by the method of Tolbert et al. (25) from 300 grams of Longstanding Bloomsdale spinach. The gradient pellet was resuspended in 0.8 M sucrose containing 20 mM sodium cacodylate buffer, pH 6.3, and 1 mM EDTA.

Fraction	3-P-Glycerate Phosphatase			
	Units	% of Total	% of 6000 x g Pellet	S.A.
Original	7,280	100	--	0.027
6000 x g fraction	2,850	38	100	0.265
All gradient fractions	Trace	--	--	--
Gradient supernatant	1,200	18.5	42	--
Gradient pellet	1,370	18.4	48	1.280

The stability and storage characteristics of the 3-P-glycerate phosphatase of the starch pellet were very good. The pellets from the preparation of peroxisomes on sucrose gradients have been stored frozen for as long as 3 months before resuspending without loss of 3-P-glycerate phosphatase activity. The resuspended particulate enzyme was stable at 4° until microbial growth started (several weeks) and the frozen suspensions were stable indefinitely. Preparations

of the solubilized phosphatase or partially purified enzyme from the starch particles were stable and could be stored frozen at -18°.

pH Optimum

The pH activity curve for the 3-P-glycerate phosphatase in the spinach starch particles had a sharp optimum around pH 5.8 (Figure 27). The addition of Mg^{++} did not effect the optimum or the activity in any way. The pH optimum is very similar to that of the soluble sugarcane 3-P-glycerate phosphatase (pH 5.9). The enzyme was stable at 4° at pH 6.3 or 7.0.

Kinetics of Enzyme

The particulate 3-P-glycerate phosphatase activity was a linear function of enzyme concentration (Figure 28). The particulate enzyme, the dialyzed particulate enzyme and acetone precipitated enzyme from the particles all showed a linear function with enzyme concentration. Normal enzyme saturation kinetics were obtained with the particulate enzyme (Figure 29-A) and the apparent K_m for 3-P-glycerate was 9×10^{-4} M (Figure 29-B).

Effect of Divalent Cations

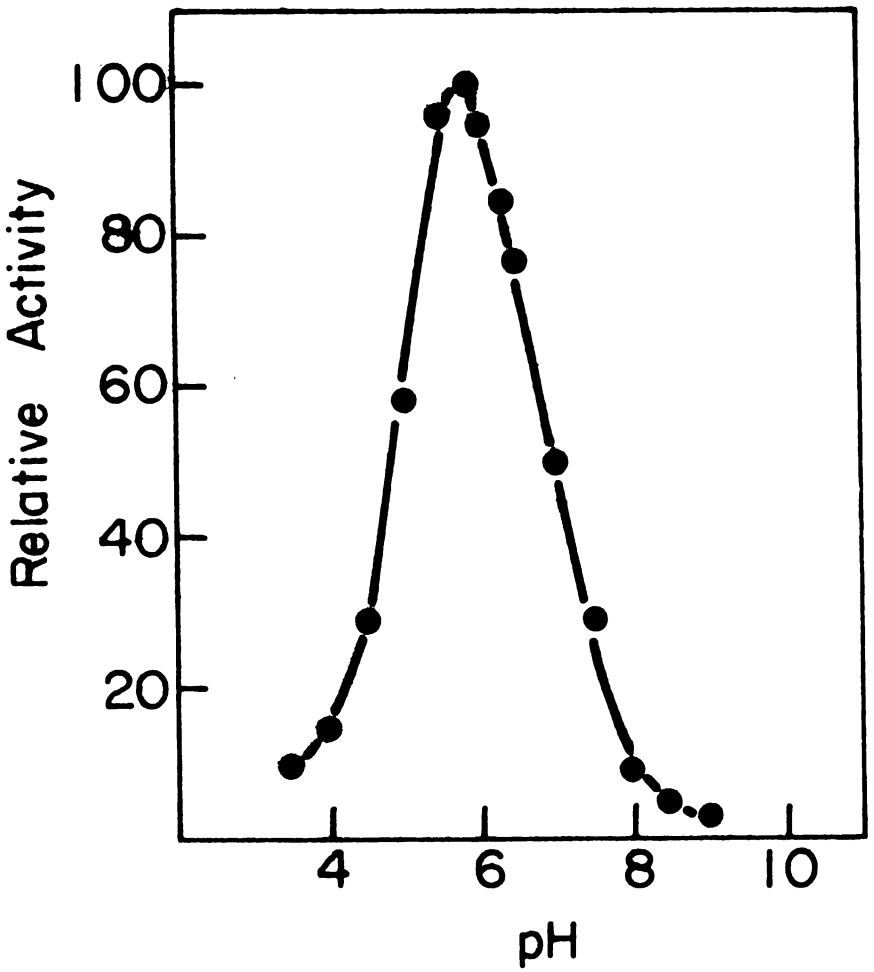
The 3-P-glycerate phosphatase in the starch particle did not show any requirement for a divalent cation (Table XX). The addition of 1 mM EDTA or dialysis against 20 volumes of 1 mM EDTA did not inhibit the enzyme activity,

Figure 27. The pH Activity Curve for 3-P-Glycerate Phosphatase in Starch Particles

The enzyme activity was assayed in the particulate form and in 3 buffer systems: 0.1 M sodium cacodylate + 0.1 M sodium acetate; 0.2 M sodium cacodylate; 0.1 M sodium cacodylate + 0.1 M glycylglycine. The activities in the different buffers were averaged for the overlapping points.

Figure 28. Linearity of the 3-P-Glycerate Phosphatase Assay

Increasing amounts of the enzyme in 3 forms were assayed. The particulate enzyme (●——●) was suspended in 0.8 M sucrose, 1 mM EDTA and 20 mM sodium cacodylate buffer at pH 6.3. The particulate enzyme was dialyzed against 20 volumes of 20 mM sodium cacodylate and 1 mM EDTA for 24 hours and then assayed (▲——▲). The acetone fractionated enzyme (■——■) had been solubilized by sonification of the starch particles and precipitated by acetone as described in the text.


Relative Activity
100
80
60
40
20
4
6
8
10
pH

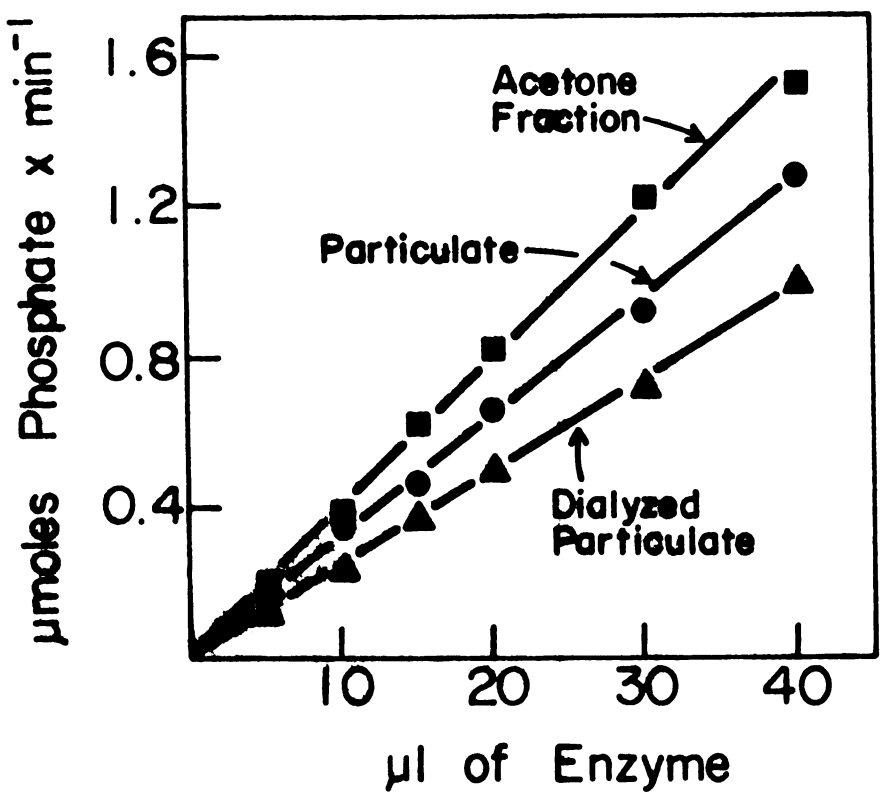
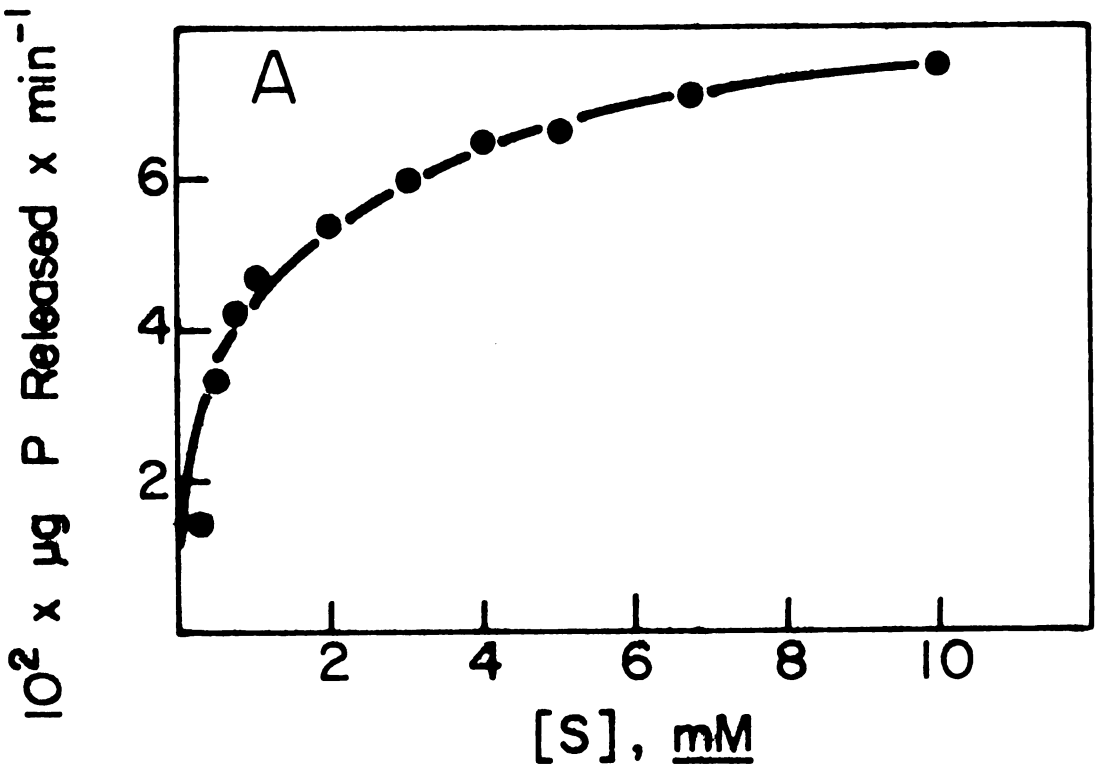

