P-GLYCOLATE PHOSPHATASE AND ACONITASE FROM TOBACCO LEAVES

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
DONALD E. ANDERSON
1969

This is to certify that the

thesis entitled

P-GLYCOLATE PHOSPHATASE AND
ACONITASE FROM TOBACCO LEAVES

presented by

DONALD E. ANDERSON

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

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Date February 19, 1969

O-169

ABSTRACT

P-GLYCOLATE PHOSPHATASE AND ACONITASE
FROM TOBACCO LEAVES

by Donald E. Anderson

In exploratory studies from this laboratory (unpublished), it was found that P-glycolate phosphatase from wheat leaves was associated with cis-aconitate, which stabilized the enzyme. Citrate or isocitrate also stabilized the phosphatase from wheat. The investigations described here extend these observations to the phosphatase from tobacco leaves, and in addition are concerned with relationships between the phosphatase, aconitase, and the tricarboxylic acids.

P-glycolate phosphatase from tobacco leaves was purified 1000 fold to a specific activity of 333 μmoles of substrate hydrolyzed per minute per mg of protein. During the last purification step, which was gel filtration chromatography, the phosphatase became unstable, as determined by heating the enzyme to 45° for 1 hour. It could be restabilized with fractions which emerged after the phosphatase from the column. The endogenous stabilizing factors in these fractions were identified as citrate, isocitrate, and cis-aconitate. Commercial

citrate, isocitrate, and cis-aconitate, as well as trans-aconitate, also stabilized the enzyme, but mono and di-carboxylic acids did not.

Cis-aconitate was found to be an inhibitor, competitive with respect to P-glycolate, of the phosphatase. The apparent K_I for cis-aconitate was 5.0×10^{-3}M when the Mg^{+2} was 2×10^{-3}M. Increasing the Mg^{+2} to 2×10^{-2}M, with cis-aconitate at 10^{-2}M, did not significantly affect the apparent K_I. The data suggest that cis-aconitate did not cause inhibition by complexing the Mg^{+2} required as an activator of the phosphatase.

In fractions from the next-to-last purification step, which was DEAE-cellulose chromatography, one of the two isocitrate peaks approximately coincided with the phosphatase peak. At this stage of purification, approximately 1 mole of isocitrate per 10 to 15 moles of amino acid of the phosphatase fractionated with the enzyme. Furthermore, comparison of the sizes of the spots on paper chromatograms suggests that more citrate than isocitrate fractionated with the phosphatase.

In most experiments, aconitase and P-glycolate phosphatase fractionated in parallel. However, when field grown tobacco leaves were kept in darkness for 3 to 4 hours before harvest and homogenization, the phosphatase and aconitase were separated during their purification. In the latter case, isocitrate no longer fractionated with

the phosphatase. The evidence suggests that the fractionation of the endogenous tricarboxylic acids with the phosphatase was closely related to the fractionation of aconitase with the phosphatase.

Although it is not known whether the parallel fractionation of the two enzymes was artifactual or of physiological significance, it is of interest that the tricarboxylic acids are stabilizers of aconitase, and that any one tricarboxylic acid would be expected to competitively inhibit the interconversion of the other two. Thus, aconitase and P-glycolate phosphatase seem to possess at least two similarities, i.e. stabilization by the tricarboxylic acids and competitive inhibition by cis-aconitate.

Partial purification and characterization of aconitase from tobacco leaves are described. In particular, partially purified aconitase was inactivated by Sephadex G-25 chromatography and was reactivated by fractions which emerged after aconitase from the column, or by sulfates or chlorides. The activation of aconitase by sulfates or chlorides is consistent with the concept that the enzyme may possess groups, other than at the active center, capable of binding citrate, isocitrate, or cis-aconitate.

In the course of the above investigations, it was discovered that P-glycolate phosphatase in fresh extracts was unstable toward dilution of 20 to 30 fold at 30° for 1 hour, and that under aerobic conditions, the enzyme in

the extracts slowly became stable toward this dilution at 30°. A variety of treatments of the extracts including mixing under air or O_2 (but not N_2 or CO_2), or a 10 minute preincubation with 10^{-2}M α-hydroxy-2-pyridinemethanesulfonate, a specific inhibitor of glycolate oxidase, quickly and completely stabilized the phosphatase toward dilution at 30°. The stabilized enzyme could be partially and rapidly reconverted to the unstable but just as active enzyme by preincubation of the oxygenated extracts with glycolate. The sulfonate completely inhibited, while anaerobic conditions enhanced, this glycolate dependent conversion. Glycolate also inhibited the stabilization toward dilution at 30° of the phosphatase by O_2. The data suggest that the phosphatase can exist in an oxidized or in a reduced state, and that in the extracts, glycolate oxidase could be involved in the interconversion between the two states.

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By

Donald E. Anderson

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1969

Dedicated

to my wife,

Adrienne

ACKNOWLEDGMENTS

I thank Dr. N. E. Tolbert for the thesis problem, and for his guidance, support, and patience during the course of the work. I acknowledge the technical help received from D. Glen Aitken, Ruth Allen, and P. L. Youngblood. The support for a period of three years of the National Science Foundation in the form of a Graduate Fellowship is acknowledged. I thank my mother, father, brothers, and sister for their financial help, interest, and patience during this research program.

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LIST OF ABBREVIATIONS

BAL	British anti-lewisite; 2,3-dimercapto-1-propanol
CM-cellulose	carboxy methyl cellulose
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DEAE	diethyl amino ethyl
FDP	fructose-1,6-diphosphate
OHPMS	α-hydroxy-2-pyridinemethanesulfonate; 2-pyridylhydroxymethanesulfonate
PGA	3-phosphoglycerate
RuDP	ribulose-1,5-diphosphate
SDP	sedoheptulose-1,7-diphosphate
TEAE	triethyl amino ethyl
TES	N-tris (hydroxymethyl) methyl-2-amino-ethanesulfonic acid
TPP	thiamine pyrophosphate

INTRODUCTION

As elaborated in the literature review, under certain environmental conditions, a substantial percent of the newly fixed $^{14}CO_2$ appears as glycolate and products of the glycolate pathway during photosynthetic CO_2 fixation. As further elaborated in the literature review, although the source of glycolate remains unknown, circumstantial evidence suggests that glycolate comes from the photosynthetic carbon cycle, that P-glycolate should be the precursor of glycolate, and that P-glycolate phosphatase should be the enzyme which catalyzes the conversion of P-glycolate to glycolate *in vivo*.

Purification of P-glycolate phosphatase from tobacco leaves by $(NH_4)_2SO_4$, calcium phosphate gel, and DEAE-cellulose chromatography yielded an enzyme specific for P-glycolate (202). Because some of the purification steps proved troublesome, a different procedure was developed based on acetone precipitation followed by DEAE-cellulose chromatography (265). When purified in this way, the phosphatase from wheat was associated with cis-aconitate, which stabilized the enzyme. Commercial citrate or isocitrate also stabilized the wheat enzyme (Tolbert and Yu, unpublished data).

The investigation described in this thesis was

inaugurated for the further purification of the phosphatase from tobacco leaves, and for a study of the relationship between the enzyme from tobacco leaves and endogenous tricarboxylic acids. The study of the latter relationship led to a study of a possible relationship between P-glycolate phosphatase and aconitase from tobacco leaves.

As discussed in the literature review, at least five other relationships may exist between glycolate metabolism and the metabolism of the tricarboxylic acids. These relationships, which are listed below, make the study of the relationships between the phosphatase and the tricarboxylic acids and between the phosphatase and aconitase of greater interest.

1. Citrate may be important for the inhibition of phosphofructokinase, which should be inhibited during the gluceogenic flow of carbon through glycolate to sucrose.

2. Evidence is reviewed which suggests that glycolate or glyoxylate may participate in inhibition of the tricarboxylic acid cycle.

3. The concentrations of α-ketoglutarate and glutamate build up significantly in tobacco leaves in the light, and it may be that these acids function catalytically in the conversion of glyoxylate to hydroxypyruvate.

4. Although isocitrate is not thought to be a precursor of glyoxylate in the leaves of higher plants, it is in algae under certain conditions. The reverse, that

glyoxylate may be a precursor of isocitrate and glutamate in tobacco leaves, is discussed.

5. Malate dehydrogenase has been found in leaf peroxisomes, which are concerned with the metabolism of glycolate and related compounds.

During the course of the investigations, it was discovered that oxygen had a pronounced effect on the stability of P-glycolate phosphatase. The investigation of the effect of oxygen on the phosphatase was of particular relevance because, as outlined in the literature review, of the known requirement for O_2 for the synthesis of P-glycolate and glycolate, and possibly for the excretion of glycolate from the chloroplast.

LITERATURE REVIEW

Glycolate Biosynthesis

Some of the research described in this thesis concerns relationships between the tricarboxylic acids and P-glycolate phosphatase, and between oxygen and the enzyme. This first section of the literature review represents an attempt to at least partially discuss some questions which were suggested by some of the results described in this thesis. Some of these questions are as follows. In tobacco, could glycolate come from the tricarboxylic acids and in particular from isocitrate? What is the most probable source of glycolate? What effect does oxygen have on the synthesis of glycolate and on CO_2 fixation? Might such effects be related to the observed effect that oxygen had on P-glycolate phosphatase? Is the phosphatase necessary for the synthesis of glycolate?

Photosynthetic CO_2 Fixation

The status of the classical photosynthetic carbon cycle has been reviewed by Bassham (15). There has been further clarification of the details, but no alteration of the framework of the classical cycle as originally conceived (260).

Recently, certain higher plants have been characterized as having a C_4-dicarboxylic acid pathway in which the first products of $^{14}CO_2$ fixation are dicarboxylic acids. P-pyruvate is carboxylated to give C_4 labeled oxalacetate which is in rapid equilibrium with aspartate and malate. It is thought that the newly incorporated $^{14}CO_2$ is transferred from one of these dicarboxylic acids to an unknown acceptor molecule to give labeled PGA. A cyclic mechanism involving oxalacetate, pyruvate, and P-pyruvate is thought to substitute for ribulose diphosphate carboxylase, which is present in inadequate amounts in these plants. The subsequent spread of label from PGA follows a pattern consistent with the operation of the classical photosynthetic carbon cycle. In these plants, enzymes of the latter cycle, other than ribulose diphosphate carboxylase, are present in adequate amounts. The high activity of P-ribulokinase in these plants is indicative of a role for RuDP, but whether it functions as an acceptor for the transcarboxylation reaction, which has been proposed, remains to be determined (109, 110, 111, 112, 221, 222).

Besides low ribulose diphosphate carboxylase activity, plants in which the C_4-dicarboxylic acid pathway represents the main carboxylation mechanism during photosynthesis are characterized by high activities of P-pyruvate carboxylase and P-pyruvate synthetase, a unique type of leaf anatomy, and high rates of photosynthetic CO_2

fixation (109). Most of the plants characterized by the C_4-dicarboxylic acid pathway belong to one of two main subgroups of Gramineae. Although these plants are monocotyledons, not all monocotyledons contain the C_4-dicarboxylic acid pathway. Furthermore, several dicotyledons have recently been found to contain this new method of CO_2 fixation (109, 111). The dicotyledonous plant Nicotiana (tobacco), which was used for most of the investigations of this thesis, fixes CO_2 by the classical photosynthetic carbon cycle (111, 117).

If the dicarboxylic acid pathway is considered a modification of the photosynthetic carbon cycle, then with but few exceptions, this cycle is the only demonstrable mechanism for the total de novo synthesis of compounds from CO_2. The fluctuations in the level of ribulose diphosphate carboxylase activity with autotrophic and heterotrophic conditions, as well as the loss of CO_2 fixing ability in a mutant of Chlamydomonas reinhardii devoid of the carboxylase, are strong evidence for its obligatory role in organisms which contain the classical photosynthetic carbon cycle (94, 260). PGA and the sugar phosphates clearly account for most of the ^{14}C found in individual compounds following a few seconds of photosynthesis with $^{14}CO_2$. Nonetheless, as pointed out by Bassham, one might ask whether or not other important pathways of CO_2 reduction not involving these compounds have been overlooked.

Such a pathway would have to include substances which are so small in concentration as not to be seen, or which are so unstable as not to be isolated by the methods of paper chromatography. These possibilities were tested by Bassham and Kirk, who compared the rate of uptake of external $^{14}CO_2$ with the rate of appearance of ^{14}C in individual compounds. With Chlorella pyrenoidosa, it was found that labeling of PGA and the sugar phosphates accounted for at least 70% of the externally measured ^{14}C uptake between 10 and 40 seconds after the introduction of $^{14}CO_2$. The small pool of ^{14}C which was not accounted for was not more than the equivalent of 5 seconds of photosynthesis. Bassham suggests that this small pool was intracellular CO_2 and enzyme-bound CO_2. If an unknown path to carbohydrates through a pool of such small size existed, the carbohydrates would have become labeled much more rapidly than the experiments show (16).

Glycolate from CO_2

The source of glycolate formation during CO_2 fixation in photosynthesis remains unknown, although under certain conditions over 50% of the total fixed carbon may be passing through this metabolite (117, 233).

Effect of Light on the Biosynthesis of Glycolate

High light intensities are required for maximum

glycolate synthesis (233). It is not synthesized in the dark by tobacco (268) or excreted in the dark by Chlorella pyrenoidosa (120).

Effect of Oxygen on the Biosynthesis of Glycolate

Evidence has been presented that the biosynthesis of glycolate by Chlorella pyrenoidosa is a function of the concentration of externally applied oxygen (21, 26, 255). For this alga, the rate of glycolate synthesis, which was nearly zero under anaerobic conditions,[1] was found to be an approximately linear function of the concentration of externally applied oxygen from 0% to 99.97% (21, 255). The synthesis of P-glycolate was also stimulated by externally applied oxygen (21). Whittingham et al (256) reported that the synthesis of glycolate in the light by Chlorella from radioactive glucose was stimulated 10 fold in 99.97% O_2 compared to the control in air. Tolbert and Zill (238) found that Chlorella pyrenoidosa would synthesize but not excrete glycolate under nitrogen[1] while it would synthesize and excrete glycolate under a 99:1 mixture of N_2 and O_2. Their data suggest that the excretion of glycolate may itself require aerobic conditions. The requirement of oxygen for the excretion of glycolate by

[1] Since these experiments were conducted in the light, some oxygen must have been present from photosynthesis. Therefore, complete inhibition of glycolate synthesis could not be expected.

Chlorella pyrenoidosa was confirmed by Miller et al (161) and Hess et al (120), but these latter experiments did not indicate whether oxygen is required at the site of excretion as well as at the site of synthesis. Some direct evidence has also been presented which suggests that externally applied O_2 is necessary for the synthesis of glycolate in the leaves of higher plants. Using OHPMS, an inhibitor of glycolate oxidase, Zelitch and Walker (274) demonstrated that the accumulation of glycolate in Nicotiana tabacum was greatly enhanced in air as opposed to N_2 containing 0.03% CO_2. The authors concluded that an anaerobic atmosphere inhibits the synthesis of glycolate in tobacco leaves (125).

Data on photorespiration indirectly suggest that oxygen is required for the biosynthesis of glycolate in the leaves of higher plants. Photorespiration (defined as the light dependent evolution of CO_2) exhibits a dependence on the concentration of externally applied oxygen, falling off to nearly zero at an oxygen concentration of zero (88). Contrary to dark respiration which is essentially saturated at very low oxygen partial pressures, photorespiration is not saturated even in an atmosphere of 100% oxygen. In tobacco, photorespiration is a function of glycolate metabolism (272). Although the CO_2 fixation by leaves of Solidago multiradiata remained high, lowering the concentration of externally applied

oxygen did not result in an accumulation of glycolate (30), which it should have if the only site for the enhancement of photorespiration by oxygen were glycolate oxidase.

Effect of CO_2 on the Biosynthesis of Glycolate

Formation of ^{14}C-labeled glycolic acid in photosynthesis is favored by low CO_2 pressure (15). For Chlorella, the maximum glycolic acid production occurs when the gas phase (at one atmosphere) contains 0.1% CO_2 (250, 256). Whittingham et al estimated that the effective CO_2 concentration at the alga surface is about one-tenth of the CO_2 concentration in the gas phase. Thus the CO_2 concentration at the cell surface, for maximum glycolate production, would be about 0.01%. The synthesis of glycolate or glycolate products in the leaves of higher plants is also favored by similarly low CO_2 partial pressures (232, 233).

Warburg Oxygen Effect

Photosynthetic CO_2 fixation and O_2 evolution in some intact higher plants are inhibited about 30% in an atmosphere of 21% oxygen and 0.03% CO_2 as compared to nitrogen and CO_2. This inhibition by oxygen, referred to as the Warburg oxygen effect, is rapidly produced and is usually rapidly and fully reversible (29, 94, 95).

There is disagreement among investigators concern-

ing the effect of light and CO_2 concentration on this inhibition by oxygen. Although it has been reported that high light intensities and low CO_2 partial pressures (conditions which increase glycolate synthesis) increase the inhibition by oxygen (95), Björkman (29) has presented data showing that light intensity is without effect on the inhibition by oxygen and that CO_2 partial pressure is probably without pronounced effect.

Björkman (29) reported that algae do not show the inhibition by oxygen, but other investigators have reported that algae do show the inhibition (95). Apparently the intensity of the oxygen inhibition is influenced by the nutrition of the algal cultures (94). Egle and Fock (74) found that the reduction by oxygen of net CO_2 uptake, though present, was much less pronounced in algae than in higher plants. A possibly related observation was made by Hess and Tolbert (118), who reported that 5 strains of algae did not metabolize glycolate under their culture conditions.

Corn, sugar cane, and Amaranthus edulis are capable of reducing the CO_2 content in a closed system to zero, which suggests that these plants lack photorespiration, a phenomenon thought to be a function of glycolate metabolism. These plants do not exhibit the Warburg oxygen effect at 21% O_2 (30).

The Warburg oxygen effect may be the result of an

increased loss of carbon as glycolate from the sugar phosphate pools of the photosynthetic carbon cycle (21, 53, 94, 255) and/or the reduction of net CO_2 uptake and O_2 evolution because of CO_2 production and O_2 uptake from glycolate metabolism.

However, the Warburg effect is not a function of temperature in the leaves of Solidago and Mimulus while photorespiration was found to be strongly affected by temperature in tobacco (30). This and other arguments led Björkman to propose that the Warburg effect is not an expression of photorespiration. Inhibition by oxygen in some ways mimics inhibition by DCMU in that both inhibitors increase fluorescence and decrease the reduction of plastocyanin. This and other data led Björkman (29) to postulate that the site of O_2 inhibition is in the electron carrier chain between the two photosystems. On the other hand, Fewson et al (81) reported that the production of ATP coupled to the reduction of $NADP^+$ is not inhibited by oxygen.

Gibbs et al (95) have reported an essentially complete (and reversible) inhibition of $^{14}CO_2$ fixation by whole chloroplasts exposed to 21% oxygen. Thus, the metabolism of glycolate by the glycolate pathway should not be the only cause of the Warburg effect, since at the most, only ½ of the newly fixed CO_2 should be evolved by such a mechanism. The finding of Tolbert et al (237)

that glycolate oxidase from spinach leaves is associated with excess catalase in the peroxisomes makes it doubtful that glycolate products are completely converted to CO_2 by reacting with H_2O_2. However, complete inhibition of CO_2 fixation by oxygen could be the result of an increased loss of carbon as glycolate from the photosynthetic carbon cycle. High levels of oxygen concurrently produce increased rates of formation of glycolate and diminished levels of the intermediates of the photosynthetic carbon cycle in Chlorella (20, 21, 53, 255).

The finding that ascorbate and cysteine, but not fructose diphosphate, reversed the inhibition of $^{14}CO_2$ fixation by 1.5% oxygen in whole spinach chloroplasts suggests that there may be an interference by molecular oxygen with some component of the photochemical act (95). Since present knowledge of the chain transferring electrons from water to NADP points to spinach ferredoxin as the carrier most readily influenced by oxygen, Gibbs et al (95) favor the hypothesis that oxygen interferes with the formation of NADPH by reoxidizing reduced ferredoxin. In the presence of saturating levels of ferredoxin and large concentrations of $NADP^+$, air does not inhibit the formation of ATP or of NADPH by spinach chloroplasts. It has been established that reduced ferredoxin is autooxidizable but that in the presence of $NADP^+$ and molecular O_2, electrons are transferred to the former rather than to

the latter. Therefore, in the presence of sufficient accepter, the formation of NADPH is not affected by oxygen (95).

After presenting and reviewing evidence on the mechanism of inhibition of photosynthesis by high partial pressures of oxygen in Chlorella, Coombs and Whittingham (53) proposed that the oxidation leading to the formation of glycolate from the sugar phosphates of the photosynthetic carbon cycle is accomplished by H_2O_2 formed by a Mehler reaction between oxygen and ferredoxin. In the presence of normal photosynthesis with CO_2, the level of reduced ferredoxin is presumed to be too low to react with O_2 to produce H_2O_2. It was postulated that in the absence of CO_2 or at very low levels of CO_2, photosynthesis would not utilize the reduced ferredoxin rapidly and its level would increase to a point where it could react with oxygen. This proposal is consistent with the finding that high light intensity and the presence of oxygen increased the rate of glycolate production (20). A possibly related observation that FMN or FAD stimulated the synthesis of glycolate from fructose diphosphate by chloroplast preparations has been made by Bradbeer and Anderson (35).