µmoles Phosphate x min⁻¹
1.6
1.2
0.8
0.4
Acetone Fraction
Particulate
Dialyzed Particulate
10
20
30
40
µl of Enzyme

Figure 29. Kinetic Plots for the Spinach Particulate 3-P-Glycerate Phosphatase

A. The initial velocity as a function of substrate concentration was measured in 0.3 ml reaction mixtures containing 0.1 ml substrate of indicated concentration, 0.15 ml 0.2 M sodium cacodylate, pH 5.9 and 0.05 ml of suspended enzyme. The reaction was terminated after 1 minute with 0.3 ml 10% TCA. The phosphate was determined by method B.

B. A Lineweaver-Burke plot of initial velocity versus substrate concentration. The apparent K_m is about 9×10^{-4} M for the particulate enzyme.


A
10^2 x µg P Released x min^{-1}
2
4
6
2
4
6
8
10
[S], mM

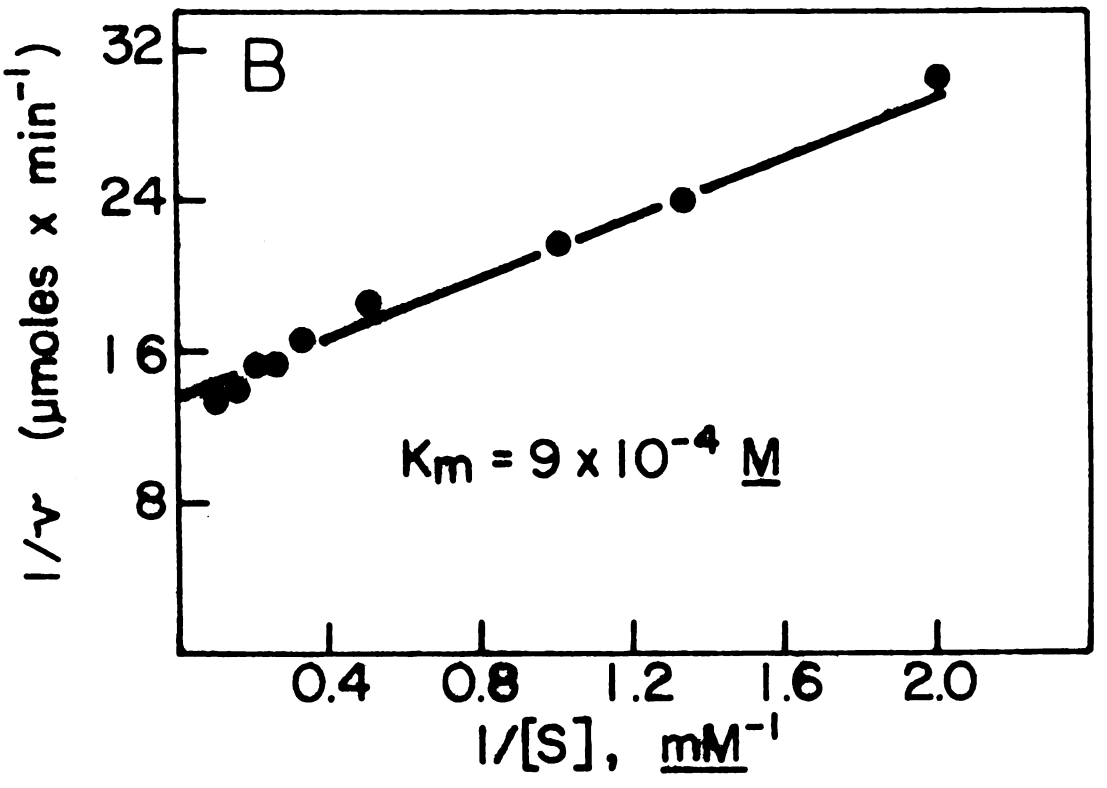

B
1/v (µmoles x min^{-1})
32
24
16
8
$K_m = 9 \times 10^{-4}$ M
0.4
0.8
1.2
1.6
2.0
1/[S], mM^{-1}

Table XX. The Effect of Divalent Cations on the 3-P-Glycerate Phosphatase in Starch Particles

Aliquots of the particles which had been dialyzed against 1 mM EDTA were equilibrated at 30° for 15 minutes in 0.5 ml of a solution containing 0.13 M sodium cacodylate buffer, pH 5.9, with 5 mM or 0.5 mM cation. The reaction was initiated by the addition of 0.25 ml substrate (10 μmoles), ran for 10 minutes at 30° and was terminated by the addition of 0.25 ml of 10% TCA.

Addition	Relative Activity	
	5×10^{-3} M Cation	5×10^{-4} M Cation
Control	100	100
Control + EDTA	102	100
$ZnSO_4$	5	15
$CoSO_4$	78	85
$CuSO_4$	14	45
$Pb(Acetate)_2$	104	99
$CaCl_2$	98	101
$MnCl_2$	102	101
$MgSO_4$	104	101
$NiSO_4$	80	101

but, in fact, increased the activity about 10%. As observed for the soluble sugarcane 3-P-glycerate phosphatase, Zn^{++} was inhibitory at 1 mM and Cu^{++} also caused considerable inhibition, greater than 50% even at 0.5 mM.

Substrate Specificity

The relative specificity of the starch particle 3-P-glycerate phosphatase (Table XXI) was similar to that for the soluble phosphatase from sugarcane leaves. The substrates were also assayed in the presence of 1 mM $MgSO_4$, with no effect on the hydrolysis of 3-P-glycerate and mixed effects on the other substrates. The partially purified particulate enzyme was not specific for 3-P-glycerate, but it is probably the preferred physiological substrate. Since there was a possibility that the nuclei of the cells may have been part of the particles, the nucleotidase and diesterase activities were investigated, but no significant activity was found by the assays used. However, the particulate enzyme may not be completely free of other phosphatase activity.

Solubilizing the Particulate 3-P-Glycerate Phosphatase

In order to adequately resuspend the starch particles, it was necessary to use approximately 0.8 M sucrose and buffer. When the particles were suspended in buffer alone and sonicated for 20 minutes, only 50% of the 3-P-glycerate phosphatase was solubilized to the extent that it was not pelleted by a 14,000 x g, 20 minute centrifugation. However

Table XXI. Substrate Specificity of the Starch Particle 3-P-Glycerate Phosphatase

Ten μmoles (13.3 mM) of all substrates were used in the assays. The Mg^{++} was equilibrated 15 minutes with the enzyme before the reaction was initiated with substrate. The enzyme was used in the resuspended particulate form.

Substrate	Relative Activity	
	- Mg^{++}	+ Mg^{++}
3-P-Glycerate (control)	100	100
P-Glycolate	4	5
2-P-Glycerate	14	15
Glucose-6-phosphate	47	40
Fructose-1,6-diphosphate	67	66
β-Glycerol phosphate	50	56
p-Nitrophenylphosphate	96	112
bis-p-Nitrophenylphosphate	9	8
Adenosine triphosphate	70	52
Adenosine diphosphate	67	62
5'-Adenylic acid	60	59
5'-Deoxyadenylic acid	4	4
3'-Adenylic acid	30	32
3'-Deoxyadenylic acid	3	8

if the starch pellet was suspended in 0.8 M to 1.3 M sucrose, 90 to 94% of the phosphatase was solubilized when sonicated. Homogenization in a glass Potter Elvejehm homogenizer did not release the enzyme from the particles, nor did repeated passage through a French pressure cell. Repeated freezing and thawing did not solubilize the enzyme. Dialysis of the suspended particles against 20 volumes of 20 mM sodium cacodylate buffer, pH 6.3, and 1 mM EDTA did not release the enzyme but did result in about a 10% increase in the activity. These procedures succeeded in breaking all whole cells and chloroplasts in the suspension. Limited solubilization of the phosphatase from the starch grains seemed to be favored at pH values above 7 (Figure 30). At pH 7.0 to 7.5 a maximum of 36% of the enzyme was released from the particles. Above pH 7.5 the stability of the particulate phosphatase began to decrease with a parallel decrease in the activity that was solubilized.

Incubation of the starch particle suspension with β-amylase at 4° in sodium acetate buffer at pH 5.5 resulted in solubilization of the 3-P-glycerate phosphatase and the concomitant release of increasing amounts of reducing sugar (Figure 31). These results tend to support the idea that the particle contains some form of starch or similar polysaccharide. The reason for loss of activity at 48 hours is not known.

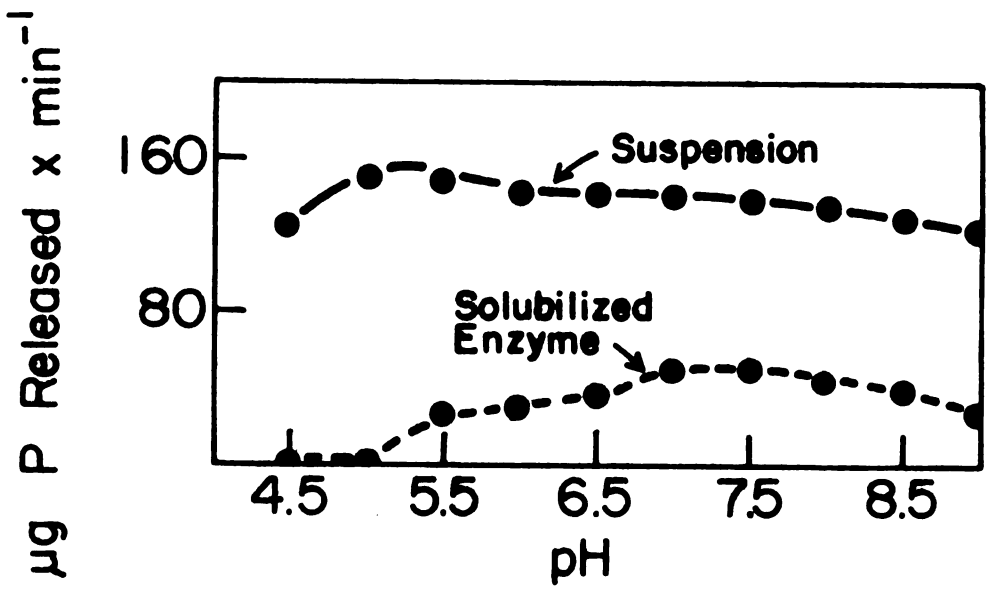
The effect of salt concentration on the solubilization of the particulate (starch grain) 3-P-glycerate phos-

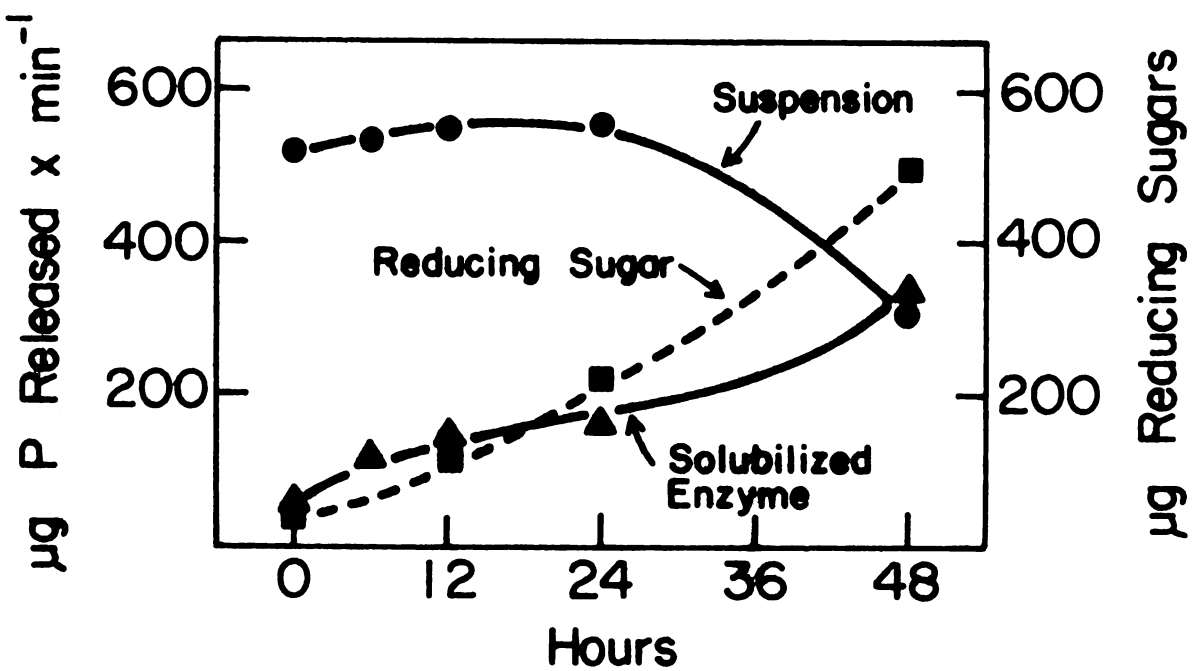
Figure 30. Solubilization of the Starch Grain 3-P-Glycerate Phosphatase as a Function of pH

The resuspended particulate enzyme was diluted 5 fold into 0.2 M buffers (cacodylate, acetate or glycylglycine) of the indicated pH and incubated for 1 hour at 4°. The particle suspension (●——●) and the supernatnat (●---●) of 10,000 x g for 20 minutes centrifugation of the suspension were assayed.

Figure 31. Solubilization of the Starch Grain 3-P-Glycerate Phosphatase by β-Amylase

Samples (0.5 ml) of the particulate 3-P-glycerate phosphatase were incubated in 50 mM acetate buffer, pH 5.5, with 700 units of β-amylase (Worthington) at 4° for a 48 hour period. The phosphatase activity of the resuspended particles (●——●) and the supernatant (▲——▲) after 10,000 x g for 20 minutes centrifugation of the suspension were assayed. Reducing sugar (■---■) was determined by Nelson's test (120).


µg P Released x min⁻¹
160
80
Suspension
Solubilized Enzyme
4.5
5.5
6.5
7.5
8.5
pH


µg P Released x min⁻¹
600
400
200
Suspension
Reducing Sugar
Solubilized Enzyme
0
12
24
36
48
Hours
µg Reducing Sugars

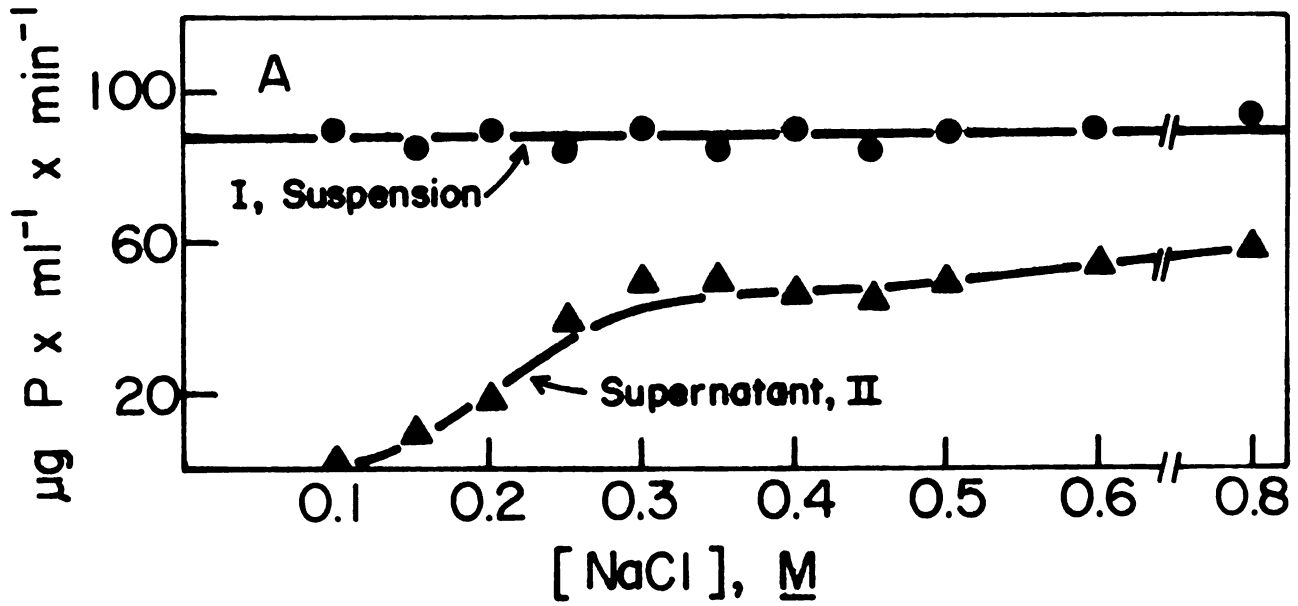
phatase is not completely established (Figure 32). Incubation of the starch particles in 0.25 M $MgCl_2$ solubilized 100% of the phosphatase (Curve II, Figure 32-B). However as the $MgCl_2$ concentration was increased to 0.35 M $MgCl_2$ or greater, the total soluble activity (supernatant) increased 15 to 20% over the total activity of the unclarified suspension in 0.35 M $MgCl_2$ (Curve I, Figure 32-B). NaCl was not as effective as $MgCl_2$. At 0.30 M NaCl, 55% of the phosphatase had been released from the particle but only 10% more of the phosphatase was solubilized even up to 0.8 M NaCl. The ionic strength of either salt for 50% solubilization of the phosphatase was the same but solubilization above that point did not coincide. Phosphatase released seems to be more dependent upon the divalent cation. The significance of the increase in activity at high $MgCl_2$ concentrations after the suspension is clarified is not understood. However it may be indicative of some control mechanism on the enzyme by the particle.