Glycolate From the Photosynthetic Carbon Cycle

The kinetics of ^{14}C accumulation in labeled glyco-

late by algae (183) and by tobacco leaves (117) suggests that glycolate is formed as a product from one or more components of this cycle. The plot of the percentage of ^{14}C in phosphate esters (183) or PGA (117) extrapolates to nearly 100% at zero time, while the percentage in glycolate extrapolates to zero before zero time.

Feeding studies with ^{14}C labeled intermediates of the photosynthetic carbon cycle have indicated that glycolate may be formed from this cycle (35, 233). However, the recoveries of glycolate from intermediates of the cycle were low. Tolbert (233) has pointed out that small yields with crude enzymes are suspect, particularly since RuDP nonenzymatically decomposes into a multitude of products including C_2 pieces. In one such feeding study, Bradbeer and Anderson (35) concluded that "since there appears to be no strong evidence in favor of a sugar phosphate being the starting point of the pathway of glycolate synthesis in green plants, serious consideration must be given to the possibility of a de novo CO_2 fixation being the major pathway." However, studies have also been made with radioactive glucose fed to Chlorella pyrenoidosa in the light. For that fraction which was converted to glycolate, the C_1 or C_6 of glucose gave rise almost exclusively to the C_2 of glycolate while the C_2 of glucose gave rise almost exclusively to the C_1 of glycolate (256). The results are consistent with cleavage between C_2 and

C_3 of an intermediate of the photosynthetic carbon cycle so that carbon 2 of the intermediate became the carboxyl of glycolate. With oxygen as the gas phase and in the presence of isonicotinyl hydrazide, as much as 50% of the glucose incorporated may be converted to glycolate (255). That glucose can be fed into the photosynthetic carbon cycle and converted to glycolate under conditions of low CO_2 concentration and high oxygen partial pressure could account for the high glycolate production reported by Warburg and Krippahl (250, 53) and for the high production of glycolate by Chlorella pyrenoidosa in the light under O_2 in the absence of HCO_3^- (120).

Calvin and coworkers found that when glycolate-1-^{14}C was administered to Scenedesmus during 10 minutes of photosynthesis with $\frac{1}{2}\%$ CO_2 in air or N_2, a pattern of photosynthetic intermediates was found similar to that obtained during photosynthesis with $^{14}CO_2$. Upon degradation of the PGA, it was found that less than 10% of the radioactivity was in the carboxyl carbon. Thus, with this alga under the conditions used, some of the glycolate was incorporated into normal intermediates of the photosynthetic carbon cycle without preliminary conversion to CO_2, since so little ^{14}C was found in the carboxyl of PGA[1] (45).

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[1]The alpha and beta carbons of PGA were found to be equally labeled, as though the pathway from glycolic acid to these carbon atoms involves a randomization of the label. This could mean that along this pathway there is a symetrical intermediate, or that an intermediate is in rapid reversible equilibrium with a symetrical compound (45).

Calvin et al and Schou et al degraded glycolate and PGA obtained from barley leaves and from Scenedesmus that had photosynthesized for a few seconds in the presence of $^{14}CO_2$ or $H^{14}CO_3^-$. The alpha and beta carbon atoms of PGA were found to be always about equal to each other in radioactivity and always less than the carboxyl carbon of PGA, until such time (1 to 5 minutes) as all three carbon atoms were completely labeled. The two carbon atoms of glycolate were always about equal to each other in radioactivity (45). Glycolate from tobacco leaves consistently showed about equal distribution of label in both carbon atoms after various short periods of photosynthesis with $^{14}CO_2$ (117).

Hess and Tolbert found that in glycolate formed by 3 different algae in the first 5 and 10 seconds of $^{14}CO_2$ fixation, the C_2 was at least twice as radioactive as C_1. A similar skewed labeling was evident between C_3 and C_2 of PGA in the same experiments[1] (118). Such a relation-

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[1]The Gibbs effect, in which carbon atom 4 of hexose is more highly labeled than carbon atom 3 while carbons 1 and 2 are more highly labeled than carbons 5 and 6, is not inconsistent with the photosynthetic carbon cycle. Consideration of the two explanations for the Gibbs effect will show that the size of the effect will depend on pool sizes and the rate of the net forward reaction as compared with the rate of the reverse reactions. It is not surprising therefore that sometimes the Gibbs effect is observed and sometimes it is not (15). Sedoheptulose phosphate from soy bean leaves which had been exposed to $^{14}CO_2$ for very short times contained about 3 times more label in carbon 3 than carbon 4, which is consistent with the Gibbs effect (15). Since carbon 3 of sedoheptulose becomes carbon 1 of pentose while carbon 4 becomes carbon 2, the labeling pattern noted by Hess and Tolbert is not inconsistent with the photosynthetic carbon cycle.

ship between the labeling of glycolate and PGA is consistent with the concept that glycolate is formed from one or more intermediates of the photosynthetic carbon cycle, and suggests as possible precursors the following moieties of intermediates of the cycle: carbons 1 and 2 of the pentose, hexose, and heptose mono and diphosphates, the terminal two carbons of these compounds, and carbons 2 and 3 of the C_3 intermediates.

Certain tropical grasses, which lack a detectable photorespiration (which has been shown to be a function of glycolate metabolism (272)), are characterized by the C_4-dicarboxylic acid pathway for CO_2 fixation (111). These plants contain a nonclassical photosynthetic carbon cycle in that ribulose diphosphate carboxylase activity is inadequate. Whether or not the reduced photorespiration in the tropical grasses is due to a changed cycle in general, or deficient ribulose diphosphate carboxylase activity in particular, remains to be demonstrated.

A C_2-TPP intermediate formed in the photosynthetic carbon cycle from fructose-6-P, sedoheptulose-7-P, and xylulose-5-P by transketolase may exist in a common pool (16). Bassham et al (17) demonstrated that carbons 1 and 2 of ribulose, fructose, and sedoheptulose were uniformly labeled; these carbons are the precursors of the C_2 moiety transferred by transketolase. Wilson and Calvin (258), following their observation of glycolate accumula-

tion at low CO_2 pressure, suggested that this glycolaldehyde moiety transferred by transketolase is the source of glycolic acid. The enzymatic oxidation of glycolaldehyde-TPP (called more precisely 2-(1,2-dihydroxy ethyl)-TPP) to free glycolate has been demonstrated using artificial oxidants (86, 116). Such a reaction has not been demonstrated with natural oxidants or with an enzyme from photosynthetic tissue. However, further work with the pig heart enzyme, hydroxypyruvate decarboxylase, has revealed that it will oxidize glycolaldehyde-TPP to glycolyl CoA plus NADH + H^+ (87). The conversion of acetyl CoA to acetyl phosphate by phospho-trans acetylase from E. coli (254) suggests that an analogous formation of glycolyl phosphate from glycolyl CoA is possible. Calvin and Bassham have speculated that glycolaldehyde-TPP may eliminate water to give acetyl-TPP which could undergo phosphoroclastic cleavage to give acetyl phosphate and TPP (45). To my knowledge, elimination of two hydrogens rather than the elements of water, and subsequent phosphoroclastic cleavage to give glycolyl phosphate has not been reported.

Sugar diphosphates are not known to be cleaved by transketolase. Furthermore, RuDP does not have the necessary trans configuration of the hydroxyl groups between carbons 3 and 4 as is required by aldolases, transaldolases, and transketolases; however, FDP and SDP do have the neces-

sary trans configuration. P-glycolaldehyde might be a precursor for P-glycolate. Aldolase has been shown to catalyze the formation of xylulose diphosphate from glycolaldehyde phosphate plus dihydroxyacetone phosphate. The reverse reaction would produce the C_2-phosphate from carbons 4 and 5 of the pentose. During photosynthesis these two carbons are also equally labeled with ^{14}C. However, no data exist which implicates xylulose diphosphate in photosynthesis (233). A transaldolase type of reaction could similarly produce the C_2-phosphate from xylulose-5-phosphate. Although the substrates for transaldolase are monophosphates, to my knowledge xylulose-5-phosphate has not been shown to be cleaved by this enzyme.

Bassham and Kirk (21) have proposed that RuDP may be the precursor of P-glycolate. Ribulose diphosphate carboxylase cleaves the pentose between carbons 2 and 3. According to these authors it may be that at some early stage in this reaction mechanism, the moiety composed of carbons 1 and 2 can be oxidized, giving rise to P-glycolate. That glycolate synthesis is favored by low CO_2 partial pressures is consistent with RuDP being the possible precursor of glycolate, since RuDP increases in concentration under this condition (15). Furthermore, high O_2 partial pressures, which favor the synthesis of glycolate, greatly decrease the pool of RuDP (20, 21).

The administration of 8-methyl-lipoic acid to

Chlorella pyrenoidosa which was fixing $^{14}CO_2$ was found to cause a rapid and great increase in the pool sizes of FDP and SDP, as though fructose diphosphatase was inhibited in vivo by this compound. The evidence suggests that ribulose diphosphate carboxylase and ATP formation were also strongly inhibited. Inhibition of ATP synthesis was accompanied by an essentially unchanging pool of RuDP even though the carboxylase was inhibited. An increase in glycolate labeling matched these changes in the concentrations of intermediates of the photosynthetic carbon cycle (19). FDP or SDP are thus suggested as possible precursors for glycolate. Although the RuDP pool did not change appreciably, the inhibition of the carboxylase is consistent with the possibility that RuDP might also be the precursor for glycolate. Lipoic, octanoic, and methyl octanoic acids produced effects similar to those of 8-methyl-lipoic acid (19, 188). Gibbs has hypothesized that the Warburg oxygen effect is a function of glycolate synthesis (94) and the reoxidation of reduced ferredoxin (95). Evidence has been presented that the fructose diphosphatase of the photosynthetic carbon cycle is activated by reduced ferredoxin but not by oxidized ferredoxin (40, 41). Loss of activation by oxidation of ferredoxin might thus give the same pattern of phosphatase inhibition and enhanced glycolate synthesis as that resulting from the application of 8-methyl-lipoic acid.

Results from experiments with light dark transients also seem to favor one of the diphosphates as a glycolate precursor. The kinetics of incorporation of $^{14}CO_2$ into glycolate by Chlorella pyrenoidosa during a light to dark transition were very much like the kinetics of incorporation into RuDP and somewhat like the kinetics of incorporation into FDP and SDP, while the curves for the other intermediates of the photosynthetic carbon cycle do not look like the glycolate curve (189).

Davies et al (56) have postulated a biosynthetic route to uniformly labeled glycolate from PGA through 3 P-hydroxypyruvate, P-glycolaldehyde, and P-glycolate but very little evidence in support of the pathway is presented. Bradbeer and Anderson (35) reported from a survey of possible sources of glycolate, that hydroxypyruvate and P-hydroxypyruvate gave good yields of glycolate with chloroplast preparations. Further investigations established that these high yields resulted from a non-enzymatic reaction which required hydroxypyruvate, Mn^{++}, FMN or FAD, oxygen, and light. The large amount of glycerate dehydrogenase found in chloroplasts has prompted Gibbs et al to propose a pathway for the synthesis of glycolate from PGA to glycerate to hydroxypyruvate to glycolate (95). A pathway for photosynthetic serine biosynthesis by chloroplasts from P-glycerate and glycerate through hydroxypyruvate has been postulated by Chang and Tolbert.

The α and β carbons of hydroxypyruvate would be uniformly labeled but less highly labeled than the carboxyl carbon (48). Hydroxypyruvate labeled in this way would be decarboxylated by hydroxypyruvate decarboxylase of pig heart to give uniformly labeled glycolyl-CoA (87). Although this enzyme has not been isolated from plant tissue, it has been reported that Scenedesmus converted hydroxypyruvate-2-^{14}C to glycolate-1-^{14}C in the light. In the same experiments, no pyruvate-2-^{14}C was converted to glycolate (160).

The C_1-C_2 fragment of any intermediate of 4 carbons or longer or the terminal two carbons of any photosynthetic carbon cycle intermediate could serve as the precursor of essentially uniformly labeled glycolate. These moieties from the erythrose, pentose, hexose, and heptulose intermediates would all be at the glycolaldehyde level and would require the removal of two electrons per molecule of glycolate synthesized. PGA, after the removal of C_1 by decarboxylation, would require the removal of 4 electrons per molecule of glycolate synthesized, while the trioses, after decarboxylation, would require the removal of 6 electrons. Thus, every possible glycolate precursor of the photosynthetic carbon cycle would require at least one oxidation step in the course of the synthesis of glycolate. On the basis of the amount of oxidation required, the C_3 intermediates do not seem to be likely

glycolate precursors. Furthermore, conversion of the C_3 intermediates to glycolate would require evolution of 1/3 of the newly fixed CO_2 by decarboxylation. That the higher algae synthesize and excrete large quantities of glycolate, yet do not consistently exhibit CO_2 exchange with the surrounding gas phase in the light (30, 74), might rule out the C_3 intermediates of the photosynthetic carbon cycle as glycolate precursors.[1] Removal of the top two carbons of erythrose-4-phosphate as glycolate would leave a remainder which is not an intermediate of the cycle. However, removal of the bottom two carbons as glycolate would leave a remainder which is an intermediate of the cycle, would not unbalance the cycle, and would obviate the need for sedoheptulose mono and diphosphate and ribose-5-phosphate. But to my knowledge there is no precedent for such an enzymatic cleavage of erythrose-4-phosphate. Removal of the terminal two carbon atoms of the pentose, hexose, and heptulose intermediates would also leave compounds which are not intermediates of the cycle. Removal of a C_1-C_2 fragment of the pentoses, hexoses, or heptuloses would leave compounds which are intermediates of the cycle, and removal of any of these

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[1] When Coombs and Whittingham fed labeled glucose to Chlorella under CO_2 free O_2 in the light, CO_2 evolution paralleled glycolate synthesis. To my knowledge, glycolate production by algae without a concomitant production of CO_2 has not been demonstrated in the same experiment.

moieties as glycolate would not unbalance the operation of the cycle. Thus, on the basis of labeling, oxidation level, conservation of carbon, the nature of the remaining compound, and the balanced operation of the photosynthetic carbon cycle, a C_1-C_2 fragment of the 5, 6, or 7 carbon intermediates seems the most likely glycolate precursor. If P-glycolate is the precursor of glycolate, a C_1-C_2 fragment of FDP, SDP, or RuDP could be considered as the most likely precursor of glycolate.

P-Glycolate as the Precursor for Glycolate

The labeling of P-glycolate at early times[1] during photosynthesis in $^{14}CO_2$ (18, 21, 271), together with the discovery of a specific and highly active phosphatase for this compound (202), suggest that the precursor of glycolate could be P-glycolate which in turn could come from carbons 1 and 2 of an intermediate of the photosynthetic carbon cycle (233). That five strains of algae were found to contain P-glycolate phosphatase but not glycolate oxidase (118), together with evidence that algae are capable of excreting large quantities of glycolate (120, 250),

[1]The inconsistent observation of P-glycolate in photosynthesis experiments is possibly a reflection of the resistance of P-glycolate phosphatase to inactivation by methanol. Ullrich (243) has reported that terminating experiments in methanol does not prevent some hydrolysis of phosphate esters, and in particular the hydrolysis of P-glycolate.

suggest that P-glycolate may well be the precursor for glycolate. A similar argument is suggested from evidence that spinach chloroplasts excrete glycolate (131) and contain P-glycolate phosphatase (230, 236, 265), but not glycolate oxidase (236, 237).

The above evidence is also consistent with the hypothesis that P-glycolate phosphatase may be part of a permease system for the excretion of glycolate from the chloroplasts (202, 260, 265). Furthermore, evidence that inorganic phosphate is transferred only slowly across the outer chloroplast membrane, and that a major portion of the phosphorylated compounds still are present in the chloroplasts after 10 minutes of photosynthesis even though much carbon has been moved into the cytoplasm during this 10 minutes, has led to the proposal that phosphatases are present in the outer chloroplast membrane[1] and oriented in such a way that carbon is released into the cytoplasm while phosphate is released back into the chloroplast (182).

Glycolate from Isocitrate

Asada and Kasai (7) reported that in tobacco leaves, there is a link between isocitrate and glyoxylate, but

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[1]P-glycolate phosphatase has not been directly studied as part of a membrane system. Adenosine triphosphatase is one example of a phosphatase which has been directly studied in membrane fragments of various tissues (32).

their evidence leads to the conclusion that glyoxylate is a precursor of isocitrate in this tissue in the light rather than the reverse. Harrop and Kornberg (107) found that isocitrate lyase was a constitutive enzyme in the Brannon no. 1 strain of Chlorella vulgaris and that the alga excreted labeled glycolate during growth in the dark on cold glucose in the presence of $H^{14}CO_3^-$. The evidence supported the view that the glycolate excreted under these heterotrophic conditions in the dark derived ultimately from the cleavage of isocitrate and not, as postulated for algae growing autotrophically in the light, from the cleavage of pentose phosphate or possibly from direct condensation of C_1 units derived from CO_2. Results obtained with [1-^{14}C] acetate, added to similar cultures growing on glucose in the dark (or on CO_2 in the light), indicated that the glyoxylate cycle did not function under these conditions. In contrast, the organism would not excrete labeled glycolate during growth in the dark on cold acetate in the presence of $H^{14}CO_3^-$, and the incorporation of isotope from [1-^{14}C] ethanol by this alga growing on ethanol aerobically in the dark was consistent with the operation of the tricarboxylic acid cycle and glyoxylate cycle. No differences were observed between the properties of the isocitrate lyase purified from cells grown on acetate and glucose. But whereas isocitrate lyase was wholly found in a soluble fraction of the organ-

ism after growth on glucose or on carbon dioxide, acetate grown cells contained a major portion of their isocitrate lyase in a dense particulate fraction. Since the excretion of labeled glycolate ceased after transfer of the cells to acetate growth medium, it can be presumed that the glyoxylate formed from isocitrate now reacted with malate synthetase and acetyl CoA as part of the operation of the classical glyoxylate cycle. The results suggest that in the Brannon no. 1 strain, isocitrate lyase participates in the glyoxylate cycle when it is incorporated into a particulate fraction but it participates in glycolate synthesis and excretion when it is in the soluble form, and that culture of the cells on C_2 metabolites is necessary for the incorporation of isocitrate lyase into the particulate fraction.

In marked contrast to the Brannon no. 1 strain of C. vulgaris, isocitrate lyase was not a constitutive enzyme in the Pearsall's strain of Chlorella or in Chlamydomonas reinhardii, and experiments with Pearsall's strain of Chlorella indicated that this alga would not excrete labeled glycolate during growth in the dark on cold glucose in the presence of $H^{14}CO_3^-$ (107). Thus, although isocitrate apparently can be an important precursor for glycolate in at least one strain of Chlorella under certain conditions of growth, isocitrate as an important precursor of glycolate would seem to be the exception rather than the rule.

Glycolate from Acetate

Two pathways have been proposed for the formation of uniformly labeled acetyl CoA from the photosynthetic carbon cycle (16).

Experiments by Goulding and Merrett (100) with ^{3}H-^{14}C-acetate revealed glycolate as an early product of its photoassimilation in Chlorella pyrenoidosa, since the total dpm for ^{3}H and ^{14}C in glycolate reached a steady state in early samples. However, the conversion of acetate to glycolate resulted in a $2\frac{1}{2}$ fold increase in the ratio of ^{3}H to ^{14}C, presumably because of simultaneous incorporation of $^{12}CO_2$ into glycolate. This significant change in ratio was cited as evidence against a direct conversion of acetate to glycolate. Data was also presented which tended to exclude the conversion of acetate to glycolate by the glyoxylate cycle or the decomposition of acetate to CO_2 and refixation through the photosynthetic carbon cycle to glycolate. The possibility of a photochemically produced reducing compound formed by removal of hydrogen from acetate reacting with carbon dioxide to produce glycolate was not ruled out by the experiments.

Jagow et al (126) have reported that in rabbits, acetic acid may be hydroxylated to glycolic acid after being bound to p-aminopropiophene by microsomal hydroxylation of (4-propionyl)-acetanilide and hydrolysis of the hydroxylation product. To my knowledge, no direct conver-

sion of acetate to glycolate by oxidation has been reported in plants.