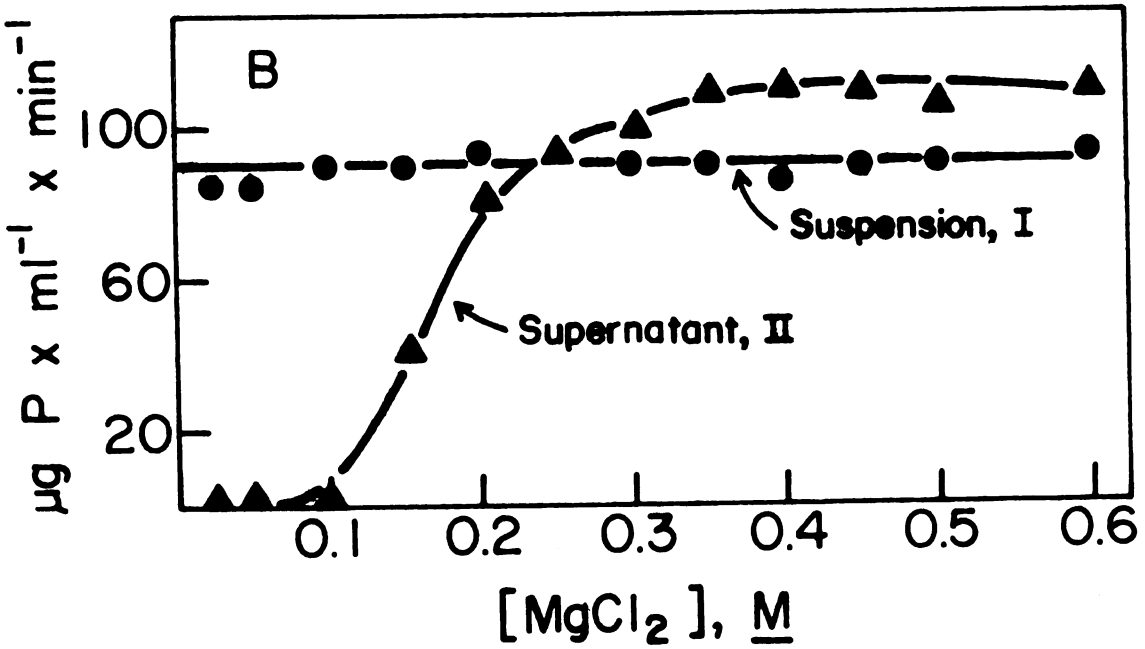
Purification of the Starch Particle 3-P-Glycerate Phosphatase

In the isolation of the starch pellet by sucrose density gradients the 3-P-glycerate phosphatase on the starch particles was enriched 46 fold. Further purification of the starch particle 3-P-glycerate phosphatase was done using solubilized enzyme, because of the large amount of information already gathered on the soluble 3-P-glycerate phosphatase from sugarcane. The starch particle enzyme was

Figure 32. Solubilization of the Particulate 3-P-Glycerate Phosphatase as a Function of Salt Concentration

To samples (0.10 ml) of the starch particles in 0.8 M sucrose, 1 mM EDTA and 20 mM cacodylate buffer, pH 6.3, were added 0.10 ml NaCl or $MgCl_2$ to give the final salt concentration. The samples were incubated for 30 minutes at 4°. Total enzyme activity is shown for the suspension (●——●) and for the supernatant (▲——▲) after removing particles by centrifuging at 10,000 x g for 20 minutes.


A
100
60
20
μg P x ml⁻¹ x min⁻¹
I, Suspension
Supernatant, II
0.1
0.2
0.3
0.4
0.5
0.6
0.8
[NaCl], M


B
100
60
20
μg P x ml⁻¹ x min⁻¹
Suspension, I
Supernatant, II
0.1
0.2
0.3
0.4
0.5
0.6
[MgCl2], M

solubilized by sonification for 20 minutes at 4° in 0.8 M sucrose, 20 mM cacodylate buffer, pH 6.3, and 1 mM EDTA. After partial purification (Table XXII) of the particulate (starch grain) phosphatase the specific activity was 10.75 (enriched 384 fold) compared to a specific activity of 740 (enriched 2530 fold) for the most pure preparations of the soluble phosphatase from sugarcane leaves.

Fractionation by acetone precipitation was performed in the same manner as that for the soluble sugarcane enzyme. A volume of reagent grade acetone (-5°) equal to 40% of the volume of the solubilized particulate 3-P-glycerate phosphatase was added dropwise to the enzyme at 4°, the system equilibrated for 20 minutes and then centrifuged for 10 minutes at 14,000 x g. The precipitate was discarded and cold acetone, equal to 20% of the volume of the solubilized enzyme, was added and the system equilibrated and centrifuged as before. The supernatant was discarded and the precipitate resuspended in 20 mM cacodylate buffer, pH 6.3, and 1 mM EDTA. The recovery of the enzyme was 61% with about a 7.5 fold enrichment.

The addition of 40 g of $(NH_4)_2SO_4$ per 100 ml of acetone-fractionated enzyme precipitated about 25% of the enzyme with no purification. Addition of more $(NH_4)_2SO_4$ equal to 20 g per 100 ml acetone-fractionated enzyme precipitated 34% of the enzyme with about 2-fold enrichment. A summary of the purification of the particulate 3-P-glycerate is presented in Table XXII. Further attempts to purify

Table XXII. Purification of the Particulate 3-P-Glycerate Phosphatase from 300 g of Spinach Leaves

Fraction	Units (μmoles/min)	Sp. Act.	% Yield	Enrichment	Total Protein (mg)
Original extract	748.0	.028	100	1	24,900
6000 x g pellet	29.0	.265	38	9.5	110
Density gradient pellet	14.0	1.29	18.6	46	10.9
Solubilization (sonicated)	13.8	1.29	18.4 (100%)	46	10.7
Acetone fractionation	8.4	9.85	11.2 (68%)	352	.86
$(NH_4)_2SO_4$ fractionation	2.9	10.75	3.0 (34%)	384	.27

the enzyme have not been made. Attempts to purify the particulate form of the enzyme as a particulate enzyme should be considered as well as purification of the enzyme after removal from the starch particle.

Further characterization of the starch-like particle itself is a definite requirement. Microscopic examination should be extended to electron microscopy, staining procedures and chemical composition data. The distribution of the starch particle must be examined especially since it was not found in all spinach leaf preparations. At present it is thought that the starch pellet could actually be residues of the starch grains which are resistant to further breakdown in the aged and stored spinach leaves from which the particles were isolated.

DISCUSSION

3-P-Glycerate phosphatase from sugarcane leaves has been purified 2530-fold with about 5% overall recovery. The enzyme stoichiometrically cleaves 3-P-glycerate to produce glycerate and inorganic phosphate. The purification of the enzyme was hampered initially by the phenol oxidases and phenolic oxidation products as well as inefficient extraction procedures for tough sugarcane leaves. The use of Polyclar AT, acid pH, EDTA and rapid acid fractionation were the most effective means of controlling the interference of phenolic compounds. The acetone, $(NH_4)_2SO_4$ and Sephadex G-200 fractionation steps were optimized with regard to pH and ionic strength so that most of the extracted protein was removed along with probably all other phosphatases, since the relative specificity did not change after the G-200 gel filtration step (Table IV). The instability of the enzyme alkaline pH and an isoelectric point of 6.8 prohibited the use of anion exchange columns for purification. Cationic exchange columns were most effective for further purification. The P-cellulose column could be operated at pH 4.0 with an additional 6-fold purification *vs* 3-fold at pH 4.5, but recovery was very poor and afterwards the enzyme was unstable even when the pH was adjusted to 5.9. The multiple peaks of phosphatase activity observed with DEAE-

cellulose or CM-Sephadex columns. It was felt that the multiple peaking on DEAE-cellulose columns was caused by operating too close to the isoelectric point.