The de Novo Synthesis of Glycolate from CO_2

Several investigators have concluded that glycolate biosynthesis may not originate with the photosynthetic carbon cycle. From electron spin resonance signals on manganese-deficient Chlorella, Tanner et al (229) suggested that glycolate is a product formed directly from the condensation of 2 CO_2 molecules. Warburg and Kripphal (250) observed that Chlorella, during 1 hour of photosynthesis, converted 92% of the fixed CO_2 into glycolate. These results, however, may be in error owing to nonphotosynthetic metabolism of the algal carbohydrates to glycolate (53, 120). Zelitch (271) reported that after CO_2 fixation by tobacco leaves for 2 to 5 minutes, the glycolate was uniformly labeled and the specific activity of these carbon atoms was greater than the specific activity of the carboxyl carbon of PGA. Therefore, he suggested that glycolate must originate from a previously undetected CO_2-fixation pathway. In contrast, the possibilities that glycolate might be labeled before PGA or by a separate CO_2-fixation process in tobacco leaves were not indicated by the data of Hess and Tolbert (117). The reverse, that PGA could be the precursor of glycolate, is consistent with their data. The latter authors point out that

Zelitch required 20 to 30 seconds to move leaf tissue from the $^{14}CO_2$ fixation vessel through air containing $^{12}CO_2$ to the killing solution and that during this time, cold $^{12}CO_2$ would have replaced a significant amount of ^{14}C from the carboxyl group of PGA. Stiller (226) has presented arguments favoring a proposal for the de novo synthesis of a C_2 fragment from CO_2, which would condense with a triose to form a pentose directly, and she has proposed that this C_2 fragment is the precursor of glycolate. However, Bassham (15) has presented convincing arguments negating Stiller's proposals. Thus, in the eucharlotic cells of higher plants and algae, there is no well documented definitive evidence for the total de novo synthesis of glycolate from CO_2. Nor in these cells is there evidence for the total de novo synthesis of any compound from CO_2 by a mechanism other than the photosynthetic carbon cycle provided that the C_4-dicarboxylic acid pathway is considered a modification of this cycle. Nevertheless, until the enzymes for glycolate biosynthesis are known in these cells, the possibility will remain that CO_2 fixation for glycolate formation differs from that for PGA formation. As Stiller (226) correctly pointed out, the slower labeling of glycolate than PGA does not rule out the independent synthesis of glycolate. The observation that glycolate can be forced to accumulate in the absence of CO_2 does not argue against the de novo synthesis of glycolate, since

such reasoning makes the invalid assumption that glycolate can be formed either de novo or not at all.

In procaryotic cells, two mechanisms other than the photosynthetic carbon cycle have been discovered for the total de novo synthesis of compounds from CO_2. A ferredoxin dependent carbon reduction cycle has been reported in the photosynthetic bacterium, Chlorobium thiosulfatophilum, by Evans et al (78) that is essentially a reversal of the citric acid cycle. Two, 3, 4, 5, or 6 carbon compounds are synthesized de novo from CO_2 by the cycle. The presence of isocitrate lyase in this organism would make possible the de novo synthesis of glyoxylate from two molecules of CO_2, but isocitrate lyase was not reported. Secondly, the total synthesis of acetate from CO_2 has been reported in the anaerobe, Clostridium thermoaceticum, even though the reductive pentose cycle does not operate in this organism. Available evidence suggests that a Co-methylcorrinoid and a Co-carboxymethylcorrinoid are intermediates in the pathway from CO_2 to acetate. It is most probable that the conversion occurs via intermediate compounds linked directly to the cobalt of the corrinoids which are enzyme bound. Therefore, no intermediate compound occurs free in solution which may be detected by the usual tracer techniques such as employed by Calvin and others in the study of photosynthesis. The intermediate cobalt complexes are degraded in the light by photolysis

(146). It is of interest that the corrinoids, which are coenzyme B_{12} derivatives, are structural analogs of chlorophyll. The de novo synthesis of acetate via corrinoid intermediates thus brings to mind the well documented CO_2 requirement for the Hill reaction, the proposal by Warburg that *in vivo* the proposed cyclic participation of CO_2 in the photochemical reactions is unbalanced so that there is a net reduction of carbon (226), and the binding of CO_2 by chlorophyll (251).

Anderson and Fuller (2) have reported that glycolate appears to be the first stable product of CO_2 fixation in the procaryotic cell *R. rubrum* cultured photoheterotrophically with L-malate, and that unlike autotrophically grown *R. rubrum*, the contribution of the photosynthetic carbon cycle to the fixation of CO_2 is nil. However, the CO_2 fixation rates through glycolate were low, so that L-malate was a far more important source of cell carbon than was carbon dioxide.

Similarities Between O_2 Evolution and Glycolate Formation

Oxygen evolution and glycolate biosynthesis share several similarities. Manganese is required for glycolate synthesis (118) and oxygen evolution (132). The culture of *Chlamydomonas* or *Chlorella* for several days in blue light resulted in the increased synthesis of glycolate and an increased chlorophyll b to a ratio (119). Chloro-

phyll b is associated with photosystem II in higher plants, which is the photosystem closest to the site of O_2 evolution (132). Tolbert (233) has reviewed other similarities which exist between glycolate synthesis and O_2 evolution.[1] However, since glycolate synthesis is greatly decreased under anaerobic conditions while CO_2 fixation and O_2 evolution are increased (21, 95, 255, 274), it would seem that glycolate synthesis is not an obligate step in oxygen evolution. Oxygen synthesis could be an obligate step in glycolate synthesis, but even here the apparent requirement for external oxygen for the synthesis of glycolate (21, 255, 274), as opposed to oxygen or oxidants generated during photosynthesis, is not consistent with the concept of a close relationship between oxygen evolution and glycolate synthesis.

The Glycolate Pathway

This pathway[2] describes the path from P-glycolate through glycolate, glyoxylate, glycine, serine, hydroxypyruvate, glycerate, and phosphoglycerate in that order. The enzymes of the pathway, except for P-glycolate phosphatase, have been thought to occur in the cytoplasm,

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[1] The effect of phosphate on glycolate synthesis was later shown by Orth et al (183) to be an effect of pH and not a specific effect of phosphate.

[2] For a figure of the glycolate pathway, see the Ph.D. thesis of Hess (116).

which is consistent with the excretion of glycolate by chloroplasts. Presumably, conversion of PGA from the glycolate pathway to sucrose could occur in the cytoplasm by the gluceogenic functioning of the appropriate enzymes, including some of the enzymes of glycolysis (233). The rather extensive evidence in favor of the present formulation of the glycolate pathway has recently been reviewed (116). The experiments of Hess and Tolbert (117) with tobacco leaves are fully consistent with the operation of the pathway in that plant. Recent discoveries that the plants which possess the dicarboxylic acid pathway for CO_2 fixation do not exhibit photorespiration (111) or inhibition of photosynthesis at high oxygen concentrations (72) suggest that the glycolate pathway may not be as important in these plants as it is in plants such as wheat, tobacco, and soybean which possess the classical photosynthetic carbon cycle and exhibit photorespiration. Similarly, the glycolate pathway as it exists in these latter higher plants may not be as important in the higher algae (116, 118).

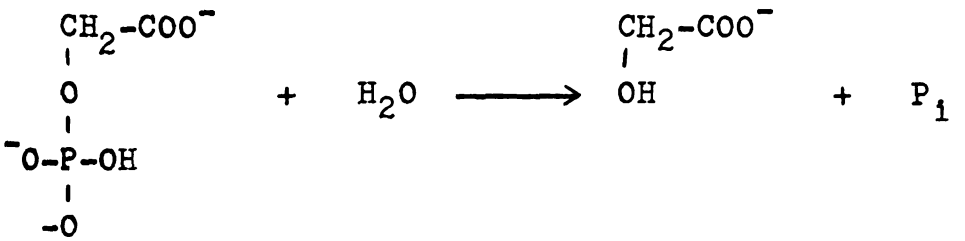
Although some of the enzymatic details for the conversion of glyoxylate to hydroxypyruvate through glycine and serine have been investigated, many remain to be elucidated. Experiments performed by Cossins and Sinha (54) with leaves of 15 day old wheat seedlings, which had been grown in a regime of light and dark periods

of 12 hours duration, provided evidence which strongly suggests that in this tissue, glycine is cleaved directly to yield N^5N^{10}-methylene-tetrahydrofolate, and that the C-2 of glycine can give rise to the C-3 of serine. It was proposed that glycine + tetrahydrofolate react to produce N^5N^{10}-methylenetetrahydrofolate + NH_3 + CO_2 and that the activated C_1 unit formed could then be utilized in the serine-hydroxymethyl transferase reaction, resulting in the formation of serine. These same authors (55), using nonphotosynthetic tissue, i.e. endosperm of 5-day-old castor bean seedlings which had been germinated in darkness for 5 days, conducted experiments which provided a background for a model of serine synthesis from glyoxylate in which half the glyoxylate is decarboxylated yielding the α carbon of glyoxylate as formate, which is then activated to a tetrahydrofolate adduct. It was proposed that the activated C_1 then condenses with glycine through a hydroxymethyl transferase reaction to form serine. Both of the above schemes for serine synthesis are in accord with the findings of Tolbert and Cohan (234) that the C-2 of glyoxylate is incorporated into the C-3 of serine in wheat and barley leaves, and with the finding of Hess and Tolbert (117) that in tobacco leaves at early times, the C_1 and C_2 of serine were uniformly labeled from $^{14}CO_2$, but C_3 was less highly labeled, presumably because in the synthesis of serine the C_3 carbon had to pass through at

least one more pool than did the C_1 and C_2 carbons.

Kinetic studies using ^{14}C and ^{15}N suggest that the primary site of ammonia incorporation in Chlorella pyrenoidosa is principally into glutamic acid, presumably via the reductive amination of alpha-ketoglutaric acid (20, 22). By the recycling of ammonia released in the conversion of glycine to serine, alpha-ketoglutarate and glutamate may thus play a catalytic role in the turnover of the glycolate pathway in the leaves of higher plants. Because alpha ketoglutarate is only one reaction removed from isocitrate, the latter hypothesis is relevant to the present thesis.

P-Glycolate Phosphatase



P-glycolate phosphatase was discovered in tobacco leaves by Richardson and Tolbert (202) in 1960. The enzyme purified from these leaves by ammonium sulfate and calcium phosphate gel fractionation was found to contain an endogenous divalent metal ion necessary for activity, and to have a pH optimum of 6.3 in the presence of this endogenous metal. After an additional DEAE purification step, the enzyme was active only toward P-glycolate and

completely inactive toward 21 other phosphate esters and anhydrides.[1] The purified enzyme, which released stoichiometric amounts of glycolate and phosphate from its substrate, exhibited a rather unique spectrum of reactivity toward sulfhydryl reagents and sulfhydryls. It was found to be strongly inhibited by p-chloromercuribenzoate but was unaffected by iodoacetate. On the other hand, cysteine and glutathione gave strong inhibition. An observation which may be related to this behavior toward sulfhydryl reagents and sulfhydryls is that at low concentrations, zinc has a considerably greater activating effect than does magnesium.[2]

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[1]The pronounced specificity of the hydrolases of phosphoryl compounds toward either polyphosphates, or phosphomonoesters, or phosphodiesters contrasts sharply with the lack of specificity shown by some phosphomonoesterases toward various phosphomonoesters (213). Thus, there is generally great specificity for one particular classification of phosphoryl compound, but often a lack of specificity within this classification. Some phosphomonoesterases however, especially those acting on sugar phosphates, are quite highly specific (69). Fructose diphosphatase from spinach chloroplasts is another example of a phosphatase active in photosynthesis which is highly specific toward its substrate (194).

[2]Zinc shows a relatively greater affinity for sulfur ligands than does magnesium (57). From the laws of chelation and atomic structure, there are reasons to believe that Cd^{++} and Hg^{++} will chelate with protein or coenzyme ligands that ordinarily chelate with Zn^{++} (216). Zn^{++} is likely to be coordinated to sulfur and nitrogen at physiological pH, but not to oxygen. Mg^{++} on the other hand is unlikely to be coordinated to anything other than oxyanions (75). It is known that zinc can bind to thiol groups in proteins (70), and sulfhydryl groups are thought to bind zinc to liver alcohol dehydrogenase (246). On the other hand, in the case of serum mercaptalbumin, zinc tends to be bound by imidazole groups (129).

Yu et al (265) discovered that P-glycolate phosphatase is present in large amounts in the green leaves of spinach, wheat, and alfalfa, as well as tobacco. The enzyme is also highly active in five of the higher algae (118). Little activity was detected in etiolated leaves or roots of wheat, but during greening of etiolated tissue in the light, the phosphatase activity developed, which would implicate the enzyme in photosynthesis. It was found that the phosphatase from all higher plants tested except tobacco was unstable toward ammonium sulfate fractionation.[1] In contrast to the tobacco enzyme purified by the method of Richardson and Tolbert, the wheat enzyme purified by acetone precipitation and DEAE-cellulose chromatography was completely inactive without added metal (265). Richardson and Tolbert (202) found that the pH optimum of the tobacco enzyme purified by their procedure was significantly shifted downwards from 6.3 in the presence of Mn^{++}, Zn^{++}, or Cu^{++}, which suggests that the endogenous phosphatase metal is not one of these. Ca^{++} is excluded by the kinetic evidence (202). Yu et al (265) found that the pH optimum of the enzyme purified by acetone precipitation and DEAE-cellulose chromatography was 6.3 with Mg^{++} as the added metal. This pH optimum is

[1]There seems to be no basis for the report that ammonium sulfate precipitation resulted in a shift in the pH optimum from 6.3 to 5.0 (265). Different metal cofactors shifted the pH optimum, however (202).

the same as that for the tobacco phosphatase purified by the method of Richardson and Tolbert which still contained endogenous metal. Taken together, these data suggest that Mg^{++} is the endogenous metal cofactor for the phosphatase.[1,2,3] On the basis of kinetic evidence, Mg^{++} or Mn^{++} were proposed as the possible endogenous metal by Richardson and Tolbert (202). The effect of Fe^{++} on the pH optimum has not been determined and kinetic data (202) does not exclude it as the endogenous phosphatase metal cofactor.

All phosphate monoesterases which have so far been studied cleave the P-O bond of the substrate (70, 213, 260). In this respect, the mechanism of phosphate monoester cleavage resembles the nonenzymatic hydrolysis of mono-ionized phosphoric esters by water. The mono-ionized phosphoric anion predominates at slightly acidic pH (127, 213).

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[1]It is of interest that Mg^{++} serves as an allosteric effector of the two photosynthetic carbon cycle enzymes, ribulose 1,5-diphosphate carboxylase (227) and fructose diphosphatase (194). Mg^{++} shifts the pH optimum of both enzymes from the alkaline to the neutral range and greatly increases the V_{max} values and decreases the K_m values of both enzymes at neutral pH.

[2]Chloroplasts isolated by the organic solvent technique were found to contain 50 to 72% of the total Mg^{++} of bean and tobacco leaves, respectively. The magnesium content of bean and tobacco leaves was determined to be 1 and 2% of the dry weight, respectively (194).

[3]The idea has been proposed that the known rapid and large scale light dependent fluxes of Mg^{++} could possibly have a bearing on the activities of various Mg^{++} dependent enzymes of spinach leaf cells (66).

The P-glycolate phosphatase isolated from wheat was found to contain endogenous cis-aconitate after it was purified by acetone precipitation followed by DEAE-cellulose column chromatography (Tolbert and Yu, unpublished data). The cis-aconitate could be removed by passage of the partially purified wheat enzyme through a Sephadex G-50 column. The enzyme without cis-aconitate was unstable but could be restabilized by adding back commercial citrate, isocitrate, or cis-aconitate. Cysteine partially stabilized the defactored wheat enzyme but was not as effective as the three tricarboxylic acids. Other acids tested were without significant stabilizing effect so that the effect of the three tricarboxylic acids appears to be rather specific. The evidence suggests that cis-aconitate may be associated with the wheat enzyme in vivo. Although an equilibrium mixture would contain about 91% citrate, 6% isocitrate, and 3% cis-aconitate (137), wheat contains almost twice as much aconitate as citrate. Only 2% of the aconitate is estimated to be in turnover pools in wheat while 15% of the citrate is estimated to be in such pools (199). The concentration of most of the intermediates of the citric acid cycle in various rat tissues was found to be of the order of 10^{-4}M. Much higher concentrations (up to 0.1M) can occur in plant tissues. Cells of baker's yeast were found to contain intermediate levels (about 10^{-3}M) of these acids (139).

Inhibition and Activation of Various Enzymes by the Tricarboxylic Acids

Citrate has been shown to inhibit a number of enzyme systems, including rat liver pyruvate carboxylase, DPNH-cytochrome c reductase, aldehyde oxidase, aspartase, and fumarase, but the effects were not specific for the tricarboxylic acid. Binding of protein bound metal at the active enzyme site appears to be the mechanism of inhibition of DPNH-cytochrome c reductase, and possibly aspartase (176). In the case of DPNH-cytochrome c reductase, the inhibition by citrate (or by pyrophosphate) was competitive with respect to cytochrome c (150). The inhibition of fumarase by citrate was competitive as was inhibition by trans-aconitate (159). Citrate was an inhibitor of inorganic pyrophosphate-glucose phosphotransferase and glucose-6-phosphatase activities which are thought to be catalyzed by the same enzyme. The inhibition was competitive with respect to the two phosphate substrates, glucose-6-phosphate and pyrophosphate, and non competitive with respect to glucose. Isocitrate was less effective and cis-aconitate was completely ineffective as an inhibitor of the latter enzyme (176). It was later found that "immediate" inhibition (i.e. no preincubation of enzyme with chelating agents) of microsomal glucose-6-phosphatase, competitive with respect to glycose-6-phosphate and pyrophosphate and non competitive with respect to glucose,

could be obtained with sodium azide, sodium cyanide, sodium oxalate, and 1,10-phenanthroline. "Immediate" inhibitions were prevented by titration of the metal binding agents with divalent cations prior to exposure of the former to the enzyme. An irreversible time dependent inhibition was noted in preincubation experiments with 1,10-phenanthroline, sodium azide, diethyldithiocarbamate, and 8-hydroxyquinoline. These observations suggest that glucose-6-phosphatase is a metaloenzyme, and that protein bound metal may participate in the binding of phosphate substrates, but not glucose, to the enzyme (175). These observations also suggest that as has been proposed for DPNH-cytochrome c reductase and aspartase, binding of protein bound metal at the active enzyme site was the mechanism of inhibition of glucose-6-phosphatase by citrate.

Cennamo et al (47) found that citrate and fumarate specifically inhibited pig heart mitochondrial malate dehydrogenase but did not inhibit pig heart supernatant malate dehydrogenase, as measured by oxalacetate reduction. Neither of the dehydrogenases were inhibited by 3.3 mM DL-isocitrate or cis-aconitate, or by the same concentration of a variety of other acids.

Although citrate competitively inhibited phosphoglucomutase (275) and intestinal alkaline phosphatase (79), the inhibition of these two enzymes could be reversed by the addition of relatively high concentrations of Mg^{++}.

It was therefore hypothesized that citrate exerted its effect by complexing the Mg^{++} required by each of the two enzymes as an activator (79, 275).

Citrate was found to be necessary for the activation of acetyl CoA carboxylase from animal tissue. Isocitrate was also very effective and malonate was effective. Fumarate was effective to an intermediate extent while cis-aconitate was completely ineffective as an activator. Activation was accompanied by an ordered aggregation of the enzyme. Phosphate was also effective in causing the ordered aggregation of the animal enzyme (103, 245). In contrast, wheat germ acetyl CoA carboxylase, unlike its counterpart in animal tissues, was not stimulated by dicarboxylic and tricarboxylic acids (43).

Deoxyribose phosphate aldolase from rat liver has also been found to be activated by di and tricarboxylic acids. Citrate, isocitrate, cis-aconitate, and trans-aconitate were about equally effective. Malate was about 2/3 as effective as the tricarboxylic acids while succinate, fumarate, and α-ketoglutarate were found to be somewhat effective in the activation of this aldolase (128). Although the purified aldolase showed a marked tendency to aggregate to form a dimer and trimer, the presence of an activating carboxylate ion did not influence the observed aggregation (104).

Very high concentrations of citrate (1-1.5M)

activated γ-glutamyl transpeptidase of kidney bean fruit. The possibility that association of subunits or dissociation to subunits occured in the presence of citrate was eliminated by the finding that the presence of citrate did not affect the sedimentation behavior of the enzyme. Other data indicate that citrate promoted a conformational change favorable for catalytic action. The activating effect was not confined to citrate alone, but was also shown by salts of some dicarboxylic acids and by EDTA (99).

Lanchantin et al (140) reported that very high concentrations of citrate (25% weight per volume) activated human prothrombin.

Citrate was found to be an important inhibitor of the phosphofructokinase from mammalian tissue and from yeast (209, 261). ATP and citrate cooperated in the inhibition of the yeast phosphofructokinase so that physiological concentrations of ATP increased the sensitivity of phosphofructokinase to inhibition by citrate (209). Similar data have been obtained for the liver enzyme (260). Isocitrate was similarly inhibitory of the yeast enzyme while α-ketoglutarate was not (209). Nevertheless, there is conflicting evidence in assessing the regulating agents in the inhibition of glycolysis under aerobic conditions (the Pasteur effect) in yeast. In contrast to the findings of Pye and Eddy, evidence has been presented that a transition from anaerobic to aerobic conditions resulted

in a great increase in the concentration of citrate plus isocitrate while the ATP concentration remained unchanged. Citrate was therefore proposed as the Pasteur effect agent in yeast (260). End product inhibition of mammalian and yeast phosphofructokinase activity by citrate (and ATP) is believed to constitute an important feedback mechanism for the flux from glucose-6-phosphate via pyruvate into the citrate cycle (176).