Stability

During purification the enzyme was stable if the pH was kept below 7. Above pH 7 the enzymatic activity decayed rapidly and irreversibly. Unlike P-glycolate phosphatase, 3-P-glycerate phosphatase did not require a tricarboxylic acid for stability, and no tricarboxylic acids were found bound to the enzyme as with P-glycolate phosphatase. Prolonged dialysis against 1 mM EDTA or passage through G-25 Sephadex did not inactivate the enzyme at any point in the purification. It was concluded that no easily removable, small molecular weight molecule is necessary for enzyme stability. High ionic strength (NaCl or Na acetate) did not effect the activity or stability, however for some unknown reason the enzyme was inactivated by $(NH_4)_2SO_4$ precipitation after the G-200 Sephadex filtration step. The enzyme was reasonably stable for short times at room temperature, both in the crude state and purified. The enzyme was very stable at all states of purity at 4° and also stable at -18° except in the $(NH_4)_2SO_4$ fraction. 3-P-Glycerate phosphatase was rapidly inactivated at temperatures above 40° with 50% loss of activity after 3 minutes at 50° compared to only 10% loss of the tobacco leaf P-glycolate phosphatase activity at 75° (Randall and Tolbert, unpublished results).

Enzymatic Properties

The sugarcane leaf 3-P-glycerate phosphatase is similar to other leaf phosphatases in many of its biochemical properties. Activity of the most pure enzyme was optimal at pH 5.7 to 6.0 depending on the buffer systems, while preparations of low purity had a pH optimum around pH 6.3. This higher optimum with the crude extract may be more characteristic of the *in vivo* situation.

The apparent K_m of 2.8×10^{-4} M for 3-P-glycerate phosphatase was similar to that reported for most other leaf phosphatases. There was no evidence for sigmoidal or multiple enzyme kinetics. The enzyme was most active at 42° which is low compared to the optimal temperature of P-glycolate phosphatase at 70°. The effect of ionic strength on the enzymatic reaction was negligible up to about 0.5 M.

The specificity of the enzyme was not absolute, but it has been designated as 3-P-glycerate phosphatase because the rate of hydrolysis of 3-P-glycerate was greater than for all other substrates examined. β-Glycerol phosphate, a typical physiological substrate used for phosphatases, was hydrolyzed at 1/8 the rate of 3-P-glycerate. PEP was the only physiological substrate which was hydrolyzed at a rate greater than 50% the rate for 3-P-glycerate. The use of p-nitrophenylphosphate (hydrolyzed at 66% of 3-P-glycerate rate) as a substrate for phosphatases is considered to be of little value to understanding their characteristics.

3-P-Glycerate is unlike the typical non-specific acid phosphatase in plants (99, 112) or E. coli (148), which hydrolyze most carbohydrate esters at about equal rates and at lower rates than for the non-physiological substrate, p-nitrophenylphosphate. The plant acid phosphatases discussed in the Literature Review usually had a preference for hydrolyzing either 3'- or 5'-nucleotides. 3-P-Glycerate phosphatase however did not rapidly hydrolyze either 3'- or 5'-nucleotides nor did it show much preference for either. The terminal pyrophosphatase activity of the phosphatase was about 1/3 or less of that for 3-P-glycerate. ATP hydrolysis at 37% of the rate 3-P-glycerate was 4-fold greater than the hydrolysis of pyrophosphate. The enzyme was inactive towards polyphosphates and it did not have an appreciable activity for converting NADP(H) to NAD(H). The diesterase activity, as measured by bis-p-nitrophenylphosphate, was zero, however RNA or DNA were not tried as substrates. The 3-P-glycerate phosphatase was not able to hydrolyze the C-P bond of three phosphonic acid derivatives.

The specificity of the enzyme showed no significant change after the second acetone fractionation. The greater than 3-fold increase in activity towards fructose-1,6-diphosphate after the first acetone fractionation could be explained as a result of removing some inhibitor. However, the enzyme lost specificity, but not total activity during aging, suggesting that some as yet unknown change was occurring in the enzyme. This phenomenon could be due to some

unfolding or proteolytic action on the enzyme thereby causing alterations in the properties of the substrate binding site. Some constituent of the enzyme preparation might also cause a conformational change in the enzyme which would open or "loosen" the active site. Since the specificity at the various purification steps was not determined on a preparation made in a very short period of time, it is difficult to assess the changes in specificity. Experiments with mixed substrates and with various amounts of P-esters indicated that the other phosphophate esters, when present with 3-P-glycerate, were slightly inhibitory with regard to total phosphate released.

The experiments using mixed substrates gave no indication of more than one phosphatase being present. The isoelectric focusing of the G-200 Sephadex fraction and the most pure enzyme resulted in only one phosphatase peak. The Lineweaver-Burk plots indicated that only one enzyme was present. These facts in addition, to the lack of change in the relative specificity during the latter stages of purification, support the conclusion that one phosphatase was present.

It was not possible to demonstrate any cation requirement by the enzyme. Nine cation complexing reagents did not significantly reduce the activity of 3-P-glycerate phosphatase. In fact, there was a slight tendency for activity to increase with these substances. The addition of various cations did not stimulate or increase the phosphatase activity.

Most divalent cations were inhibitory, especially Zn^{++}, Sn^{++}, Cu^{++}, and Co^{++}. Inhibition by Zn^{++} has been used as a characteristic feature of this particular phosphatase, when comparing it to P-glycolate phosphatase or alkaline phosphatase which are most active with Zn^{++}. The salts of Pb^{++} and Hg^{++} were typically inhibitory. The monovalent cations had no effect and no anion effects were detected. With respect to divalent cations, 3-P-glycerate phosphatase is probably more like the typical acid phosphatases of E. coli and yeast, which do not require cations for activity. It does pose an interesting mechanistic question why two phosphatases, such as 3-P-glycerate phosphatase and P-glycolate phosphatase (requiring a divalent cation), each specifically hydrolyzing closely related substrates, can be so different.

The 3-P-glycerate phosphatase was not greatly affected by alkylating agents. Only p-chloromercuribenzoate at 10^{-3} M gave as much as 24% inhibition. The phosphatase was typically inhibited by fluoride, L(+)tartrate and molybdate. The tartrate inhibition was competitive. L(-)tartrate and meso-tartrate were not significantly inhibitory. It appears that L(+)tartrate inhibits those phosphatases without cation requirements, perhaps suggesting some unique nature of the active site. In contrast to P-glycolate phosphatase, cysteine and glutathione did not inhibit the 3-P-glycerate phosphatase. The tricarboxylic acids were also slightly inhibitory.

Inhibitors of glycolate metabolism were ineffective

on 3-P-glycerate phosphatase activity. The use of α-hydroxy-2-pyridinemethanesulfonate *in vivo* usually results in an accumulation of glycolate by inhibiting glycolate oxidase. This compound caused an accumulation of glycerate in photosynthesis experiments in sugarcane (74). However, our attempts to find a glycerate oxidase in sugarcane or corn leaves were negative.

A number of analogs of 3-P-glycerate were investigated in attempts to find an inhibitor of the enzyme which might be usable for physiological studies, but none were found. A number of metabolites of glycerate and 3-P-glycerate in addition to the products of the photosynthetic C_4-pathway were not effectors of the phosphatase. L-Aspartate at 10^{-3} M provided a 10% stimulation of the phosphatase activity. Phosphonic acid derivatives (Table IX) also produced a 10% stimulation at saturating substrate concentration. The reasons for the slight stimulation by L-aspartate and the phosphonic acids are not known. One would reasonable expect the phosphonic acids to inhibit competitively. The stimulation by L-aspartate might be indicative of some means of *in vivo* control since both 3-P-glycerate and L-aspartate are major, initial products of CO_2-fixation in C_4-plants.

The kinetics of inhibition by glycidol-P were typical of competitive inhibition, but no phosphate was released from glycidol-P. The enzyme was irreversibly inhibited by glycidol-P. Alkylation of the active site by the glycidol or epoxide moiety must prevent the enzyme from completing

the hydrolysis, thus, this compound appears to bind at or near the active site. The inhibition and alkylation may indicate a similarity of enzymatic mechanism for the phosphatase and for triose isomerase and enolase reactions which are also inhibited irreversibly by glycidol-P (135).

Physical Properties

3-P-Glycerate phosphatase is quite large. It was slightly retarded on G-200 Sephadex with a 1.4 to 1 ratio of the elution volume to void volume, from which a molecular weight is estimated to be in the 150-200,000 range. The sucrose density gradients with low ionic strength produced evidence of a range of active forms or aggregating forms. The system was probably in a rapidly associating-dissociating state, and the most prevalent form was around 13 S or around 300,000 deltons. Increasing the ionic strength to 0.25 M with KCl resulted in only one form of the enzyme with a 8.0 S value or around the 160,000 molecular weight range. This latter value is in agreement with the estimate from the G-200 Sephadex column. Whether or not the multiple aggregates or the associating-dissociating phenomenon is significant is difficult to predict at this time. The phenomenon could be due to low protein concentrations or some interferring protein that has not yet been removed. The tendency to aggregate may conceivably have physiological significance. In vivo aggregation could control the rate of hydrolysis of the substrate, although the enzyme in the

various forms was active. Some undetected factor may also be necessary for such a control mechanism. The diurnal variations in the enzyme (discussed below) could possibly be a manifestation of this aggregation phenomenon, one form predominating during the more active period for the enzyme. The effects of various metabolites on the aggregated forms has not been examined.

The SDS-polyacrylamide electrophoresis experiment produced two polypeptide bands from the most pure enzyme preparation. The major band had an approximate molecular weight of 51,000 and the minor band was about 62,000. The molar ratio of the two bands was 4.3 to 1. If both of these were subunits, it is difficult to imagine a combination of these two units that would give a molecular weight in the 140-200,000 range. One polypeptide could be a contaminant. If this were the case, one would have a tendency to pick the 51,000 unit chain as the phosphatase polypeptide. This would suggest an enzyme of 3 or 4 polypeptide chains. If the smaller peptide chain was the result of cleavage of a 10-11,000 molecular weight peptide from 62,000 pieces, one wonders at the incompleteness of the process. Non-identical subunits in a ratio of 4 to 1 or 3 to 1 is not impossible, but an odd number of subunits (4 to 1) would be very unusual. The fact that the enzyme will not migrate on regular polyacrylamide gel electrophoresis (5% gels) can be considered to support the idea that the enzyme is quite large (aggregated) at low ionic strength. In the three systems tried

there were no detectable protein bands on the gels, even though standards were easily detected. The lack of detectable protein on the gels indicates that the 3-P-glycerate phosphatase preparation was probably quite pure but not necessarily homogeneous. This suggests that two small polypeptide bands in the SDS-polyacrylamide were from one enzyme or the contaminating protein was bound closely to the phosphatase.

Physiological Considerations

The results of the survey for the phosphatases showed that the 3-P-glycerate phosphatase was most active in C_4-plants but was also found in significant amounts in C_3-plants. The rate of 3-P-glycerate hydrolysis in C_3-plants was 0.7 to 9 μmoles per mg chlorophyll per minute as opposed to CO_2 fixation rates of 1 to 4 μmoles CO_2 per mg chlorophyll per minute (16). Thus there is enough 3-P-glycerate phosphatase even in C_3-plants to hydrolyze most of 3-P-glycerate formed. In the C_4-plants the enzyme is consistently 2- to 3-fold more active than the C_3-plants and still as great as, or greater than the rate of photosynthesis. These facts support the idea that the 3-P-glycerate phosphatase is closely involved in the photosynthate metabolism in C_4-plants.

The enzyme seems to be localized in the leaf tissue and is 20-fold more active there than in the stem and root. The very low levels of 3-P-glycerate hydrolysis by extracts of stem and roots can easily be attributed to acid phosphatases of these tissues. Limiting the enzyme to leaf tissue

does not necessarily link the enzyme to photosynthesis. Therefore the specific location in the leaf may help delineate its function. As described in the Literature Review, sugarcane has two types of chloroplast containing cells, the mesophyll cells and parenchyma or bundle sheath cells. Appendix A presents the results of experiments that concluded that the 3-P-glycerate phosphatase is mainly in the mesophyll cells as compared to the bundle sheath location for the P-glycolate phosphatase. The mesophyll cells contain the chloroplasts with enzymes unique to the C_4-pathway of CO_2 fixation (Table 1). Different lines of evidence were presented to indicate that the phosphatase was not in any particulate fraction from sugarcane leaves. Chloroplast isolation procedures specifically designed for sugarcane leaves were unsuccessful in localizing the 3-P-glycerate phosphatase with the mesophyll chloroplasts. Other differential centrifugation procedures in buffered, isotonic media failed to locate the enzyme with peroxisomes or any other subcellular body. These results contrast with those of Matile *et al.* (149) who have presented evidence that there is considerable acid phosphatase in spherosomes or dictyosomes of higher plants.

Non-aqueous density fractionation procedures located the 3-P-glycerate phosphatase in the non-chloroplastic fractions of the leaf, in complete agreement with the acid phosphatase distribution in sugarcane leaf tissue report by Slack *et al.* (41). This does not necessarily mean that the

enzyme is not involved in the pathway of carbon metabolism during photosynthesis. There is increasing evidence (54, 57, 150) that the flow of carbon between the chloroplasts of the mesophyll cells and chloroplasts of bundle sheath cells is very rapid and obligatory to the total photosynthetic process. PEP carboxylase is believed to be located in the peripheral reticulum of the mesophyll chloroplasts for easy access to the CO_2 and to facilitate the transport of oxaloacetate or malate to the bundle sheath chloroplasts. Triose isomerase and glyceraldehyde-P-dehydrogenase are in both types of chloroplasts. 3-P-Glycerate, the product of the "transcarboxylation reaction," is distributed about evenly between the two chloroplast types. These facts all lend support to rapid movement of carbon compounds between the cells. A phosphatase could conceivably regulate the direction of carbon flow or create a concentration gradient of a particular compound in a metabolic sequence.

Further evidence for the involvement of the 3-P-glycerate phosphatase in photosynthesis or in related metabolism in C_4-plants is supported by its formation during the biogenesis of the chloroplasts. The enzyme activity increased 10-fold on a fresh weight basis and 4-fold on a protein basis after etiolated tissue was illuminated. Slack et al. (151) reported very little change in acid phosphatase levels in etiolated corn and sorghum leaf after exposure to light but the enzymes involved in photosynthesis increased 4- to 12-fold. The increase in 3-P-glycerate phosphatase activity

during greening of etiolated tissue is the same phenomenon as increases in P-glycolate phosphatase activity (76), which is found on or in the chloroplasts. The evidence supports a function for 3-P-glycerate phosphatase in the photosynthesis of C_4-plants.

Speculations on the Function(s)

The exact function of 3-P-glycerate in leaves is not known. Since this phosphate ester is a primary product of photosynthetic CO_2 fixation and a key intermediate in glucose synthesis and photosynthate metabolism, numerous regulatory functions for the phosphatase can be invisioned.

Regulation of the photosynthetic carbon pathways: Certainly any hydrolysis of 3-P-glycerate as it is formed during CO_2 fixation will curtail the rate of photosynthesis by slowing the regeneration of RuDP. 3-P-Glycerate also could exert product inhibition on the RuDP carboxylase; thus, hydrolysis of 3-P-glycerate in this case could spped-up photosynthesis. Whether the phosphatase functions for such regulation *in vivo* has not been tested. It is known that during short-term $^{14}CO_2$ photosynthesis by many plants, considerable carboxyl-labeled glycerate is formed.

Serine formation: P-Glycerate and glycerate are metabolic precursors for serine formation in plants. Aside from synthesis in the glycolate pathway, evidence supports the predominance of the non-phosphorylated pathway of serine synthesis in green leaves. Serine, besides being an essential amino acid, can be a precursor of glycine and C_1 syn-

thesis. A major flow of carbon to the C_1 pools must occur during plant growth.

Regulation of starch synthesis: 3-P-Glycerate is an allosteric activator of ADP-glucose pyrophosphorylase (152) which forms the substrate the starch synthetase. Regulation of the pool size of 3-P-glycerate should have a profound influence on the rate of starch formation.

Carbon transport: 3-P-Glycerate phosphatase may function as part of a transport mechanism for photosynthate between the two different cell types in leaves of C_4-plants such as sugarcane. Several additional factors must be considered for such a function.

(1) 3-P-Glycerate phosphatase is generally more active in C_4-plants than in C_3-plants.

(2) 3-P-Glycerate phosphatase and glycerate kinase in C_4-plants are primarily located in the mesophyll cells, along with the enzymes for the C_4-pathway of CO_2 assimilations (Table 1).

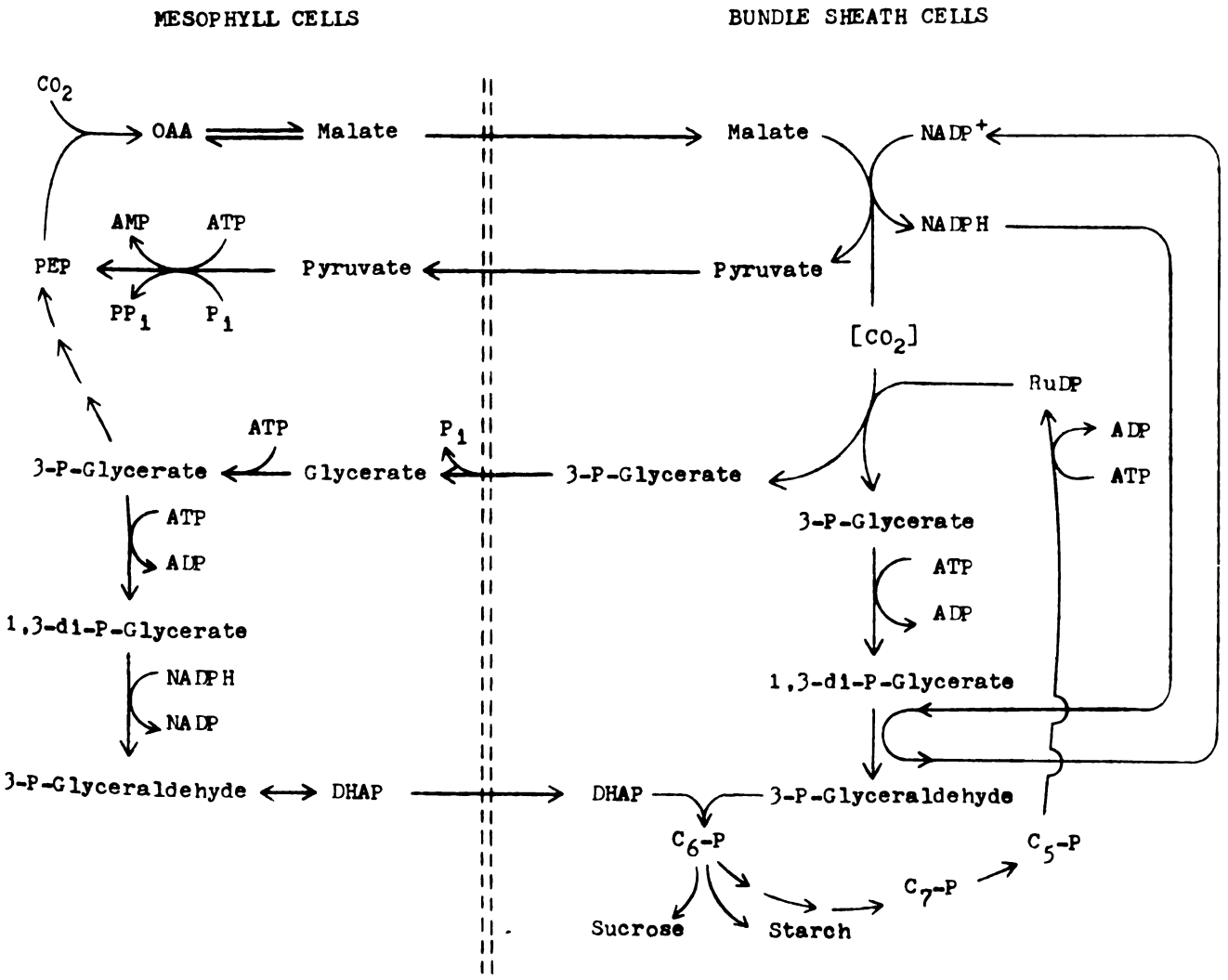
(3) According to Andrews (54) the "transcarboxylation" reaction and 3-P-glycerate should be formed in the bundle sheath chloroplasts where there is inadequate reducing power.