Switching mechanisms in the light and in the dark would seem to be required for fructose diphosphatase and phosphofructokinase from plants. The net effect of the uninhibited operation of both enzymes in the light would be activity equivalent to an ATPase resulting in the hydrolysis of photosynthetically produced ATP (20). Chloroplasts are supposedly not capable of glycolysis, (94) and to my knowledge there is no evidence for a phosphofructokinase in the chloroplasts. Fructose diphosphatase activity in the light is required in the chloroplast for the operation of the photosynthetic carbon cycle and presumably in the cytoplasm for the gluceogenic synthesis of sucrose from glycolate. In gluceogenesis, the balance between phosphofructokinase and fructose diphosphatase plays an important role in control (260). Two fructose diphosphatases have been reported, one in the chloroplast, and the other apparently in the cytoplasm (226). The phosphofructokinase of the cytoplasm would have access to

fructose-6-phosphate from the cytoplasmic phosphatase. Furthermore, since many of the intermediates of the photosynthetic carbon cycle seem to be exchangeable with the cytoplasm (20), even the chloroplast phosphatase could act with the cytoplasmic kinase to give ATPase activity if it were not for inhibition of the kinase in the light. Light dark kinetic studies with algae cells indicated that there was an inhibition of phosphofructokinase in the light and a release from inhibition in the dark. Although ATP has been shown to be an inhibitor of phosphofructokinase in mammalian and yeast cells, the level of ATP remained essentially constant in these light and dark experiments with algae (20, 189).

Phosphofructokinase from carrots was completely inhibited by 10 mM ATP and almost completely by 10 mM citrate. ATP and citrate showed a distinctly synergistic inhibition. Inorganic phosphate partially reversed the inhibitions by ATP and citrate, but unlike the enzyme from adipose tissue, citrate inhibition was not alleviated by AMP, ADP or 3',5'-AMP (61). Experiments with phosphofructokinase from the leaves of Brussels sprout yielded similar results. The authors concluded that P_i is probably the positive and citrate the negative effector for the plant enzyme (62).

Aconitase

Data is presented in the results section concerning similarities between aconitase and P-glycolate phosphatase, and a possible association between the two enzymes. This section of the literature review, on aconitase, represents an attempt to discuss some questions which were suggested by some of the results. Some of these questions are as follows. Is aconitase found outside of the mitochondria *in vivo*? Might the enzyme have functions other than participation in the tricarboxylic acid and glyoxylate cycles? Is the enzyme under metabolic control? Is aconitase stabilized by the tricarboxylic acids? How stable is aconitase to freezing and thawing, precipitation by $(NH_4)_2SO_4$ or acetone, and acid or alkaline conditions of pH? Might there be an evolutionary relationship between aconitase and P-glycolate phosphatase?What anions activate aconitase? What are the K_m values for the tricarboxylic acids? What is the effect of trans-aconitate on the enzyme? Does the enzyme have binding sites, other than the active site(s), for its substrates? What is its pH optimum? What are the effects of cations, sulfhydryl reagents, and inhibitors on the enzyme? What is the substrate specificity of aconitase? What is its mechanism of action?

Introduction and History

Aconitase (or cis-aconitate hydratase, or citrate (isocitrate) hydrolyase, E.C. 4.2.1.3) is an enzyme catalyzing the interconversion of citrate, cis-aconitate, and isocitrate, and was discovered by Martius and Knoop in 1937 (156). It was first found in higher plants (kidney beans, cucumber seeds) by Martius in 1939 and was later shown to occur in leaves of cabbage by Jacobsohn and Soares in 1940, and of rhubarb by Morrison and Still in 1947 (11).

The Function of Aconitase

The two most obvious requirements for cell maintenance and growth are a source of energy and a source of carbon skeletons. In aerobic organisms, including microorganisms and higher plants, both energy and carbon skeletons are supplied by the reactions of, and ancillary to, the citric acid cycle (33). In this cycle, the pathways of protein, fat, and carbohydrate catabolism are united.

Oil bearing seeds make use of the glyoxylate cycle for the conversion of fat to carbohydrate during germination. Other than enzymes of the citric acid cycle, isocitritase and malate synthetase are required to establish the cycle (23). In higher plants, isocitritase is present in those tissues which are actively converting fats to carbohydrates, and the malate synthetase activity of oil

seeds rises sharply during germination (33). Some malate synthetase activity has been detected in mature leaves of tomato, tobacco, and barley by Yamamoto and Beevers, but Carpenter and Beevers were unable to detect significant isocitritase in extracts of the leaves of tobacco, barley seedlings, or bean seedlings (270). Although the glyoxylate cycle is not thought to be in leaves, some speculation continues on this possibility. Thus Asada and Kasai (7) suggest that isocitritase may function *in vivo* in tobacco leaves, and evidence has been presented which suggests the possible participation of the glyoxylate cycle in *Sedum* leaves (200). The glyoxylate cycle is also necessary for the replenishment of the intermediates of the citric acid cycle when a 2 carbon compound such as acetate serves as the sole carbon source for growth. Such an application of the glyoxylate cycle is well established in *E. coli* (135), and under the appropriate conditions acetate apparently induces the operation of the glyoxylate cycle in several algae (167).

Aconitase seems to be an indispensible enzyme in the citric acid cycle, the glyoxylate cycle, and the ferredoxin dependent carbon reduction cycle (p. 32). There is evidence that the path through aconitase may be the only path available between citrate and isocitrate. Aconitaseless mutants have been isolated from the yeast, *Saccharomyces*, which are capable of converting various

substrates to the level of acetate, but which are incapable of degrading two carbon substrates to CO_2 via the citric acid cycle. The block at aconitase leads to citrate accumulation when glucose is metabolized. The inability to reach α-ketoglutarate via the citric acid cycle is expressed as a total growth requirement for glutamate in the presence of 18 other common amino acids (180). Mutants of Bacillus subtilus, devoid of aconitase, were asporogenic, and transfer of the defective aconitase gene to sporogenic strains by transformation caused the recipient strains to become asporogenic (228).

Aconitase is often required even under anaerobic conditions. An anaerobic citric acid cycle has been observed in the photosynthetic bacterium, Rhodospirillum rubrum (92, 93). All the reactions of the citric acid cycle, with the exception of 2-oxoglutarate dehydrogenase, have been demonstrated in the green obligately photosynthetic anaerobic bacterium, Chloroseudomonas ethylicum, where they act in a biosynthetic capacity (44). The anaerobic bacterium, Clostridium kluyveri, possesses aconitase and other enzymes of the upper half of the citric acid cycle, which are necessary for the synthesis of glutamate from citrate and precursors of citrate (224).

The Intracellular Location of Aconitase

In rabbit brain, aconitase is localized in the

mitochondria (214). In rat liver, however, over 80% of the aconitase activity is found in the soluble fraction after differential centrifugation in cold 0.25M sucrose (65, 197). The relatively small percentage of aconitase which is associated with rat liver mitochondria cannot be leached out by repeated washings with cold 0.25M sucrose (65). Schneider (214) pointed out that since small molecules such as citrate are kept within the mitochondria during isolation, it seems unlikely that large molecules such as aconitase should leak out, and the fact that citrate rather than any other citric acid cycle intermediate is found in the mitochondria might be interpreted in favor of a low level of aconitase in mitochondria (197). A case can be made for a functional role of a naturally occuring extramitochondrial aconitase. It may act in conjunction with extramitochondrial isocitrate dehydrogenase, in a shuttle of tricarboxylic and dicarboxylic acids to and from the mitochondria, to regulate the rate of oxidation. At the same time, aconitase and isocitrate dehydrogenase may function as a reductive system for extramitochondrial NADP (197). It is well documented that citrate is excreted from the mitochondria of mammalian tissues in significant quantities (9, 167).

Thus, although there is suggestive evidence in favor of a functional extramitochondrial aconitase in mammalian tissue, we cannot decide at present whether it

is an artifact of preparation or not (197).

Histochemical methods conducted with the human parasite, Trichomonas vaginalis, were able to demonstrate aconitase only in some of the organisms, where aconitase appeared in some of the cytoplasmic granules (presumably mitochondria) and along the nuclear membrane and parabasal body (218).

Eighty-five to ninety-five percent of the aconitase activity was found in the supernatant fraction after crude extracts from the leaves of broad bean, cabbage, and mustard were centrifuged at 40,000 g for periods up to one hour (11). Similarly, Pierpoint (193) found that most of the aconitase activity of tobacco leaf extracts was found in the supernatant fraction after differential centrifugation. On the other hand, Brummond and Burris (38) found that aconitase from the leaves of three weeks old Lupinus albus (Lupine), which had been ground in 0.2M sucrose, was found entirely in the fraction centrifuged down by 25,000 g for 20 minutes. In a preliminary communication, Breidenbach and Beevers (36) reported that glyoxysomes, isolated from castor bean endosperm by sucrose gradient centrifugation after isolation in a sucrose containing solution, contained all the enzymes needed for the net synthesis of succinate from acetyl CoA, with the exception of aconitase. The glyoxysomes were separated from the mitochondria, but the aconitase was present in neither the mitochondria nor

the glyoxysomes; rather it was found in the soluble fraction. In a note added in proof in a later, more complete report on glyoxysomes from the same tissue source, the authors (37) reported that "By providing SH protectants in the sucrose gradient, we have now shown that aconitase is indeed associated both with mitochondria and glyoxysomes."

Chloroplasts do not contain a cytochrome c oxidase system,and the prevailing view seems to be that chloroplasts do not contain a citric acid cycle (94). Leech (143) and Zelitch (270) have reported that some of the cellular malate dehydrogenase from photosynthetic tissue was associated with chloroplasts. More recently, Mukerji and Ting (168) found that malate dehydrogenase was associated with chloroplasts from cactus. Both aqueously and nonaqueously isolated chloroplasts, after washing and purifying by aqueous or nonaqueous gradient centrifugation, were found to contain the enzyme. However, Yamazaki and Tolbert (262), using the technique of isopycnic centrifugation in a sucrose gradient, found that part of the malate dehydrogenase from the leaves of 6 different plants fractionated with the peroxisome and mitochondrial fractions, but not with the chloroplast fraction. Ogren and Krogmann (177) and Leech (143) have presented evidence that isocitrate dehydrogenase may be present in chloroplasts. Although the absence of

cytochrome c oxidase in chloroplasts should rule out the classical functioning of tricarboxylic acid cycle enzymes which might be in the chloroplast, such enzymes could be used for the purpose of biosynthesis.

The Possible Role of Aconitase in Metabolic Control

A common type of metabolic control involves regulation of an enzyme catalyzing the first reaction after a metabolic branch point (8). In animal tissues, citrate is an important precursor of fatty acid synthesis (167), so that in these tissues, citrate is at a metabolic branch point. In plants, the importance of citrate as a precursor for fatty acid synthesis is unknown (167). Not all controlled enzymes have their substrates at a metabolic branch point. For example, the affinity of fumarase (an enzyme similar to aconitase) for fumarate is very strongly decreased by ATP even though fumarate is not known to occupy a metabolic branch point of any significance (8).

Evidence that the conversion of citrate to glutamate is blocked in the light in mung bean leaves has been obtained by Graham and Cooper (101). Cook and Carver (52) found that light decreased the activity of aconitase, but not isocitrate dehydrogeanse, by 50% in green Euglena, but not in the permanently bleached mutant. On the other hand, when pyruvate 3-^{14}C was supplied to the cut ends of wheat seedlings in the light, the label in glutamate con-

firmed the operation of the Krebs cycle in this tissue in the light (172).

A possible physiological role of glyoxylate in the control of the citric acid cycle is suggested by the work of Ruffo et al (205, 206), Payes and Laties (187), and Laties (141). These workers have demonstrated that oxalomalate, the product of the condensation of glyoxylate and oxalacetate under physiological conditions, and γ-hydroxy-α-ketoglutarate, the decarboxylation product of oxalomalate, are remarkably effective inhibitors of citrate oxidation. Both compounds are competitive inhibitors of aconitase, so that control by these compounds would not be an example of the type of control in which the concentration of a modifier affects the substrate affinity of an enzyme. Nevertheless, oxalomalate at 1 mM inhibits aconitase and isocitrate dehydrogenase almost completely (206). Zelitch and Barber (273) found that glycolate or glyoxylate inhibited the oxidation of Krebs cycle acids by particles from Spinach leaves. Marsh et al (154) found that light had no detectable effect on the turnover of the citric acid cycle in the alga, Scenedesmus obliquus. However, Hess and Tolbert (118) presented evidence suggesting the absence of glycolate oxidase, and an incomplete glycolate pathway in algae. The enhanced production of glycolate in the light may partially explain the inhibition of the Krebs cycle that is sometimes observed in some plants in

the light (73, 273).

The work of Hoch et al (123) and Forrester et al (88, 89) provides evidence that dark respiration may indeed be inhibited in the light. But the work of Santarius and Heber (210) suggests that the inhibition of glycolysis and of dark respiration in leaf cells in the light may be because of the changed ratio of ATP to ADP, both of which are permeable to the chloroplast membrane. Furthermore, the work of Graham and Walker (102) demonstrates that the ratio of malate to oxaloacetate is significantly increased in mung bean leaves in the light as a consequence of the increased ratio of NADH to NAD, and Mudd (167) has suggested that the apparent inhibition of the tricarboxylic acid cycle in the light may be due to the diversion of acetyl CoA to fatty acid synthesis in the presence of ample reductant. Citrate synthetase, which seems to be the locus of the slow rate determining step for the turnover of the citric acid cycle (139), is inhibited by high ATP concentrations in tobacco and in mammalian tissue (8), and the concentration of pyruvate, which is freely permeable to the chloroplast membrane, is known to decrease in the light and increase in the dark (210). Graham and Walker (102), using mung bean leaves, found that the concentration of citrate, which had been labeled by the dark fixation of $^{14}CO_2$, decreased significantly after 30 minutes in the light and again increased after 30 minutes in a following dark period,

while the concentration of the labeled glutamate changed relatively little during the same transitions.

Thus, control of the catabolic functioning of the citric acid cycle by the inhibition of aconitase and/or isocitrate dehydrogenase would seem inconsistent with the latter findings, and at least partially superfluous in view of the possible control of glycolysis and dark respiration by other mechanisms. Dark respiration may be completely inhibited in the light in corn (89), yet the negligable photorespiration in this plant (89), together with evidence that photorespiration is a function of glycolate metabolism (272), suggest that glycolate metabolism may not be important in the control of dark respiration in corn.

The possible inhibition of aconitase in the light, which is suggested by the work of Graham and Cooper (101), Cook and Carver (52), Ruffo et al (205, 206), Payes and Laties (187), Laties (141), and Zelitch and Barber (273), either by the condensation products of glyoxylate and oxalacetate or otherwise, might be for a less obvious reason than the control of the catabolic functioning of the citric acid cycle.

The concentrations of glutamate and alpha ketoglutarate in tobacco leaves were found to follow diurnal variations. The maximum glutamate concentration per gram fresh weight, which occured between 10 A.M. and 2 P.M., was

about 4 times greater than the minimum which occured between midnight and 4 A.M. (173). The maximum alpha ketoglutarate concentration per gram dry weight also occured during daylight hours (174). Burns et al (42) have recently obtained labeling patterns for glutamate in the leaves of Nicotiana rustica after exposure of the plants to $^{14}CO_2$ in the light. The investigators thought that the labeling data for glutamate were not easily explained by a combination of the photosynthetic carbon cycle and tricarboxylic acid cycle.[1] The data were also not compatible with 4 other pathways to glutamate which had previously been proposed. The authors pointed out that their data could be explained by assuming a rapid formation of symmetrically labeled glycolate and its subsequent conversion to glutamate, either via glyoxylate, oxalomalate, γ-hydroxy-α-ketoglutarate, and α-ketoglutarate, or by condensation of glyoxylate with pyruvate to give γ-hydroxy-α-ketoglutarate directly. Since it is assumed that isocitrate lyase is absent in the leaves of higher plants, the proposal of Burns et al (based on experiments with tobacco leaves in the light) might explain the observations of Asada and Kasai (7) that

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[1]The data and proposals of Burns et al, which were in a preliminary communication, have not been confirmed by the authors or by groups from other laboratories. The labeling times were 3 minutes and 18 minutes, which seem long for kinetic labeling experiments.

glyoxylate was a precursor of isocitrate in tobacco leaves in the light, and that the labeled isocitrate was apparently not converted to labeled citrate.

The Purification of Aconitase

To date, most work has been done with aconitase from pig heart. It remains as one of the major metabolic enzymes which has not been obtained as a stable homogeneous preparation. The purification of aconitase has been greatly hampered by its apparent instability (162). Krebs and Eggleston found that glycerol stabilized crude enzyme extracts, but Buchanan and Anfinsen reported that glycerol was without effect in stabilizing purified preparations of aconitase (39). Substrate was found to be the most effective stabilizer of aconitase (5, 39, 114, 162). Henson and Cleland (114) found that for 20-fold purified beef liver aconitase, both citrate and ammonium sulfate were required for good recovery of activity after storage at -20°.

Aconitase from pig heart has been purified 24-fold by Morrison (162) by low temperature ethanol and ammonium sulfate fractionation combined with heat fractionation. Citrate was included in all purification steps except the first two ethanol fractionations. Electrophoresis of the final preparation permitted its purity to be estimated at 75-80%. When the enzyme was

dissolved in 4×10^{-3}M citrate, it was stable for 15 minutes at any temperature up to 51°. From 51° to 55.5°, 15 minutes of heating destroyed the activity so that no activity survived at 55.5°.

Aconitase from mustard leaves has been purified about 200 fold by ammonium sulfate fractionation, DEAE-cellulose chromatography, and gel filtration chromatography (185). Citrate was included in all purification steps. The best preparation was homogeneous in the ultracentrifuge with $s_{20} = 4.7S$. Sedimentation of the enzyme after partial decay of activity provided no evidence that its instability is due to a major change in the protein structure. The final specific activities of the mustard aconitase were roughly 10% of those obtained with the pig heart aconitase purified by Morrison.

Marked loss of pig heart aconitase activity occured when the pH of the clarified crude extract was adjusted below pH 5.0 (162). The pig heart enzyme was unstable toward freezing at the earliest stage of purification, but the purified enzyme was unaffected by repeated freezing and thawing or by freeze drying (162). In contrast, the 200-fold purified aconitase from mustard was totally inactivated by freezing or freeze drying (185).

Morrison reported that the fractionation of purified aconitase from pig heart with acetone resulted in a marked loss of activity even though carried out between

-5° and -10° (162). The previous purification included two ammonium sulfate fractionations; however the final preparation had been dialysed against 0.004M citrate to remove salt. This finding is in contrast to that of Buchanan and Anfinsen (39) who reported that aconitase may be precipitated by alcohol or acetone without loss of activity, provided the temperature is kept sufficiently low. These authors used ammonium sulfate fractionation as a last step after fractionation by alcohol. They also reported that such high concentrations of alcohol are required to precipitate the enzyme in the presence of salts that denaturation results. Thus, it may be that aconitase from pig heart is stable to organic solvents before ammonium sulfate treatment, but not after, even if the salts are removed.

Evidence that Aconitase is One Enzyme

Morrison found that during the purification of aconitase from pig heart, the ratio of the activities, measured by the conversion of isocitrate to citrate and cis-aconitate to citrate, stayed constant. Furthermore, the partially purified aconitase, when further isolated as an electrophoretically homogeneous protein, was capable of converting both isocitrate and cis-aconitate to citrate (162). Morrison's results were a confirmation of the same findings by Buchanan and Anfinsen (39). In

addition, the latter authors reported that the activities of the enzyme, with respect to both citrate and isocitrate formation from cis-aconitate, decayed at the same rate. Similarly, during the 200-fold purification of aconitase from mustard, the ratio of the activities, measured by the conversion of citrate and of isocitrate to cis-aconitate, stayed constant, and these two activities decayed at the same rate (185).

Ogur et al (180, 181) have isolated two mutants (glt_{1-1} and glt_{2-1}) from the yeast, Saccharomyces, which exhibited a blocked citric acid cycle due to the lack of aconitase and which were dependent on added glutamate. When the two mutant strains were mated, glutamate independent hybrids were always obtained. Tetrad analysis of the four-spored asci obtained from these diploid hybrids showed a high frequency of prototrophic recombinants and the independent assortment of the glt_1 and glt_2 markers predicted for unlinked genes. Attempts to obtain complementation *in vitro* by mixing cell free preparations from the two mutants were negative. Although the work of Ogur et al suggests that aconitase in yeast may include two proteins, the work of Tomizawa (240) with isolated aconitase from yeast suggests that it is one protein. Neilson (169, 170, 171) has reported an enzyme from *Aspergillus niger* which catalyzes the interconversion of cis-aconitate and citrate, but which is inactive in the interconversion

of cis-aconitate and isocitrate. The enzyme (E.C. 4.2.1.4 aconitic hydrase, citrate hydrolyase, also called citrate dehydratase) is also present in Penicillium chrysogenum and to a lesser extent in cucumber leaves (171). Aconitase is present in Aspergillus niger but can be separated from aconitic hydrase (169, 170, 171). To my knowledge, no enzyme has been reported which catalyzes only the interconversion of cis-aconitate and isocitrate.

The Effect of Ions

The pH optimum of aconitase isolated from mammalian tissue is influenced by the ions present in solution. For the isocitrate to citrate, isocitrate to cis-aconitate, and cis-aconitate to citrate reactions, the pH optima were the same in the presence of a particular buffer and were altered to the same extent in different buffers. The range in the pH optima for the 4 buffers tested was 7.5 to 8.6, while the pH optimum with no buffer present was estimated at 8.0. For each of the three reactions, the absolute rates were approximately the same at the pH optimum for each buffer (165). Thomson et al (231) found that aconitase isolated from beef liver had a pH optimum of 7 in 0.02M glycine buffer, but that the pH optimum was shifted to 8 when 0.2M NaCl was included. Furthermore, the maximum activity with NaCl present was 1.4 times greater than the maximum activity when it was absent.

Peters (191) found that increasing amounts of NaCl dramatically increased the activity of aconitase from pig heart, while KCl at low concentrations increased the activity and at high concentrations decreased the activity. Aconitase isolated from mustard is reported to have a pH optimum of 8.0 at low isocitrate concentrations, and 8.5 at high isocitrate concentrations. Increasing the isocitrate concentration also had an activating effect (185).

pH Optimum

For the interconversion of the tricarboxylic acids, rat liver mitochondria exhibited a biphasic pH optima curve, with optima at pH 5.8 and about 7.3 (65). Aconitase which could be released from the mitochondria exhibited the same single alkaline pH optimum as the soluble aconitase. The authors favored the idea that the lower pH optimum was a consequence of increased permeability of substrate through the mitochondrial membrane, although the alternative that the structural binding of aconitase may alter its pH optimum was not excluded.

The Effect of Metals, Chelating, and Reducing Agents

Iron binding agents such as o-phenanthroline and α,α'-bipyridyl inhibit the animal aconitase, which is consistent with the concept that the native enzyme contains

iron which is necessary for activity (64). Chelating agents affect the animal aconitase only after preincubation. The inhibition can be reversed by excess Fe^{++} but not by excess substrate (115). Thus, it appears that substrate prevents inhibition by chelating agents but will not reverse it.

Dickman and Cloutier (64) found that aconitase from pig heart, which had lost activity during purification, could be reactivated by preincubation with Fe^{++} plus cysteine or ascorbate. No other metal would substitute for iron. Glutathione was about half as effective as the other two reducing agents. Morrison (162, 163) confirmed and extended these findings with the enzyme from pig heart. With respect to its ability to convert both isocitrate and cis-aconitate into citrate, the addition of Fe^{++} and cysteine increased the two activities equally. The activating effect of Fe^{++} and cysteine during the early stages of purification was small and remained reasonably constant. After dialysis, the activation was greatly enhanced.[1] Fe^{++} and cysteine can, therefore, completely replace the dialyzable prosthetic group. It would appear that not only is iron capable of activating

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[1]The dialysis was performed against 0.004M citrate. Apparently, citrate was required to maintain the enzyme in a stable configuration even while it became inactive from loss of Fe^{++}.

pig heart aconitase, but it is also the metal with which the enzyme is associated in vivo (162). Buchanan and Anfinsen (39) reported that cysteine alone, in the initial stages of the purification, showed a stabilizing effect on pig heart aconitase, but as the purification of the enzyme continued, cysteine alone strongly inhibited the enzyme.[1] A similar observation was made by Herr et al (115) who found that when cysteine alone was preincubated with a freshly prepared enzyme solution, a marked loss of activity occured.

The activation of pig heart aconitase in the presence of reducing agent (10^{-2}M) by Fe^{++} followed a Michaelis Menton saturation curve, suggesting that one iron is bound per active site to form the active enzyme (163). The K_A for Fe^{++} in the presence of cysteine is 3.9×10^{-6}M and it is 1.7×10^{-5}M in the presence of ascorbic acid. The activation of the enzyme in the presence of Fe^{++} (5×10^{-4}M) by cysteine, thioglycolate, and ascorbate also followed Michaelis Menton saturation curves, suggesting that one molecule of reductant is required per active site to form the active enzyme. The

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[1] Dickman and Cloutier (64) found that cysteine alone activated pig heart aconitase, but it was later found by Morrison (162) that the preparation of cysteine which gave similar results to those of Dickman and Cloutier contained appreciable amounts of iron. Morrison (163) found that cysteine alone had no activating effect on the inactive purified dialyzed aconitase. He also found that it was not necessary to add Fe^{++} and cysteine as stabilizing agents (162).

K_A values are 2.3-3.6 x 10^{-3}M for cysteine, 4.6 x 10^{-3}M for thioglycolate, and 1.2 x 10^{-3}M for ascorbate. The amounts of the reducing agents present, and the fact that the redox potentials are such that essentially all the iron present, for all concentrations of reductant, was in the Fe^{++} state, suggest that the reductants must have a function in addition to keeping iron in the Fe^{++} state. The V_{max} was dependent on the nature of the reductant, suggesting that not only Fe^{++} and enzyme, but also reductant, participate in the active complex, a concept which is strengthened by the finding that the dissociation constant of the Fe^{++} enzyme complex varies according to the reducing agent present. The kinetic evidence that only one reductant was required per active site and the finding that arsenite does not inhibit aconitase suggest that the function of the reductant is not the cleavage of S-S on the enzyme (163).

Speyer and Dickman (223) postulated that Fe^{++} and reducing agent take part in an enzyme-metal-substrate-reducing agent complex and also that Fe^{++} acts as an electron acceptor or Lewis acid which aids in lowering the activation energy in the removal of OH^- from citrate or isocitrate. But whether Fe^{++} participates in the dehydration mechanism directly or indirectly is still unknown (203). The picture is further complicated by evidence that the pig heart enzyme reconstituted by the

addition of iron and reducing agent may be different from the native enzyme (191).

Bacon et al (10) grew mustard plants in nutrient solutions containing different concentrations of iron. The leaves of those plants receiving the least iron, which always showed signs of iron deficiency, exhibited about one-half the aconitase activity per gram of leaf as those from non-deficient plants. Simultaneous measurements of malate dehydrogenase and fumarase showed that these activities were not significantly affected by iron deficiency. The aconitase activity of extracts from the deficient plants could not be increased by treatment with Fe^{++} and cysteine, and mixtures of extracts from normal and deficient plants did not show an activity greater than the sum of the activities measured separately. The authors postulated that the lower aconitase activity in iron deficient plants is due to a lack of the enzyme system as a whole and not merely to a lack of iron (10). Palmer (185) found that the 200-fold purified aconitase from mustard was not activated by Fe^{++} and cysteine. Under certain conditions, partially purified preparations could be activated to some extent. Dialysis of the enzyme against citrate buffer did not lead to any measurable loss in activity. Cysteine alone had no effect on the stability of the plant aconitase. Preincubation with o-phenanthroline caused a slow loss of activity compared to the

control, which the author postulated may have been due to a nonchelation effect.[1] The purified enzyme did not contain a significant amount of iron. Palmer (185) concluded that iron does not play a direct role in the activity of plant aconitase and supported the earlier conclusion that the lower activity of aconitase in iron deficient leaves is because of an indirect effect on the enzyme (10).

Rahatekar and Rao (198) reported on the Fe^{++} plus cysteine dependent activation of aconitase from bacteria, molds and plants. The activation was measured using crude preparations which had been concentrated by ammonium sulfate precipitation. Aconitase from E. coli was activated 2.3 to 8-fold by Fe^{++} plus cysteine, the enzyme from Aspergillus niger 1.8-fold, and the aconitase from pea (Pisum sativum) and cholai (Vigna catjang) three day old etiolated seedlings 1.2 to 2.3-fold. The aconitase from cholai cotyledonous tissue, grown in tissue culture, was stimulated 3 to 9 fold. The aconitase from all the sources tested showed significant inhibition by phenanthroline. These activations, though significant, are relatively small compared to those which have been reported for the aconitase from animal tissue (163).

Growth of Aspergillus niger on a chemically defined

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[1] Glutamate dehydrogenase is inhibited by o-phenanthroline and also by analogs of this compound which do not possess chelation properties (264).

medium containing no manganese yielded a mycelium that apparently was free of both aconitase and aconitic hydrase, and which gave an accumulation of citric acid. When the medium was made 3 μg percent in manganese, the mycelium contained aconitic hydrase activity but lacked aconitase activity. Doubling the manganese concentration in the medium to 6 μg percent yielded a mycleium with some aconitase activity along with aconitic hydrase activity. When manganese was added to extracts from the mycelium grown on the manganese deficient medium, the two enzyme activities were still absent. Thus manganese is apparently required for formation of the two enzymes rather than for direct activation (170). The aconitase of Chlorella vulgaris has been reported to show a dependence on manganese similar to the dependence found with Aspergillus niger (28). These same investigators reported that an iron deficient culture medium for Chlorella increased the aconitase activity, which is in contrast to the effect of iron deficient growth conditions on the aconitase of mustard.

Morrison (162) reported that incubation of the Fe^{++}-cysteine activated aconitase from pig heart with phosphate buffer at pH 7.4 at 30° essentially completely inactivated the enzyme in 30 minutes, presumably due to the formation of the insoluble iron phosphate complex. The water control lost little activity. Similar results were obtained

with veronal-acetate, borate, and glycero-phosphate buffers at pH 7.4. The inactivation by incubation with buffers could be completely prevented with substrate (162, 165). Therefore, when it was desired to maintain activity, reactions were initiated by adding enzyme to the buffer substrate mixture (165). In contrast to Morrison's findings, Dickman and Cloutier (64) found that phosphate did not inactivate the iron-cysteine activated enzyme from pig heart. However, they did find that phosphate prevented the activation of aconitase by iron plus cysteine, and that bicarbonate had this same effect. Phosphate buffer had no inactivating effect on the aconitase of higher plants compared to Tris buffer (11).

Inhibitors

p-Mercuribenzoate at 0.00053 mM or $HgCl_2$ at 0.0011 mM inhibited aconitase 50%. Iodoacetate at 10.0 mM or arsenite, a reagent for vicinal sulfhydryls (84, 85, 217), at 10.0 mM gave no inhibition of aconitase. The enzyme had been activated by Fe^{++} and ascorbate and the inhibitors were at the concentrations indicated during the 5 minutes of preincubation at the reaction temperature of 30°. Inactivation of aconitase from pigeon breast muscle with p-mercuribenzoate could be reversed with glutathione (63). Aconitase from pig heart is strongly inhibited by cyanide and sulfide, possibly because of the presence of Fe^{++} as

a cofactor, and is also inhibited by low concentrations of Cu^{++} ions (5, 63). Inclusion of Cu^{++} with manganese in the growth medium prevented the formation of both aconitase and aconitic hydrase in Aspergillus niger (170). Ten minutes of preincubation of aconitase from mustard with 1 mM iodoacetate had no effect on the activity (185). Aconitase in centrifuged extracts of broad bean, cabbage, tomato, mustard, Heracleum, Hosta, and Sambucus lost 50-75% of its activity when preincubated with 1-10 mM cyanide for 30 minutes at 0^{o} (11).

In a study of aconitase from rat kidney, Fanshier et al (80) found that of the four possible fluorocitrates, the isomer from the enzymatic synthesis starting with fluoroacetyl CoA and oxalacetate is the inhibitory species. Two kinetic mechanisms are responsible for the inhibition of this aconitase by fluorocitrate: (a) direct competitive inhibition, detectable by initial rate measurements, and (b) time dependent progressive inhibition. With sonic extracts as the enzyme system, fluorocitrate inhibited the in vitro conversion of citrate to cis-aconitate, isocitrate to cis-aconitate, and citrate to isocitrate, but for some reason did not produce direct competitive inhibition of cis-aconitate to α-ketoglutarate when the aconitase reaction was coupled to isocitrate dehydrogenase. Peters (191) reported that the time dependent progressive inhibition of the pig heart aconitase by fluoro-

citrate could be partially reversed by high concentrations of substrate. Sycamore aconitase is inhibited by fluorocitrate at a much higher concentration (about 2000-fold) than that needed to inhibit pig heart aconitase. The inhibition is qualitatively similar to the inhibition of pig heart aconitase in that the inhibition is slow to develop, requiring 10 to 20 minutes at 25° to reach a maximum, but once developed, the inhibition of the plant aconitase can be largely reversed by high concentrations of substrate (242).

Substrate Specificity

Of the substances present in biological material, only isocitrate, citrate, and cis-aconitate are substrates for aconitase (63, 139, 219). There appear to be no significant competing reactions in crude pig heart tissue extracts which might lead to error in the assay of aconitase (5).

Binding of Tricarboxylic Acids to Aconitase

Henson and Cleland (114) reported that citrate, which was added during purification for stability purposes, remained bound to beef liver aconitase even after passage through Sephadex G-25. The amount of citrate that was bound to the enzyme was sufficient to require removal before kinetic experiments could be performed.

The activities of aconitase (165, 191, 231) and fumarase[1] (158) are both known to be affected by the presence of various anions in the assay solution. Massey (158) found that the effect of anions on fumarase was not a direct effect at the active center. He suggests that both activating anions and substrate can combine with groups on the protein other than at the active center. Palmer, using the argument of Massey, postulated that the apparent activating effect on purified mustard aconitase by isocitrate was by combination with groups on the protein other than at the active center (185).

The K_m Values for the Substrates and the K_I for Trans-aconitate

The K_m of beef liver aconitase for citrate was found to be 0.95×10^{-3}M, for threo-Ds-isocitrate 1.39×10^{-4}M, and for cis-aconitate 0.99×10^{-4}M (231). The K_m values of pig heart aconitase (164) and rabbit liver aconitase (239) were found to be of the same order, and the K_m values determined by Racker (196) are likewise of the same order. However, the K_m values of beef liver aconitase which were determined by Henson and Cleland (114) are an order of magnitude lower than those determined by the other investigators. Every investigator

[1] Fumarase is thought to be an enzyme similar to aconitase (164, 179).

found that the K_m for cis-aconitate is the smallest, the K_m for isocitrate is intermediate, and the K_m for citrate is the largest.

Bacon et al found the Lineweaver and Burk plots obtained with crude aconitase preparations from higher plants to be not linear (185). At low substrate concentrations, Palmer (185) found the K_m of purified mustard aconitase for citrate to be 4.4×10^{-3}M, for threo-Ds-isocitrate 1.5×10^{-4}M, and for cis-aconitate 1×10^{-4}M. These values are of the same order as those quoted by most investigators for the enzyme from animal tissue. But the K_m values of the purified mustard aconitase were found to be a function of substrate concentration. The K_m for isocitrate was found to increase 100-fold to 15×10^{-3}M at high concentrations.

Trans-aconitate is a competitive inhibitor of aconitase from animals and plants (5, 63, 231). The K_I of beef liver aconitase for trans-aconitate was found to be 7.0×10^{-4}M which is about the same value as the K_m for citrate in the same experiments (231). The isomer threo-Ls-isocitrate was found to have a K_I of about 4×10^{-3}M (231).

Substrate as a Competitive Inhibitor of Aconitase

Product inhibition is usually competitive inhibition (149). For the conversion of any one tricarboxylic

acid, the other two tricarboxylic acids should be competitive inhibitors of aconitase. Tomizawa (241) demonstrated that DL-isocitrate-2-^{14}C competitively inhibited the formation of unlabeled citrate from unlabeled cis-aconitate.

The Mechanism of Action of Aconitase

Stereospecificity

Water can be added in four ways to cis-aconitate to give the 4 stereoisomers of isocitric acid. The two possible trans additions yield the two threo enantiomorphs, while the two possible cis additions yield the two erythro enantiomorphs. The "natural" isomer of isocitric acid is threo-Ds-isocitrate[1] (248).[2] From the known geometry of cis-aconitate (151, 152) and the known configuration of the threo-Ds-isomer, the trans nature of the dehydration of isocitrate to cis-aconitate and of the reverse reaction is established (90) (Figure 1C).

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[1]Until 1959, only threo-Ds-isocitrate had been found in living organisms. In 1959, erythro-Ls-isocitrate was discovered as the fermentation product of Penicillium. The evidence suggests that the path to this fermentation product does not include aconitase (208). To my knowledge, there is no evidence for any aconitase in nature having as substrate any isomer other than the threo-Ds form.

[2]Much confusion exists in the early literature with regard to the nomenclature and absolute configuration of the isocitrate isomers. The review by Vickery (248) summarizes the history up until 1962. Since 1962, there has been little confusion.

Figure 1

The Stereochemistry[1] of the Aconitase Reactions (106)

A. The absolute configuration of enzymatically formed citric acid. Hanson and Rose, using citric acid with one of the methylenes containing one tritium, and with a known absolute configuration with respect to the center carboxyl group, the -OH group, and the methylene group which contained tritium, found that all the tritium was released to water by aconitase from pig heart. Thus, the stereochemical relationship between the center -COOH group, the -OH group, and the methylene containing the hydrogen exchangeable by aconitase is established in absolute terms. Since the portion of the molecule derived from oxalacetate is known to bear the hydrogen exchangeable by aconitase, the stereochemical course of citric acid biosynthesis is also established in absolute terms. The symbol $\sim$ indicates that the configuration of the carbon atom bearing tritium was unknown.

B. The configuration at the oxalacetate derived methylene group of citric acid (the determination of which hydrogen exchanges with the medium in the presence of aconitase). The configuration at the oxalacetate-derived methylene group of citric acid had been correlated with the fumarase reaction by Englard (76), and the trans nature of the fumarase reaction had been established by Gawron et al (90) and by Anet (4). The stereochemical knowledge of which methylene group of citric acid is derived from oxalacetate lead Hanson and Rose to summarize Englard's experiments as shown. When Englard treated the two citric acids with pig heart aconitase and isocitrate dehydrogenase, the deuterium from fumaric acid -2,3-D appeared in NADPH while the deuterium from D_2O did not. The hydrogen which exchanges with the hydrogen of the medium in the presence of aconitase is therefore known and it is established that the hydration of cis-aconitate to citrate in the presence of pig heart aconitase is trans.

C. The stereochemistry of the aconitase reactions. The hydration of cis-aconitate to form citrate and the hydration to form isocitrate are both trans. Furthermore, a given double bond carbon atom is always attacked from the same side, i.e. the alternative approaches of the proton must be from opposite sides of the cis-aconitate plane (106).

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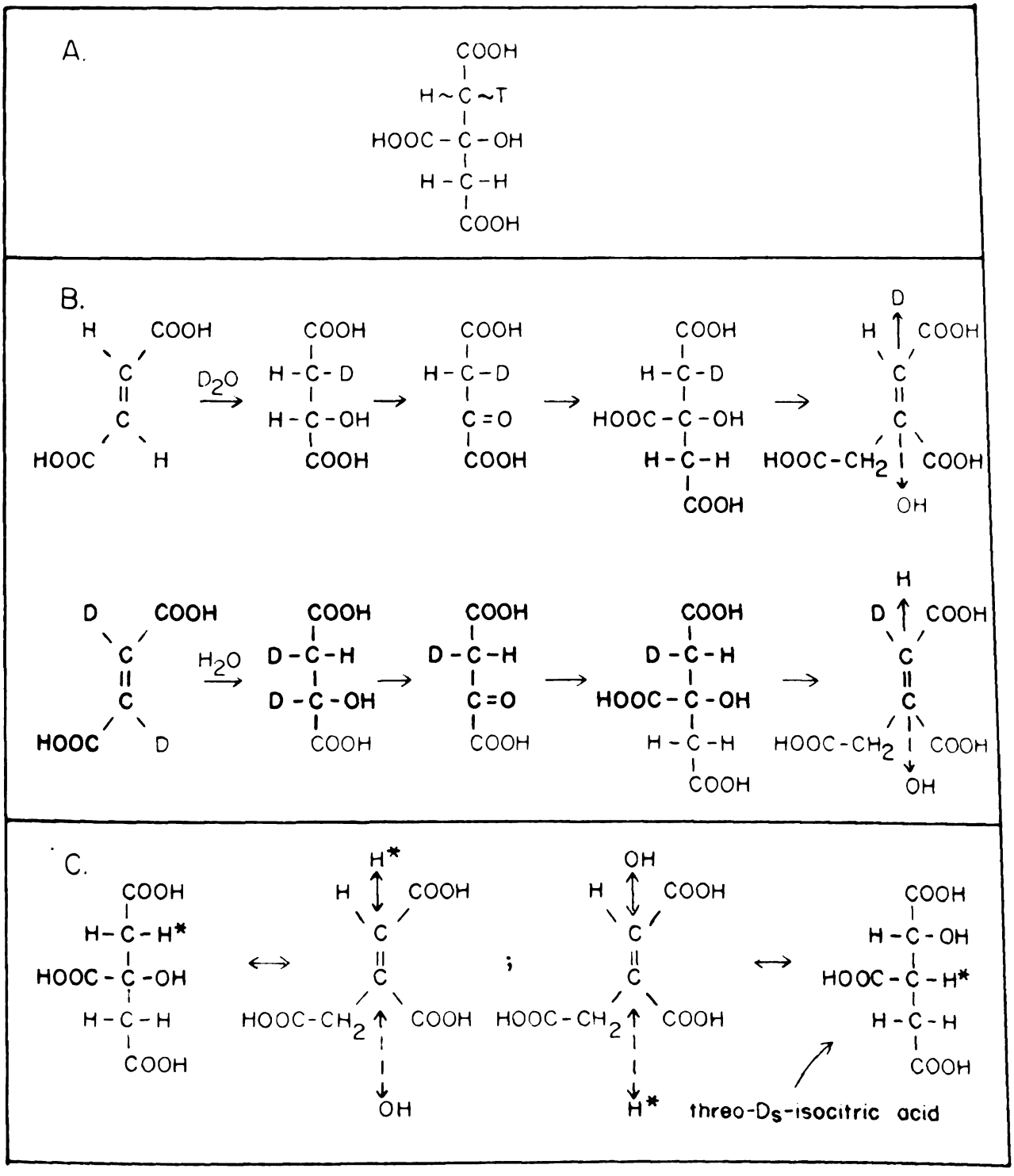
[1]The stereochemical formulas of this figure are Fisher projection formulas.