(4) At least half of the 3-P-glycerate produced by "transcarboxylation" in C_4-plants is found in the mesophyll cells along with about half of the NADP-glyceraldehyde-P-dehydrogenase and triose isomerase (41).

The 3-P-glycerate could be moving to the mesophyll cell and/or chloroplast for further reductions or metabolism to regenerate the primary CO_2 acceptor (PEP) for the C_4-pathway. With regard to this, I would like to postulate that 3-P-glycerate phosphatase may be a part of a permease or transport system to move carbohydrate from the bundle sheath cells into the mesophyll cells or mesophyll chloroplasts. This scheme is represented in Figure 33 in combination with part of the data from Hatch and Slack (16) concerning the two photosynthetic pathways in different types of cells. 3-P-Glycerate phosphatase would be part of a facilitative diffusion process by hydrolyzing 3-P-glycerate thereby creating a natural diffusion gradient for carbon flow towards the mesophyll cells and chloroplasts. The concentration of 3-P-glycerate, an inhibitor of RuDP carboxylate, would be kept low in the bundle sheath preventing inhibition of the carboxylase, and the carbon would be moved to the location of adequate reducing power in the mesophyll cells.

If the mesophyll chloroplasts are more permeable to glycerate than 3-P-glycerate, the phosphatases would be facilitating transport into the chloroplast as well as the cell. Glycerate in the mesophyll chloroplast would be phosphorylated by the glycerate kinase located in the chloroplast and then reduced and isomerized to DHAP. The DHAP would then be available for conversion to the other intermediates of the C_4-pathway. Hatch and Slack (16) have

Figure 33. Proposed Intercellular Movement of Newly Fixed Carbon Between Bundle Sheath and Mesophyll Cells



suggested on the basis of other data that DHAP could be a major transport form of carbon from the mesophyll cells to the bundle sheath cells.

Substitute for glycolate pathway in peroxisomes: DeDuve (153) has emphasized that peroxisomes occur in tissue capable of gluconeogenesis, though how the particle functions in this manner is not known. 3-P-Glycerate phosphatase in the C_4-plants could be a substitute for a gluconeogenic function of the glycolate pathway or plant peroxisomes. Glycerate is the major product of the glycolate pathway in leaf peroxisomes and the glycerate is subsequently converted to sucrose. Through the action of the 3-P-glycerate phosphatase, photosynthate can be converted directly to glycerate. In effect the phosphatase has bypassed the glycolate pathway and the loss of 25% of the carbon as CO_2 via peroxisomal respiration and the hydrolysis of an extra phosphate ester, since two P-glycolate molecules must be hydrolyzed to make one glycerate. If glycolate metabolism or peroxisomal metabolism is essential for photosynthesis and polysaccharide formation in C_3-plants, then 3-P-glycerate phosphatase could be serving this same essential function in a more efficient manner for C_4-plants. The reciprical relationship between 3-P-glycerate and P-glycolate phosphatases in the soybean varieties also would support 3-P-glycerate phosphatase as a substitute for the glycolate pathway as a glycerate forming system. The total possible glycerate formation by both systems is about the same among the 15 soybean varieties.

Diurnal Variations in Activity

The diurnal variations of the phosphatase activity may also be reconciled with an enzyme involved in photosynthesis. Phosphatase activity increased 50% in the latter part of the daylight when the photosynthetic activity has decreased. Even at the periods of lowest 3-P-glycerate activity, this activity was as high as the PEP-carboxylase activity reported by Hatch and Slack (16). The 50% increase in the phosphatase activity late in the afternoon could be indicative of its role in regulating the metabolic fate of photosynthetic carbon pools.

There are several possible implications of the diurnal variation in the 3-P-glycerate phosphatase activity. During the waning hours of daylight the plant could be recalling carbon from storage (i.e. assimilatory starch) for further metabolism for maintenance, growth and additional transport. The assimilatory starch is mainly located in the bundle sheath chloroplasts and when the carbon from this starch is reassimilated into the metabolism of C_4-plants, the soluble phosphatase could be instrumental in creating a gradient for 3-P-glycerate flow from the bundle sheath cells to the mesophyll cells. This flow of 3-P-glycerate out of the bundle sheath chloroplasts would also reduce the concentration of 3-P-glycerate and in turn starch formation should be curtained. The mesophyll cells must also have a large portion of the enzymes for further metabolism of the carbon from starch, to such metabolites as pyruvate or acetyl CoA for

the Krebs cycle and lipid synthesis.

Glycerate is also the major product of the glycolate pathway and assuming that there is significant metabolism through this pathway in C_4-plants, the condition favoring glycolate biosynthesis decrease in the later part of the day. Thus it is reasonable that the need for glycerate formation could be covered by increased hydrolysis of 3-P-glycerate. Since the glycerate from the glycolate pathway is probably metabolized to sucrose (73), the increased phosphatase action late in the day would also permit sucrose formation and continue the drain of carbon out of the photosynthetic fixation cycles.

Photosynthetic Efficiency

Photosynthesis is obviously a major determinant of crop yield. Crop species with the highest rates of photosynthesis (sugarcane, sorghum) are also the highest yielding species. Photorespiration or peroxisomal respiration (82), which does not appear to be coupled to any energy conserving mechanism, reduces the net photosynthetic efficiency of a plant. Consequently the efforts of investigators attempting to use CO_2 fixation rates as an index of yield potential are often inaccurate. P-Glycolate phosphatase provides the substrate for photorespiration and therefore has the potential of being the limiting enzyme. Glycolate oxidase, as shown by Curtis *et al.* (147), is probably not a limiting enzyme in photorespiration.

The results of our survey for the two phosphatases in various plants appeared sufficient to support conclusions that these two enzymes could be indicative of photosynthetic efficiency. 3-P-Glycerate phosphatase appears to be more important to the metabolism of the photosynthate of C_4-plants than C_3-plants. It is reasonable to believe that the two phosphatases are reciprocally related in function in the two types of plants. The 3-P-glycerate phosphatase could be considered a marker for the more efficient photosynthesizing plants or plants having lower levels of CO_2-photorespiration. These conclusions coupled with the evidence that 3-P-glycerate phosphatase action may substitute for the glycolate pathway were the basis for experiments with the soybean varieties. Soybeans have high levels of activity for both phosphatases.

The soybean plant is a typical C_3-plant with photorespiration which supposedly makes it less efficient. The hypothesis was that 3-P-glycerate phosphatase would be a marker for more efficient soybean plants. The relative activities of the phosphatases might provide a biochemically based index for yield potential of a plant in early stages of varietal development by the plant breeder.

The results of investigations on the 15 soybean varieties were the opposite to those predicted. With increasing photosynthetic rates the level of P-glycolate phosphatase activity increased and 3-P-glycerate activity decreased. Hopefully, this correlation may yet provide a

biochemical index for photosynthetic potential. Efforts to substantiate the correlation are hampered by lack of fully established functions for both glycolate metabolism and the 3-P-glycerate phosphatase.

The results of this soybean varietal experiment are inconclusive by itself but it does provide a basis for further experiments such as similar assays for the peroxisomal enzymes. The relative levels of the phosphatases may provide a rapid enzymatic assay for determining relative photosynthetic or growth efficiency. Dr. W. L. Ogren at the University of Illinois is pursuing this idea, and from other physiological variations he thinks there is a positive correlation between growth and amount of peroxisomal activity (personal communication). The function of P-glycolate hydrolysis (glycolate pathway) versus 3-P-glycerate hydrolysis and the differences in subsequent metabolism are confusing. But if the differences in metabolism that result can be linked to grain output or composition, an additional method can be added to the means of evaluating new varieties. There is precedent for linking enzyme levels with grain output. Hageman et al. (154) have found that the level of nitrate reductase in the wheat leaves is positively correlated with protein levels in the grain of the wheat.