A.
B.
C.
threo-Ds-isocitric acid

The stereochemical handling of citrate by aconitase was more difficult to elucidate. In tissues or organisms so far studied (pig heart, rat liver, pigeon liver, and Clostridium kluyveri), when citrate is formed from oxalacetate and acetyl-CoA by citrate synthase (E.C. 4.1.3.7, condensing enzyme), and is then treated with aconitase to form cis-aconitate and threo-Ds-isocitrate, the $-CH_2-COOH$ ends of the latter compounds are derived from acetyl-CoA, while the other atoms are derived either from oxalacetate or water (76, 106, 147, 225, 257). These findings are in accord with the proposal of Ogston (178, 179) that it is possible for an asymmetric enzyme which attacks a symmetrical compound to distinguish between its identical groups. The elucidation of the stereochemical handling of citrate by aconitase is shown in Figure 1. Gawron et al (90), using the methodology of organic chemistry, had earlier arrived at the same conclusions.

A Single Active Site and the Nature of the Common Enzyme-Intermediate Complex

Morrison (164), using pig heart aconitase, found that the rate of formation of cis-aconitate from a combination of citrate and isocitrate was half the sum of the rates obtained with each hydroxy acid separately, which would be expected for a single site catalyzing both reactions. In more extensive but similar investigations, Tomizawa reached a similar conclusion experimenting with

aconitase from pig heart, bacteria, and yeast (240), and from rabbit liver (239). With aconitase from rabbit liver, Tomizawa also found that the three K_m values were specific to the starting substrates regardless of the reaction by which they were measured, and that the activation energy was the same (about 12 kcal/mole) starting with any of the three substrates. Both these findings were considered evidence for a single active site.

A mechanism in which an intermediate enzyme complex is common to all three substrate interconversions is suggested by the kinetics, but not unambiguously (6).

Tomizawa (241) found that when the rabbit liver enzyme[1] was presented with a mixture of DL-isocitrate-2-^{14}C and cold cis-aconitate, the specific activity of citrate formed after a certain period was greater than that of cis-aconitate. Speyer and Dickman (223), using aconitase from pig heart,[2] found that citrate formed from isocitrate in deuterowater contained considerably less deuterium than citrate formed from cis-aconitate. Both experiments suggested that cis-aconitate need not be an obligate free intermediate. Rose and O'Connell (204), using pig heart aconitase,[2] have also presented evidence that cis-aconitate need not be an obligate free inter-

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[1]The enzyme was purified but no mention was made of activation.

[2]These preparations were purified then reactivated with iron and cysteine.

mediate. The conversion of 3-T-isocitrate to citrate at early times gave T-citrate with no dilution of tritium. Similarly, when the conversion of 2-T-citrate to isocitrate was brought to completion by trapping the isocitrate with isocitrate dehydrogenase and converting the α-ketoglutarate to glutamate with glutamate dehydrogenase, the amount of tritium found in the glutamate (about 20% of that which had been in the citrate) agreed with the prediction based on the relative initial rates of formation of isocitrate and cis-aconitate from citrate (about 4 cis-aconitates per isocitrate). Furthermore, when a mixture of citrate labeled with deuterium at the aconitase specific C-2 position, and citrate labeled with deuterium in the C-4 position, was acted upon by aconitase and the conversion was brought to completion by trapping the isocitrate, analysis of isocitrate for singly and doubly deuterated species showed that about 20% of the conversion from citrate to isocitrate (i.e. the amount corresponding to the initial rate of formation of isocitrate as a percent of the total initial rate of isocitrate plus cis-aconitate formation) occured with intra molecular transfer of hydrogen.

These same authors (204) also found that at high concentrations of cis-aconitate, some of the tritium of 2-T-citrate that was normally found in the water was diverted to isocitrate, and that this conservation of tritium was due to an intermolecular transfer since in

the presence of high concentrations of cis-aconitate, tritiated 2-methyl hydroxy acids[1] gave rise to tritiated isocitrate. These observations established that when free cis-aconitate is formed from citrate or isocitrate, the proton is retained by the enzyme for a finite time after the dissociation of cis-aconitate. Thus, the participation of the enzyme in the abstraction of a proton from the hydroxy acids during the formation of free cis-aconitate was definitively established, and the participation of this same mechanism for intramolecular hydrogen transfer is suggested.

Using isocitrate containing ^{18}O at C-2 and tritium at C-3, Rose and O'Connell found that conversion to citrate through aconitase occured with substantial retention of tritium but no retention of the ^{18}O-labeled hydroxyl group.

The stereochemical restrictions of the aconitase mechanism (Figure 1-C) have led to the alternative hypotheses that either cis-aconitate fits the active site in both a heads or tails fashion, or the protonating and hydroxylating groups of the enzyme are interchangeable. In keeping with the complete retention of the substrate hydrogen in the direct interconversion of the hydroxy acids, it is possible to exclude any dilution of the substrate proton by hydrogens of the pig heart enzyme. Thus, $-NH_2$ is excluded but $-S^-$, $-COO^-$, or imidazole are not excluded as

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[1]These synthetic acids together with synthetic α-methyl-cis-aconitate are substrates for aconitase (91).

possible functioning bases. For the same reason, two interchangeable enzyme protonating groups seem unlikely. Direct conversion of one hydroxy acid to the other could occur through cis-aconitate in which the required 180° rotation occurs with the acetate group attached as a pivot to the enzyme, or alternatively through cis-aconitate bound in the sense of being partly confined but free to rotate in a space within the enzyme at the active site, and free to reassociate with the protein about as frequently as it dissociates into the substrate containing medium (204). The enzyme-cis-aconitate complex in which the cis-aconitate is free to rotate would represent an intermediate enzyme complex common to all three substrate interconversions.

The Lag in the Interconversion of the Hydroxy Acids

Much controversy has existed concerning the observation and interpretation of a lag in the citrate to isocitrate or the reverse conversion.[1] No lag was observed by Martius and Lynen (157) or more recently by Thomson et al (231). A lag was observed by Krebs and Holzach (138), Tomizawa (240), and Morrison (164). The disagreement

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[1]All the evidence which is presented here concerning a lag has been obtained with aconitase from animal tissue, specifically pig heart, beef liver, beef heart, and rabbit liver.

between investigators concerning the observation of a lag may be explained by the observation of Krebs and Holzach (138) that dilution of the enzyme and a relatively low temperature draw out the time curve and favor the detection of a lag. The observation of a lag caused Krebs and Holzach to postulate that cis-aconitate was an intermediate in the conversion of either hydroxy acid to the other. Speyer and Dickman (223) pointed out that if the initial rate of interconversion of the two hydroxy acids were greater than zero, this amount of interconversion would be direct, i.e. cis-aconitate would not be an obligate free[1] intermediate. Comparison of the later maximum rate of interconversion with the initial rate would allow an estimate of the proportion of interconversion that proceeded through free cis-aconitate. Tomizawa (240) had earlier obtained evidence which fit the proposal of Speyer and Dickman. The initial rate of citrate formation from isocitrate, though considerable, did not become maximum until the pool size of cis-aconitate, expressed as a percent of total substrates, became maximum. The data of Rose and O'Connell (204) suggest that the initial rate of formation of one hydroxy acid from the other, compared to the initial

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[1]In those cases where the meaning seemed clear, I have employed the words free (non enzyme bound) and obligate in this discussion of the lag, even though most of the original authors did not.

rate of formation of free cis-aconitate, is a valid indication of the percent of direct conversion in which cis-aconitate is not a free intermediate. Much discrepancy exists in the literature concerning the ratio of the initial rates of formation of cis-aconitate and one hydroxy acid from the other hydroxy acid. For example, Henson and Cleland (114) reported that the initial rates of formation of citrate and cis-aconitate from isocitrate were equal, Speyer and Dickman (223) reported that for the same reaction the initial rate of cis-aconitate formation was 2 to 2½ times greater than the initial rate of citrate formation, while Rose and O'Connell (204) reported that for the same reaction the initial rate of cis-aconitate formation was 4 times greater than the initial rate of citrate formation.[1] Morrison (164) actually observed a zero initial rate for the conversion of citrate to isocitrate, indicating that under his conditions, cis-aconitate was an obligate free intermediate. It would seem that cis-aconitate, depending upon the conditions employed, is to a greater or lesser extent a free intermediate in the interconversion of the hydroxy acids, and that under limiting conditions, cis-aconitate may be an obligate free intermediate. However, as Morrison (164) pointed out, even

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[1]The presumed absence of any initial lag in the interconversion of the two hydroxy acids is of doubtful validity (6). Complete absence of any lag would exclude the transient formation of free cis-aconitate in the interconversion of the two hydroxy acids.

the observation of a zero initial rate for the conversion of citrate to isocitrate is not proof that separate sites are required for the citrate to cis-aconitate and cis-aconitate to isocitrate conversions. That the initial rate for the conversion of the two hydroxy acids by the animal aconitase has been observed not to be zero by most investigators would suggest (in agreement with the other evidence already presented) that just one active site is responsible for the aconitase reactions.

The variation in the ratio of the initial rates of formation of cis-aconitate and one hydroxy acid from the other hydroxy acid seems like a natural extension of the mechanism proposed by Rose and O'Connell, since the catalysis of the interconversion of the two hydroxy acids via free cis-aconitate would be the same as the catalysis of the direct interconversion in the sense that cis-aconitate, which is free to rotate, would be the intermediate in both cases.

Rates of Conversion of Cis-aconitate to the Other Two Tricarboxylic Acids

The initial rate for the conversion by the animal aconitase of cis-aconitate to citrate has been found by most investigators to be equal or nearly equal to the initial rate for the conversion of cis-aconitate to isocitrate (114, 164, 223, 231, 239). Some investigators found that the aconitate to isocitrate conversion was

slightly favored, being as much as 1.2 times faster than the aconitate to citrate conversion (223, 239). Thus free cis-aconitate has about an equal chance to be converted to either hydroxy acid.

Kinetic Evidence for at Least 3 Central Complexes

Using beef liver aconitase, Henson and Cleland (114) found that the relative maximum velocities for the disappearance of the three substrates were as follows: 3.1 for cis-aconitate, 1.7 for isocitrate, and 1.0 for citrate. Similarly, the maximum velocity for the appearance of a given product depended upon which of the other two substrates was used. If only one central complex of enzyme and substrate were formed, the maximum velocity for the appearance of a given product would have been independent of which substrate was used. The authors concluded that the minimum number of central complexes which exist during catalysis is three.

Rate Limiting Steps

Englard and Colowick (77) discovered that as the result of equilibration in the presence of pig heart aconitase in 85% D_2O, 0.546 atoms of deuterium were incorporated per molecule of citrate rather than 0.85. From this the K_H/K_D was calculated as 4.7, which led the authors to suggest that the making of the C-H bond is rate limiting in the

hydration of cis-aconitate, while in the dehydration of citrate, the breaking of the C-H bond is not rate limiting, since if it were, the isotope effect should not have been observed. Using deuterated citrate and isocitrate in the presence of beef liver aconitase, Thomson et al (231) provided direct evidence that in the dehydration of these acids, the rate limiting step is the removal of the hydroxyl rather than the hydrogen. Although several authors favor an enzyme bound carbonium ion intermediate (77, 223, 231) rather than a carbanion or a concerted mechanism,[1] the sequence of bond cleavage has not yet been determined (204).

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[1]The possibility that cis-aconitate is an obligate intermediate (either free or enzyme bound) (204) suggests that two rather than one such intermediate ions or mechanisms may exist.

MATERIALS AND METHODS

Plants

Tobacco plants (Nicotiana tabacum) were grown in the field,[1] the greenhouse, and a controlled environment chamber (Sherer-Gillett). The Swiss chard plants (Beta vulgaris cicla), which were used for a few of the experiments, were grown only in the greenhouse and a controlled environment chamber. For tobacco plants which were to be grown in the field, seeds were planted around the middle of April. The seedlings were grown in the greenhouse until about the first of June when they were transplanted to the field, at which time they were about 4 inches tall. The plants which were grown in the greenhouse and controlled environment chamber were maintained on Hoagland's solution (122). The controlled environment chambers provided about 3500 ft-c of continuous light from Westinghouse cool white super high fluorescent bulbs (F96T12/CW/SHO) and from a few 60 watt incandescent bulbs. Typically, the lights were on in the chambers from 7 A.M. to 7 P.M. and

[1]Some of the field grown tobacco plants were fertilized with the following commercial mixture: 8-16-24 + 4% MgO. Thus, the mixture comprised the following by weight: 8% N, 16% P_2O_5, 24% K_2O, and 4% MgO. This fertilizer was made and applied essentially as recommended by Bowling et al (34).

off from 7 P.M. to 7 A.M., and the temperature was maintained at about 27° when the lights were on and 21° when the lights were off.

Preparation of Extracts from Leaves

Exceptions to the following general procedure are noted in the results section. All the leaves which were used were harvested from adult plants. The size of the leaves and the location on the plants from which the leaves came are given in the results section. Because some of the experimental observations were found to be a function of the time of the day and/or the season of the year or the method of culture, the date and time of harvest are noted for the pertinent experiments in the results section. Leaves from the field were used during the summer and leaves from the greenhouse or growth chamber were used during the rest of the year.

The harvested leaves were placed upright in a container with their petioles in about 1½ inches of tap water. They were then taken to the laboratory where they were washed with tap and/or distilled water. Following this, the leaves were drained of water, deribbed, and the blades were weighed. All subsequent operations were done at 0°-4°. The leaf blades were homogenized by a Waring blendor, or a Hobart meat grinder, or a mortar and pestle as described in the results section. The mixture was

squeezed through 2 layers of cheesecloth and then centrifuged at 15,000 g for 20 minutes. The precipitates were discarded and the supernatant solutions were saved. Aqueous solutions prepared in this way are called "extracts" in the results section.[1]

About 15 minutes elapsed between the time of harvest and the time of homogenization for those leaves which were harvested from the field, and about 7 to 8 minutes elapsed between the time of harvest and the time of homogenization for those leaves which were harvested from the greenhouse or controlled environment chamber. In the 6 or 7 minutes during which the leaves were in the laboratory building, no attempt was made to shield them from exposure to the artificial light (10-100 ft-c from fluorescent lights).

Measurement of Light Intensities

The light intensities were measured in foot candles with a Weston Model 756 Illumination Meter. The intensities

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[1] In two of the previous papers, the purification of the phosphatase was considered to start with the crude sap after filtration through cheesecloth. The initial centrifugation to give what is now defined as the extract resulted in a 2-fold purification while about 15% of the activity of P-glycolate phosphatase was centrifuged down with the pellet (3, 202). When an extract which had been centrifuged was adjusted to pH 8.3 and recentrifuged, the purification was only 1.02-fold (data not shown). Thus, the 2.16-fold purification by the first pH 8.3 adjustment of the crude sap followed by centrifugation (202) was probably mostly because of the centrifugation, not the pH adjustment.

at the times the leaves were harvested were the maximum intensities noted at those times.

Water and Temperature

Deionized distilled water was used throughout. Unless otherwise noted, purification and storage of the enzymes were at 0-4°.

The Gassing of Enzyme Preparations

The iced and stoppered side arm test tube, or the Thunburg tube, or the double side arm Warburg flask, which contained the cold enzyme preparation , was evacuated to about 15 mm Hg (H_2O pump). The desired gas was admitted till the pressure equaled atmospheric pressure as indicated on a mercury manometer, then the preparation was shaken about 20 times. This cycle was repeated at least 2 more times. The desired gas was left at atmospheric pressure over the cold enzyme preparation.

Mixing of Enzyme Preparations by "Buzzing"

Enzyme preparations in a test tube were vigorously mixed for 2.0 minutes with the help of a vortex mixer. The aliquots were always small enough that the vortex easily went to the bottom of the test tube and the liquid formed a layer of about 1/8 inch thick on the walls of the test tube. The "buzzing" itself was at an ambient temper-

ature of 20-25° with the result that the temperature of the samples rose from about 1° to 12° at the end of the 2 minutes. The samples were immediately recooled in an ice bath. "Buzzed" preparations are those which were treated by this procedure.

NaCl Determinations

NaCl concentrations were measured using a Barnstead Purity Meter, Model PM-4. The proteins and other electrolytes of plant origin which were eluted from ion exchange columns did not noticeably affect the purity meter readings.

Protein Determinations

260/280 Method (249)

Using a 1 cm light path, the absorbance values of a solution were determined at 260 and 280 mμ. The protein concentration in mg/ml was then taken from a nomograph.[1] The protein concentration may also be estimated by the following formula (142):

$$\text{Protein concentration (mg/ml)} = 1.55\ D_{280} - 0.76\ D_{260}$$

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[1]Distributed by: California Corporation for Biochemical Research, 3625 Medford St., Los Angeles 63, Calif.

where D_{280} and D_{260} are optical densities at 280 and 260 mμ, respectively.

A_{280} Method (67)

Using a 1 cm light path, the absorbance of a solution was determined at 280 mμ. The absorbance gave the protein concentration directly in mg/ml. A modification of this method was used with effluents from chromatographic columns. The effluents were continuously monitored at 253.7 mμ and the transmittance was recorded as a function of time. An event marker operated by the fraction collector marked the recording every time a tube was changed. The method was intended to provide a recorded, qualitative, comparative measure of the protein in each fraction.

Lowry's Modified Folin-Ciocalteu Method (148)

To 1.0 ml of protein solution was added 5.0 ml of a solution made up by combining 50 ml of 2% Na_2CO_3 in 0.1N NaOH with 0.5 ml of 2.0% sodium tartrate and with 0.5 ml of 1.0% $CuSO_4 \cdot 5H_2O$. After mixing, the 6.0 ml was allowed to sit at room temperature for 10 minutes or more. Then 0.5 ml of 1.0N Phenol Reagent was added rapidly with immediate mixing. The Phenol Reagent was made by diluting 2.0N Phenol Reagent (Fisher Scientific Co., Fair Lawn, N. J.) 1:1 with H_2O. After allowing the

color to develop for 30 minutes or more at room temperature, the absorbance was read at 660 mμ. The result was compared with a standard curve. Crystaline bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) served as the standard. The standard curve, with the y axis being absorbance and the x axis being protein concentration, showed some downward curvature. It is known that the color is not strictly proportional to protein concentration (142).

When it was desired to use more than 1.C ml of protein solution, 2.5 ml of a solution made by combining 25 ml of 4% Na_2CO_3 in 0.2N NaOH with 0.5 ml of 2.0% sodium tartrate and with 0.5 ml of 1.0% $CuSO_4 \cdot 5H_2O$ was used. With this modification, up to 3.5 ml of protein solution could be used, with the difference made up with H_2O. The final volume after addition of the phenol reagent was 6.5 ml.

Another modification was the reduction of all volumes to 1/20 of their normal size. The absorbance of the final 0.325 ml was then read in a Beckman D.U. spectrophotometer using a micro cuvette.

Unless noted otherwise, protein concentrations were determined by the Lowry's modified Folin-Ciocalteu method.

Inorganic Phosphate by a Modified Method of Fiske and Subbarow (83)

To a 1.0 ml aliquot of the aqueous P_i solution, ideally containing between 3 and 30 µg of phosphorous, were added 8.0 ml of molybdate reagent and 1.0 ml of elon reducing agent. The 10.0 ml was thoroughly mixed, allowed to stand at room temperature for at least 20 minutes, then the absorbance was read at 660 mµ. The result was compared with a standard curve. The standard curve was perfectly linear up to at least 30 µg of phosphorous in the 1.0 ml aliquot. The molybdate reagent consisted of 50 ml of 10N H_2SO_4 plus 100 ml of 2.5% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ plus 650 ml of H_2O. Although the reducing solution based on 1-amino-2-napthol-4-sulfonic acid (83) was found to yield 15-20% more color, the simpler elon reducing agent consisting of 3 g of $NaHSO_3$ and 1 g of elon (p-methylaminophenol sulfate) in 100 ml of H_2O (50) was the one routinely used. The phosphorous standard solution, 0.1N in H_2SO_4, was made up to 40.00 µg of phosphorous/ml using Fisher certified reagent KH_2PO_4, which had been dried at 130° for 24 hours and cooled to room temperature in a dessicator over anhydrous $CaSO_4$ before weighing.

Inorganic Phosphate by an Isobutanol Benzene Extraction Method[1]

To a 2.0 ml aliquot of the aqueous P_i solution, containing no more than 8 µg of phosphorous, was added 0.50 ml of acid molybdate solution consisting of 6% ammonium molybdate (Malincrodt analytical reagent $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$) and 2N H_2SO_4. The 2.5 ml was thoroughly mixed and allowed to stand 5 minutes. Then 2.5 ml of isobutanol benzene (1:1 v/v) was added and the 5.0 ml was thoroughly mixed by vortex mixer for 45 seconds.[2,3] After the phases separated, 1.0 ml of the upper isobutanol benzene phase was syringed into a 1 ml cuvette, 10 µl of absolute EtOH was added and mixed thoroughly,[4] and the absorbance was read at 310 mµ in a Beckman D.U. spectrophotometer. The result was compared with a standard curve (Figure 2).

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[1]The method used is essentially the one used in the laboratory of Dr. Loran L. Bieber (Biochemistry Dept., Michigan State U., East Lansing, Mich.), which in turn is based on the work of M. De Luca et al (59), Martin and Doty (155), and Berenblum and Chain (27).

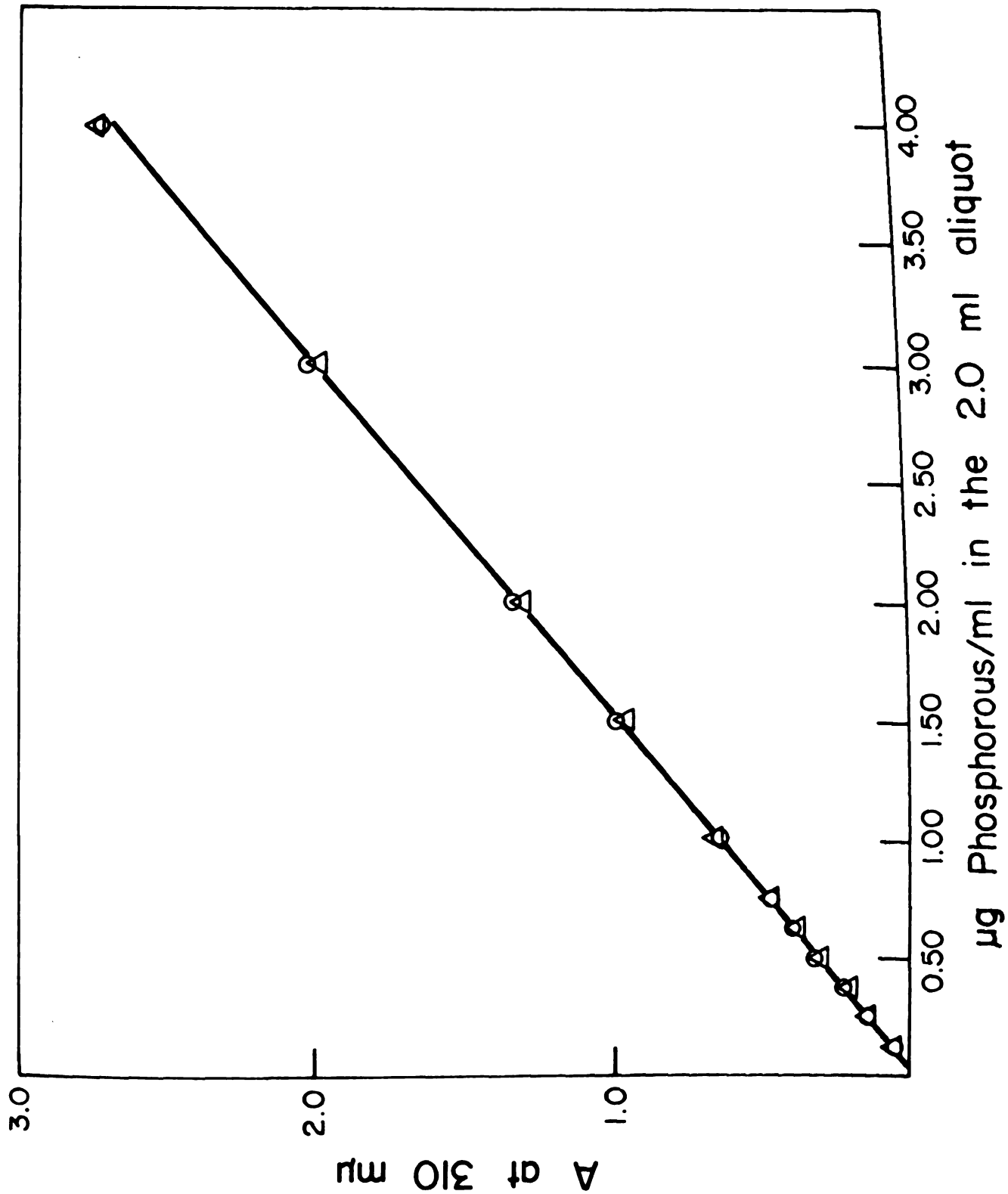
[2]All solutions and operations were at room temperature. Sharper phase separation may be obtained if everything is kept at 0° (personal communication from Dr. Loran L. Bieber).

[3]Sharper phase separation was obtained when small (1.4 cm internal diameter x 15 cm high) rather than medium or large sized test tubes were used.

[4]The ethyl alcohol was necessary to dissolve droplets of water (27) which sometimes remained in small amounts in the isobutanol benzene phase.

Figure 2

Inorganic Phosphate by the
Isobutanol Benzene Extraction Method


A at 310 mμ
3.0
2.0
1.0
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
μg Phosphorous/ml in the 2.0 ml aliquot

Determination of Total Phosphorous (51)

To a suitable aliquot (about 10 µl) of the phosphorous solution was added 0.5 ml of 10N H_2SO_4. The mixture was heated for 60 minutes at 130-160° in a sand bath. After allowing the solution to cool, 2.0 ml of water was slowly added and the solution was heated in a boiling H_2O bath for 10 minutes to hydrolyze the polyphosphates. Then 1.0 ml of the 2.5% ammonium molybdate solution was added, followed by enough H_2O to bring the volume to 9.0 ml. The concentrations of H_2SO_4 and ammonium molybdate were then the same as for the modified method of Fiske and Subbarow for the determination of P_i. The remainder of the procedure, including the addition of 1.0 ml of elon reducing agent, was as directed by the modified method of Fiske and Subbarow.

Determination of Glycolic Acid (144)

To a 0.2 ml aliquot of the aqueous glycolate solution containing not more than 20 µg of glycolic acid was added 2.0 ml of a 0.02% solution of 2,7 dihydroxynaphthalene in concentrated sulfuric acid. This solution was thoroughly mixed and placed in a boiling water bath for 20 minutes, with a marble over the top of the test tube. The solution was allowed to cool, then 4 ml of 2N sulfuric acid was added and mixed. After allowing the solution to cool again, the absorbance was read at 530 mµ. The

amount of glycolic acid was read from a standard curve, which was linear between 0 and 20 μg.

Enzyme Assays

Standard P-glycolate Phosphatase Assay (3)

A solution of 0.50 ml of 0.20M cacodylate[1] (97) (hydroxydimethyl arsine oxide, $(CH_3)_2AsO(OH)$), pH 6.3, 0.60 ml of 0.010M $MgSO_4$, 1.0 ml of 0.010M P-glycolate, pH 6.3, and enough H_2O to bring the final reaction volume to 3.0 ml, was brought to 30° in a water bath. The reaction was initiated with enzyme which had been kept at 0°. After 10.0 minutes, the reaction was stopped by adding 1.0 ml of 10% trichloroacetic acid. After the removal of denatured protein by centrifugation (with purer enzyme preparations, the centrifugation was omitted), the P_i was determined in a 1.0 ml aliquot using the modified Fiske and Subbarow method. Two controls, one containing enzyme and 10% trichloroacetic acid only and the other containing substrate, cacodylate, $MgSO_4$, and any other additives used in the assay, were run to correct for P_i not liberated in the reaction. Quarter sized assays with a reaction volume of 0.75 ml (for pure enzyme) and half sized assays

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[1] pK = 6.19 in aqueous solutions at 25°. Handbook of Chemistry and Physics, Forty-Fourth Edition. 1962/1963. Charles D. Hodgman, ed. The Chemical Rubber Publishing Co., Cleveland, Ohio.

with a reaction volume of 1.5 ml (for crude enzyme) were more commonly used than the 3.0 ml full sized assay.

As long as no more than 40% of the substrate was hydrolyzed, the enzyme remained saturated for the full 10 minutes of the reaction (Figure 3). When somewhat more than 40% of the substrate was hydrolyzed, the result could be corrected upward to the extended straight line, which gave the amount which would have been hydrolyzed had the enzyme been fully saturated. Whenever this correction was checked by repeating the assay with half the enzyme concentration, the amount of phosphate released was accurately predicted.

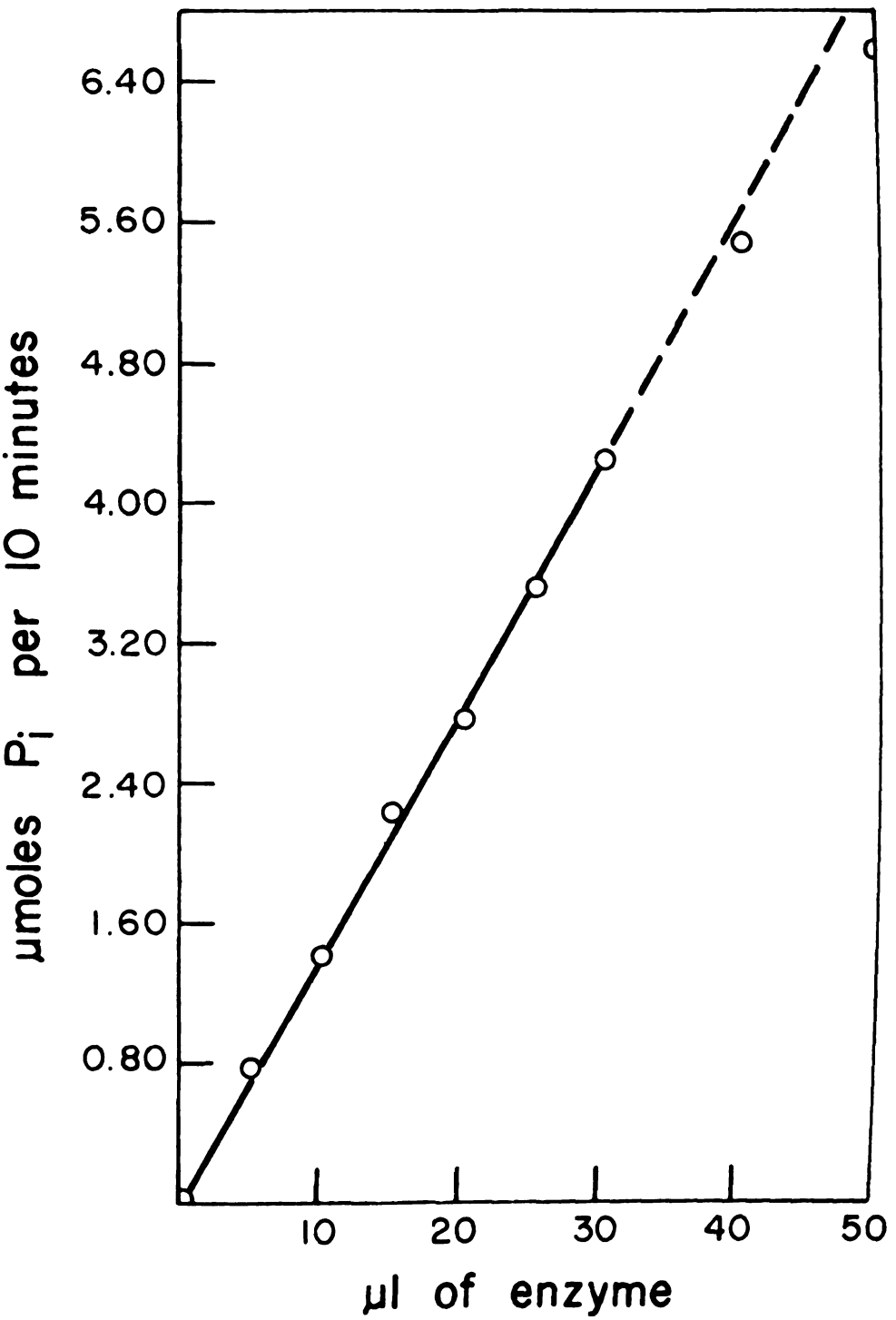
P-glycolate was obtained as the tricyclohexylammonium salt from General Biochemicals, Chagrin Falls, Ohio, as a special preparation for us. It was used in this salt form after adjustment to pH 6.3, or it was converted to the free acid with Dowex-50 (H^+), and then adjusted to pH 6.3 with base. The concentration was checked by exhaustive enzymatic hydrolysis, after which the concentration could be adjusted by dilution. When tested with a partially purified enzyme preparation, the Na^+, K^+, and tricyclohexylammonium forms of the substrate gave identical reaction rates.

A number of buffers without phosphorous, including Tris-acetate and maleate, were checked for their ability to hold the pH during the reaction and for their effect

Figure 3

P_i Released in the Standard P-glycolate Phosphatase Assay as a Function of the Concentration of Enzyme

An extract from tobacco leaves was prepared by Waring blendor. The enzyme was purified from this extract by one acetone fractionation. Complete hydrolysis of the substrate gave 10.00 μmoles of P_i.


6.40
5.60
4.80
4.00
3.20
2.40
1.60
0.80
µmoles P_i per 10 minutes
10
20
30
40
50
µl of enzyme

on the reaction rate. Maleate gave the highest reaction rate and no change in pH during the reaction. The pH in Tris-acetate buffer changed from 6.3 to 5.7 in the reaction time and the reaction rate was considerably less than with the maleate buffer, possibly due to the unfavorable change in pH. Cacodylate also prevented any pH change during the reaction and allowed rates 90% as great as with maleate. Because of the structural similarity between maleate and cis-aconitate, which was to be investigated as a stabilizing factor, cacodylate was adopted as the standard buffer.

The reaction velocity showed a Michaelis Menton dependence on the concentration of Mg^{+2} (Figure 4A).[1] At the reaction concentration of 2×10^{-3}M which is the one used in the standard P-glycolate phosphatase assay, the system was essentially saturated with Mg^{+2}. When the data of Figure 4A are plotted by the method of Lineweaver and Burk (145), the plot is a straight line (Figure 4B). This suggests that during the phosphatase reaction, one Mg^{+2} is required per active site of the enzyme. The

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[1] After two $(NH_4)_2SO_4$ steps plus a calcium phosphate gel purification, the tobacco enzyme had significant activity without added metal (202). By contrast, the wheat enzyme, after one acetone precipitation followed by DEAE-cellulose chromatography, was completely inactive without added metal (265), and the tobacco enzyme after one acetone fractionation was very dependent on added Mg^{++} (Figure 4A).

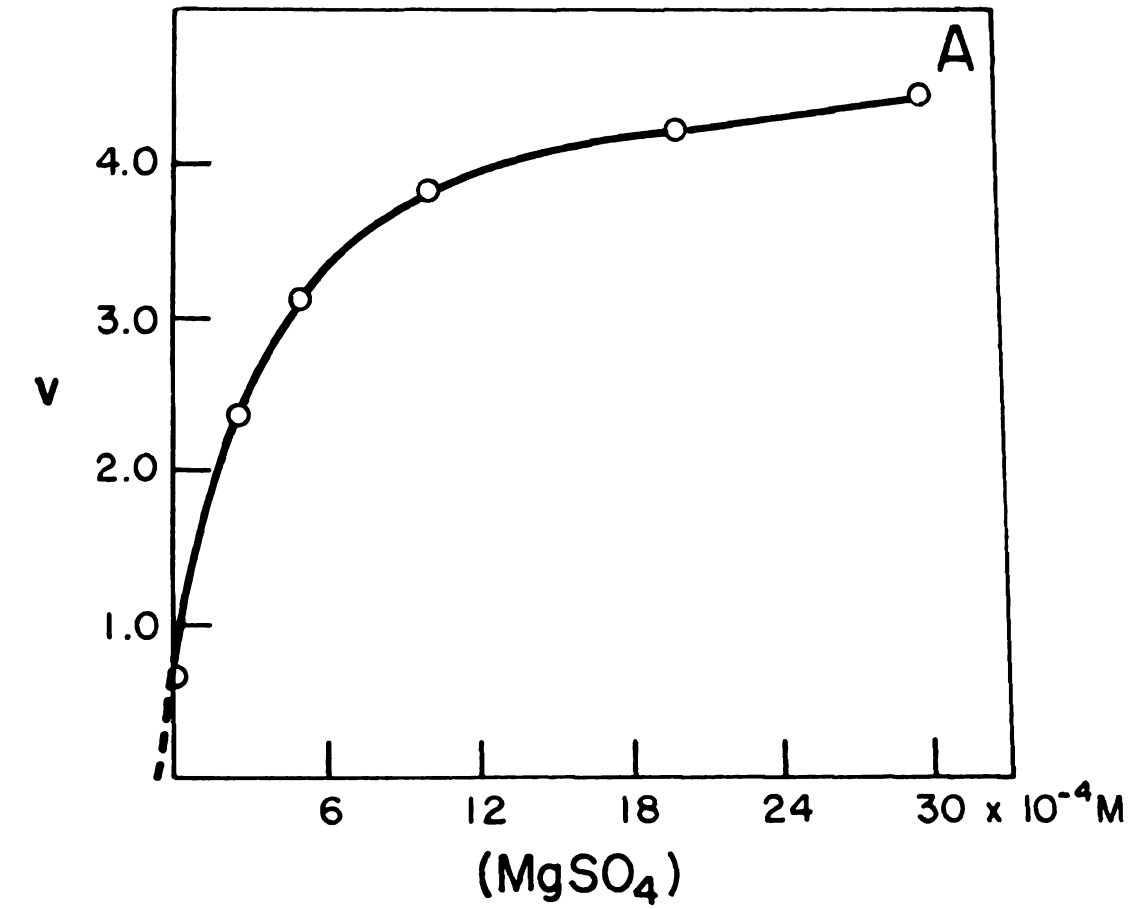
Figure 4

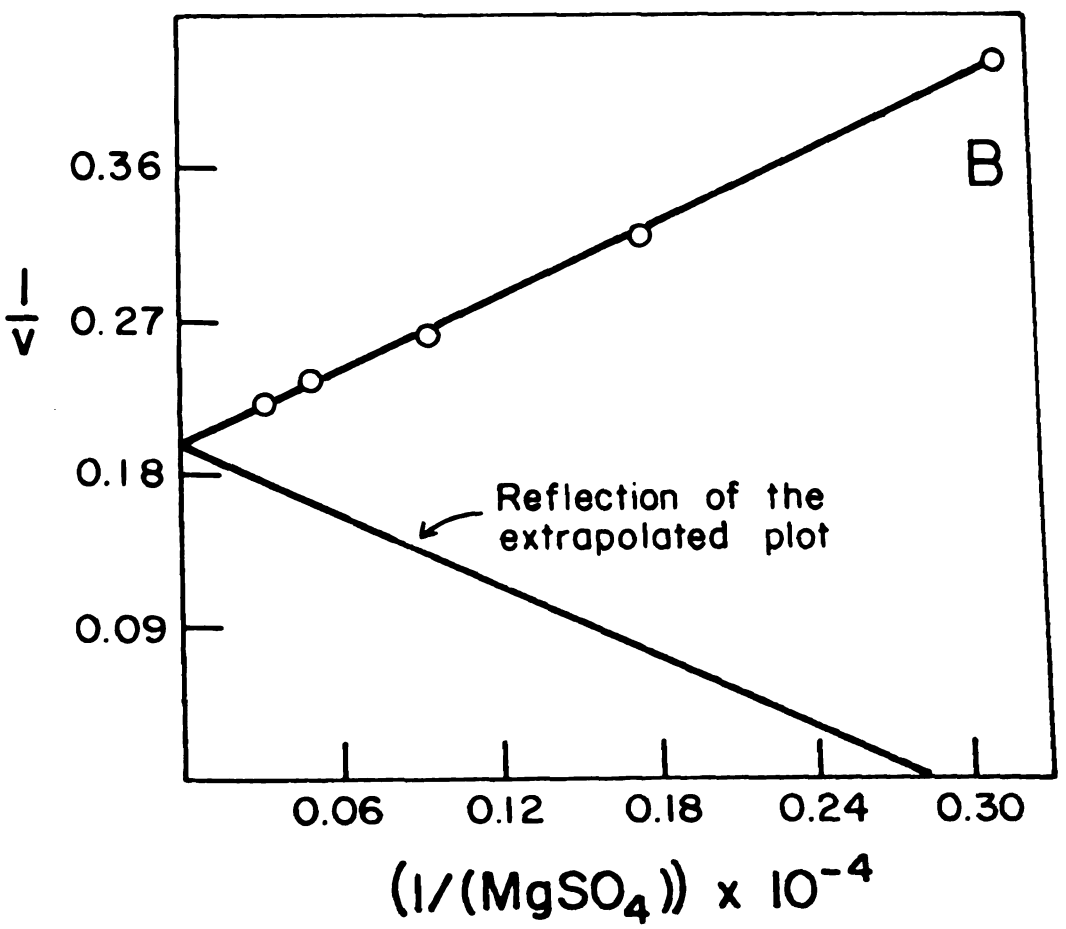
The Effect of Mg^{++} on P-glycolate Phosphatase

The standard P-glycolate phosphatase assay was used except that the concentration of $MgSO_4$ was varied. The enzyme which was used was prepared as described for Figure 3. Each reaction mixture contained 30 µl of enzyme. The enzyme was saturated with substrate during the 10 minute reactions at 30°.

v = velocity in µmoles of P-glycolate hydrolyzed per 10 minutes per 3 ml reaction mixture.

Figure B: The v value from Figure A which was obtained even without added $MgSO_4$ was not used. The other ($MgSO_4$) values from Figure A were each increased by 0.65×10^{-4}M. The reciprocals of these corrected values were plotted. The 0.65×10^{-4}M correction is an estimate obtained by extrapolation (dotted line of Figure A) and is based on the assumption that the activity without added $MgSO_4$ was because of endogenous metal which was not all removed by the acetone fractionation.