Discussion of the Particulate 3-P-Glycerate Phosphatase

Investigation of the particulate or starch grain 3-P-glycerate phosphatase is being continued. The biochemical

properties determined to date are essentially identical to those of the soluble sugarcane 3-P-glycerate phosphatase. The known properties of the particle itself are limited. The particle is probably polysaccharide in nature in that its density is greater than 2.3 M sucrose and because β-amylase released reducing sugars concomitant with the release of the enzyme. The binding of the enzyme is likely to be ionic, since the high ionic strength solubilized it. But the high ionic strength also could possibly be instrumental in plasmalyzing some closed membrane system. Either possibility could be reconcilable with the fact that the enzyme can also be solubilized by extended sonification.

The question still to be answered is whether or not the particulate form of the enzyme is of physiological origin or an artifact of isolation such as non-specific binding. If the particulate form of the enzyme is of <u>in vivo</u> origin, its possible function(s) is highly speculative.

A possible function of the particulate 3-P-glycerate phosphatase is an involvement with the regulation of starch synthesis or degradation. Such a function is based upon reports that the control of the biosynthesis of assimilatory starch in spinach leaves is mediated by 3-P-glycerate (152). ADP-Glucose pyrophosphorylase, the enzyme which makes the substrate for the starch synthetase, is an allosteric enzyme activated 50-fold by 2×10^{-5} M 3-P-glycerate (152). The 3-P-glycerate concentration thus becomes the fine control on starch synthesis. If the concentration drops due to

curtailment of photosynthesis which provides the 3-P-glycerate and ATP, starch synthesis stops and will not begin until the pools of the intermediates of the carbon reduction cycle are full. Hydrolysis of 3-P-glycerate by the phosphatase would lower the concentration of 3-P-glycerate, inactivate the synthetase and provide P_i for a phosphorylase degradation of starch. Inorganic phosphate also inhibits the ADP-glucose pyrophosphorylase (152). The effect of 3-P-glycerate on starch phosphorylase is not known. Whether or not the particulate 3-P-glycerate phosphatase does function in this manner to control starch formation and degradation is admittedly speculative at this time. For plants there is lack of knowledge with regard to regulatory mechanisms and intermediates such as cyclic AMP. Key phosphate esters such as 3-P-glycerate and specific phosphatase and kinase systems could possibly be a manifestation of the plant's regulatory requirements.

Best success in isolating the starch-like particles with phosphatase activity was obtained with stored spinach leaves in which starch-rich particles should have been depleted. Normally destarching of leaves is accomplished by 3 days of darkness. Little is known about any residual particle with primer starch and the necessary enzymes for starch synthesis. As isolated from old stored leaves the particle carrying the phosphatase could be the primer or limit dextrin of the starch grain or the membrane surrounding the starch grain or both. Regulation of the phosphatase

may involve the physical properties of particle, and perhaps the enzyme is inactive *in vivo* in either the bound or soluble form.

SUMMARY

3-P-Glycerate phosphatase from sugarcane leaves was isolated and purified 2530-fold. The purity of the enzyme is uncertain, but no protein bands could be detected using three different sets of electrophoresis conditions. The enzyme stoichiometrically hydrolyzes D-3-P-glycerate to D-glycerate and inorganic phosphate. The enzyme was not absolutely specific, but it was at least 2-fold more active with 3-P-glycerate than most other substrates. PEP was hydrolyzed at 0.66 of the rate of 3-P-glycerate. Optimal enzymatic activity was between pH 5.7 and 6.0. The enzyme required no divalent cations or other detectable cofactors nor was it inhibited by EDTA. The apparent Michaelis constant for 3-P-glycerate was 0.28 mM. The purified phosphatase had an isoelectric point at about pH 6.8 and did not electrophorese in 3 different buffering systems and pH's. The enzyme was stable at -18° or 4° indefinitely, and at room temperature for brief periods. Over 50% of the enzyme was inactivated by incubation at 50° for 3 minutes.

The 3-P-glycerate phosphatase was inhibited by typical phosphatase inhibitors, L(+)tartrate, molybdate and fluoride. Glycidol-P inhibited the enzyme irreversibly without concomitant release of inorganic phosphate. Three phosphonic acid derivatives, phosphonoacetate, 2-amino

phosphonoacetate and 2-amino-3-phosphonopropionate were not hydrolyzed by the enzyme but stimulated (10%) the enzymatic activity with substrate at saturating concentrations. L-Aspartate stimulated the phosphatase to a similar degree as the phosphonic acids.

The enzyme showed an aggregation phenomenon on sucrose density gradients of low ionic strength. On high ionic strength sucrose gradients, the enzyme centrifuged in one form at approximately 8.0 S, with an estimated molecular weight in the 160,000 unit range. SDS-Polyacrylamide electrophoresis yielded a major band at 52,000 molecular weight units and a minor band at 62,000. The molar ratio of the two bands was 3.6 to 1.

The enzyme is located in the leaf tissue of sugarcane. Different lines of evidence localized the sugarcane 3-P-glycerate phosphatase in the cytoplasm of the mesophyll cells and there was no evidence of the sugarcane enzyme being particulate. The enzyme activity increased at least 4 fold on a protein basis and 10 fold on a chlorophyll basis during the greening of etiolated tissue. On sunny days the enzyme activity displayed a diurnal variation with at least a 50% increase in activity during the late daylight hours and early darkness.

The 3-P-glycerate phosphatase was generally more active in plants with the C_4-pathway of CO_2 assimilation but the activity in C_3-plants was enough to account for any glycerate formed. P-Glycolate phosphatase was generally

most active in plants with the C_3-pathway of CO_2 assimilation but significant and sufficient activity was found in C_4-plants to account for any glycolate formed. The levels of the activities of P-glycolate phosphatase and 3-P-glycerate phosphatase were correlated positively and negatively respectively to the rates of CO_2 fixation in 15 varieties of soybeans (Glycine max. L. Merrill). These phosphatases may be used as indices of photosynthetic potential.

A particulate or starch grain 3-P-glycerate phosphatase from spinach leaves was discovered and partially characterized. The enzyme was pelleted through the 2.3 M or 2.5 M sucrose layer of discontinuous sucrose gradients used for preparation of peroxisomes. Because of its density, a positive reaction of the material to KI-I_2 reagents and the release of the phosphatase and reducing sugar by β-amylase, the pellet and particles to which the enzyme was bound were tentatively termed the starch pellet and starch particles.

The biochemical characteristics of the particulate 3-P-glycerate phosphatase were similar to the soluble enzyme from sugarcane leaves. Optimal activity was around pH 5.8 and it required no divalent cations or other cofactors. The enzyme exhibited normal hyperbolic kinetic plots with an apparent Michaelis constant of 9×10^{-4} M for 3-P-glycerate. The phosphatase was not absolutely specific but hydrolyzed 3-P-glycerate about 1.5 times faster than the other physiological substrates. The particulate phosphatase was enriched 46-fold over the crude spinach extracts.

Solubilization of the starch particle 3-P-glycerate phosphatase was possible by extended sonification treatment, 0.35 M $MgCl_2$ or incubation with β-amylase. Passage through a French pressure cell, homogenization in a Potter-Elvjehm, repeated freezing and thawing, changing the pH and extended hydrolysis were ineffective in releasing the enzyme from the particles. The solubilized 3-P-glycerate phosphatase from the starch particles was partially purified by acetone and $(NH_4)_2SO_4$ fractionation. The partially purified enzyme was enriched 384 fold with a specific activity of 10.8 and a 34% recovery.

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A P P E N D I C E S

APPENDIX A

ENZYMES OF THE GLYCOLATE PATHWAY IN PLANTS WITHOUT CO_2-PHOTORESPIRATION

D. W. Rehfeld, D. D. Randall, and N. E. Tolbert

Canadian Journal of Botany, Vol. 48, p. 1219.

APPENDIX B

Plant		Variety
Cotton	Gossypium hirsatum L.	---
Tobacco	Nicotiana tabacum L.	Maryland Mammoth
Spinach	Spinacia L.	Longstanding Bloomsdale
Sunflower	Helianthus L.	Mammoth Russian
Wheat	Triticum vulgare L.	Thatcher
Alfalfa	Medicago sativa L.	---
Tomato	Lycopersicon Mill.	Big Boy
Bean	Phaseolus vulgaris L.	Sonalac
---	Atriplex patula hasta	---
Sudan grass	---	Piper
Sudum	---	---
Sorghum	Sorghum halepense (L.) Pers.	---
Pigweed	Amaranthus hybridus (L.)	---
Sugarcane	Saccharum	CL 41-223
Corn	Zea mays L.	Michigan 500
---	Atriplex rosea	---
Crabgrass	Digitana sanquinalus	---
Burmuda	Cynodon dactylon (L.) Pers.	---
Bluegrass	Poa compressa L.	---
Bent Grass	Agrostis tenuis	---

Plant		Variety
Merion bluegrass	Poa pratenis	---
White oak	Querus alba L.	---
Red maple	Acer rubrum L.	---
Mountain ash	Sorbus americana marsh	---
Cottonwood	Populus del toides marsh	---
American elm	Ulmus americana L.	---
Red spruce	Picea rubens saxy	---


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