A
4.0
3.0
V
2.0
1.0
6
12
18
24
30 x 10⁻⁴M
(MgSO4)


B
0.36
1/V
0.27
0.18
0.09
Reflection of the extrapolated plot
0.06
0.12
0.18
0.24
0.30
(1/(MgSO4)) x 10⁻⁴

apparent K_A for Mg^{+2} was found to be 3.5×10^{-4}M.[1]

H^+ concentration is a factor that must be considered in the Fiske and Subbarow method for the determination of P_i (83). Using the modified Fiske and Subbarow method, 1.0 ml aliquots made up with 3/4 ml of the phosphorous standard solution had the same absorbance values with 1/4 ml of 10% trichloroacetic acid as with 1/4 ml of H_2O. Thus, the amount of 10% trichloroacetic acid used in the standard P-glycolate phosphatase assay had no effect on the determination of P_i by the modified Fiske and Subbarow method.

One unit of enzyme is defined as the amount which catalyzes the hydrolysis of 1 μmole of substrate per minute under the conditions of the standard P-glycolate phosphatase assay. Specific activity is units per mg of protein. Since a unit was previously defined in terms of μgrams of phosphorous released in 10 minutes (202, 265), it is necessary to divide previous units and specific activities by 10 x 30.975 or 309.75 if comparison is desired.

Aconitase Assays

The procedure which was used is based on that of

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[1]The apparent K_A must be considered an approximation for two reasons. First, the enzyme was only purified about 10-fold by the acetone fractionation. Secondly, the enzyme preparation showed some activity even without added $MgSO_4$ (Figure 4).

Racker (196). A similar procedure was used by Bacon et al to measure the aconitase activity in a variety of plants (11).

Citric acid (Nutritional Biochemicals Corp.), isocitric acid (Calbiochem, A grade, DL-isocitric acid · $\frac{1}{2}H_2O$, allo free), and cis-aconitic acid (Calbiochem A grade) were obtained as the free acids. Solutions of these acids were adjusted to pH 7.5 with sodium or potassium hydroxide before use.

Assay protocols varied and are given in the results section. Reaction rates were measured by following the change in absorbance at 240 mμ in a Beckman D.U. spectrophotometer which was equipped with a Gilford cuvette positioner, Gilford optical density converter, and a Sargent recorder. The temperature was controlled at 25° in the constant temperature cell holder supplied with the spectrophotometer. Two controls, one containing enzyme but no substrate, and the other containing substrate but no enzyme, were run to establish blank rates which were subtracted from the measured reaction rates to give the aconitase reaction rates.

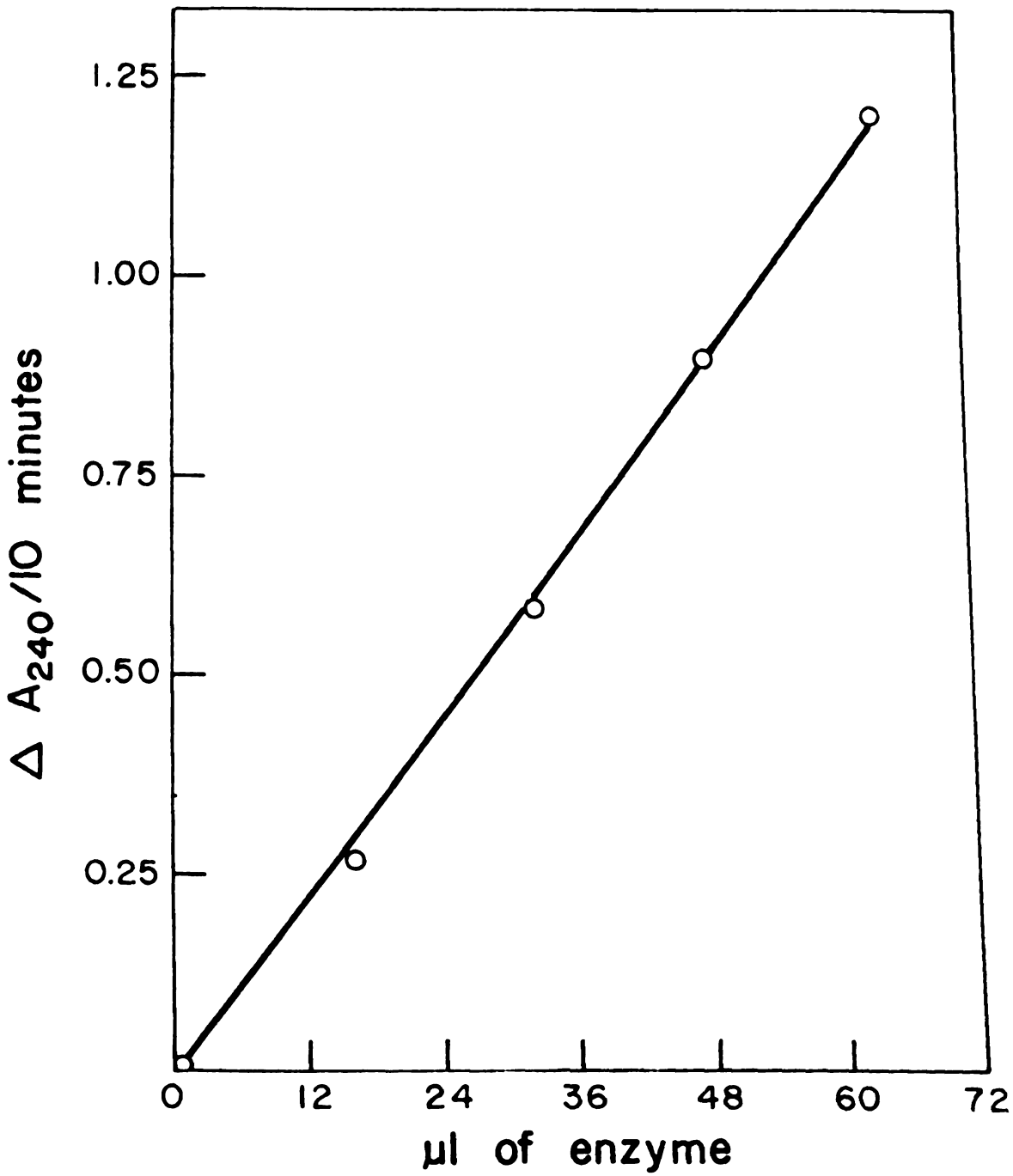
The effect of the enzyme concentration on the reaction velocity is shown in Figure 5.

The millimolar extinction coefficient at 240 mμ, which may be calculated from an absorbance of 0.71 for 0.20 mM cis-aconitate (196), is 3.55 mM^{-1} cm^{-1}.

Figure 5

Initial Reaction Rates for Aconitase as a Function of the Concentration of Enzyme

The enzyme which was used had been purified by one acetone precipitation and had come from an extract which had been prepared by mortar and pestle using Swiss chard leaf tissue. The reaction mixtures included 0.33 ml of 0.10M TES (98), pH 7.5, 0.10 ml of 0.10M $MgSO_4$, 0.020 ml of 0.01M sodium cis-aconitate, pH 7.5, and enough water to bring the reaction volumes to 1.00 ml. The reactions were initiated by the addition of cis-aconitate. The reaction rates were constant for at least the first $1\frac{1}{2}$ minutes.


1.25
1.00
0.75
0.50
0.25
Δ A240/10 minutes
0
12
24
36
48
60
72
µl of enzyme

Recovery and Yield

The term recovery is used to mean the sum of the units in all the fractions of an isolation step divided by the total units submitted to the fractionation, multiplied by 100 to convert to a percent. Yield is calculated on the basis of the amount of enzyme in pooled fractions which was saved or purified further.

The Stability of P-glycolate Phosphatase at 45°

Enzyme in a test tube was placed in a 45° water bath for 1 hour, after which it was quickly cooled by immersing the tube in an ice bath. The activities of the heated preparation and a control held in the ice bath the entire time were then measured in units per ml of enzyme. The ratio of the activities, expressed as a percent, is the "stability at 45°" in this communication.

The Stability of P-glycolate Phosphatase toward Dilution at 30°

A reaction mixture for a standard P-glycolate phosphatase assay, less substrate and enzyme, was prewarmed to 30°. At zero time, the assay amount of cold enzyme was added. After 1 hour at 30°, the phosphatase reaction was initiated with the standard amount of substrate, which was also at 30°. The reaction time and temperature, the method of terminating the reaction, and

the assay for P_i were as described for the standard P-glycolate phosphatase assay. The phosphatase activity from such an assay is the "activity after dilution at 30°." The control consisted of a standard P-glycolate phosphatase assay. Thus, for the control the reaction was initiated with cold enzyme which was added to the otherwise complete reaction mixture, which was at 30°. The activity after dilution at 30° and the control were measured in μmoles of P_i per minute per ml of enzyme. The ratio of the activities, expressed as a percent, is the "stability toward dilution at 30°" in this thesis.

The inactivation of P-glycolate phosphatase as a function of the time the enzyme was diluted at 30° is shown in Figure 6.

In some experiments, stability toward dilution at 30° was studied as a function of the time after homogenization. For the controls, the post homogenization times were from the time of the homogenization to the times at the start of the reactions. For the activities after dilution at 30°, the post homogenization times were from the time of the homogenization to the times at the start of the 1 hour of dilution at 30°.

The Enzymatic Determination of Isocitrate (219)

A mixture which contained 0.70 ml of 0.10M TES (98), pH 7.5, 0.15 ml of 0.02M $MnCl_2$, 0.040 ml of 5×10^{-3}M

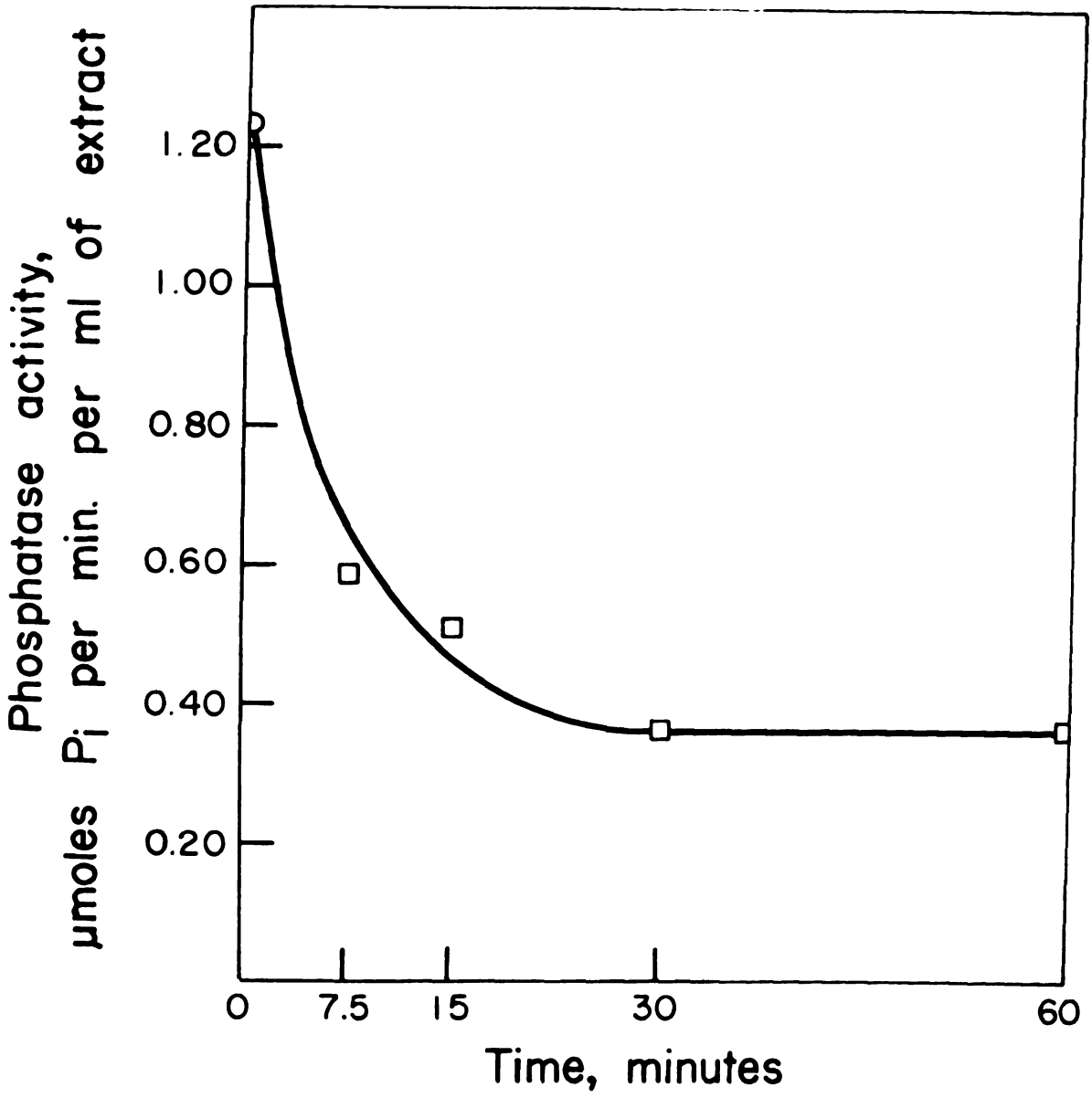
Figure 6

Inactivation of P-glycolate Phosphatase

One tobacco leaf was harvested on 11-2-1964 from the second growth of a plant which had been grown in the field and then transplanted back to the greenhouse about Oct. 1. The leaf blade tissue was homogenized in a mortar and pestle with two weights of cold 0.1M cacodylate, pH 7.5.

- ○ Phosphatase reaction initiated with cold fresh extract.[1]
- □ P-glycolate phosphatase reaction mixtures without substrate or enzyme were prewarmed to 30°. At zero time, aliquots of cold fresh extract[1] were added to these reaction mixtures. For each assay, the extract was diluted 20-fold during the dilution at 30°. After the designated times, the phosphatase reactions were initiated with 1.0 ml of substrate which was at 30°.

[1]The extract had been centrifuged by increasing the force quickly to 12,000 g and then immediately allowing the centrifuge rotor to coast to a stop.


Phosphatase activity,
μmoles P_i per min. per ml of extract
1.20
1.00
0.80
0.60
0.40
0.20
0
7.5
15
30
60
Time, minutes

$NADP^+$ dissolved in the 0.10M TES, pH 7.5, the desired amount of the unknown solution or of 5×10^{-4}M stock isocitrate,[1] pH 7.5, and enough water to bring the final reaction volume to 1.20 ml, was mixed in a 1.5 ml cuvette. The blank absorbance was recorded at 340 mμ for 5 minutes using a Beckman D.U. spectrophotometer which was equipped with a Gilford OD converter and a Sargent recorder. Then approximately 1/5 μl of highly purified isocitrate dehydrogenase from pig heart (Sigma Type IV), which had been held at 0°, was added and the complete mixture was remixed.[2,3] The increase in absorbance at 340 mμ, which

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[1]The stock isocitrate solution (Calbiochem, C grade, dl + allo) contained all four optical isomers. 5×10^{-4}M was the total concentration.

[2]A blank run without isocitrate present showed that the isocitrate dehydrogenase which was added made no measurable contribution to the absorbance at 340 mμ.

[3]The Type IV isocitrate dehydrogenase which was used was shown to be free of aconitase. When 7 μl of 0.01M pH 7.5 Na cis-aconitate was substituted for isocitrate in a reaction mixture, there was no detectable change in absorbance in 8 minutes. Following this, 7 μl of the stock isocitrate solution, which was about 1.25×10^{-4}M in threo-Ds-isocitrate, was added and the absorbance increased rapidly as expected. The stock cis-aconitate solution which was used in this control was used for aconitase assays before and after the experiments for the enzymatic determination of isocitrate and was therefore known to be active as a substrate for aconitase. There was nothing in the reaction mixtures which should have prevented a linked assay from working (219). In a different experiment, when some crude isocitrate dehydrogenase from pig heart (Sigma Type I) was tested for aconitase using the procedure just described for testing the Type IV isocitrate dehydrogenase, it was found to possess significant aconitase activity. Thus, the method which was used is a valid check for the presence of aconitase.

took 3-5 minutes, was recorded and the final absorbance was recorded for about 5 minutes after the reaction had ceased. Then in those cases where no reaction or only a small reaction had occurred, the reaction mixtures were tested by adding a small amount of stock isocitrate and noting the rapid increase in absorbance. The temperature was controlled at 25° in the constant temperature cell holder supplied with the spectrophotometer. Using the known molar extinction coefficient of $6.22 \times 10^{3} M^{-1} cm^{-1}$ for NADPH at 340 mμ (124, 136), the change in absorbance was converted to the concentration of isocitrate in the reaction mixture. From this value and the known dilution of the unknown in the reaction mixture, the concentration of isocitrate in the unknown sample was calculated.

The procedure as described was used for the experiment shown in Figures 16 and 17 of the results section. For the experiment shown in Figure 39, the procedure was the same except all volumes were scaled down to ¼ and the reactions were run in a 0.5 ml cuvette.

Paper Chromatography

General

Whatman No. 1 filter paper was used throughout. Spots were applied by repeated applications of 1 μl each. Before respotting, each spot was dried by a stream of room temperature air from a hair drier. When compounds were to be checked by cochromatography, they were mixed before application. Chromatography containers were pre-equilibrated

with the solvent to be used. Where necessary, extra papers were included during the run to maintain equilibration. Pre-equilibrations and runs were made at room temperature. As many as 4 dosages were tried for each compound with a given solvent system and method of detection. Results reported were from the most optimum of the dosages tried.

Solvent Systems

t-Butanol-water-picric acid system (105): 80 ml of t-butanol plus 20 ml of H_2O plus 4 grams of picric acid.

Phenol-water system (25): 80 ml of phenol plus 20 ml of H_2O. The phenol used was Mallinckrodt analytical reagent which was approximately 88% phenol by weight, so that the final solution was about 70% phenol by volume.

n-Butanol-propionic acid-water system (25): Fresh solvent was prepared from equal volumes of two solutions, A and B. Solution A contained 1246 volumes of n-butanol and 84 volumes of H_2O. Solution B contained 620 volumes of propionic acid and 790 volumes of H_2O.

n-Butanol-ethyl acetate-formic acid system (215): 100 ml of n-butanol plus 100 ml of ethyl acetate plus 100 ml of formic acid.

Ether-acetic acid-water system (60): 130 ml of diethyl ether plus 30 ml of glacial acetic acid plus 10 ml of H_2O.

Detection of Compounds on Paper

Organic Phosphates (13, 24, 105)

The phosphate spray solution was made as follows: 5 ml of 60% (weight by weight) perchloric acid, 10 ml of 1.0N HCl, 25 ml of 4% (weight by volume) ammonium molybdate, and 60 ml of H_2O (13, 105). The dry chromatograms were lightly sprayed with the phosphate spray solution, dried in a prewarmed oven at about 90° for 1 minute, and then uniformly irradiated with a G.E. Germicidal lamp (2537 Å) from 10 cm for 10 minutes to develop the blue color characteristic of all organic phosphates (13). By this method, inorganic phosphate produces a yellow green color (13).

Acids

The dry chromatograms were sprayed with alkaline bromocresol green made by mixing 400 mg of the dye with one liter of 95% ethyl alcohol and titrating the solution to a blue color with NaOH (60). Acids were detected as yellow spots against the blue background. To eliminate the yellow background which was sometimes encountered after spraying the chromatograms, it was necessary to use one or more of the following treatments, none of which noticeably reduced the sensitivity. Some of the less intense yellow spots were not visible until after one or

more of these treatments.

n-Butanol-propionic acid-water solvent system: 0.10 ml of 6N NaOH was added per 100 ml of the butanol-propionic acid-water solvent (108). This modification gave better visualization of acid spots of lower Rf values.

After chromatography in some of the solvent systems, it was advantageous to autoclave the chromatograms to remove traces of acid solvents. The chromatograms were placed on edge in a stainless steel basket which was raised off the floor of the chamber by means of two horizontal test tubes. The autoclave was programmed for "dry goods," i.e. after sterilization, steam was immediately and automatically vented from the chamber. The programmed sterilization temperature was 100° and the sterilization time was 3 minutes. The drying time (venting) was programmed at 5 minutes.

When one or the other or both of the previous two treatments did not elininate all the yellow background, the remaining background could be converted to a blue color by spraying lightly with 3×10^{-3}M Na_2CO_3.

Detection of ^{14}C

The dry chromatograms were exposed to Kodak No-Screen X-ray film for about 1 to 2 weeks. Dating the chromatograms with radioactive ink before exposure to

the X-ray film provided for accurate later matching between the developed films and the chromatograms. Occasionally, the radioactivity was first counted on the chromatograms using a Nuclear Chicago Scaler (Model 161A) with a thin window (DuPont Mylar film) gas flow counter. Helium, passing through ethanol at 0°, provided the gas for the counting chamber. The time to expose the X-ray film was inversely proportional to the cps with 20 cps requiring a week's exposure. The counting efficiency was estimated at 10% (116).

Purity and Characterization of P-glycolic Acid

This compound was synthesized for this project by General Biochemicals, Chagrin Falls, Ohio, and was obtained as the tricyclohexylammonium salt.

An 8 µl aliquot containing 125 µg of the free acid (converted from the tricyclohexylammonium salt with Dowex 50 H^{+}) gave a single spot (detected by the phosphate spray-UV lamp method) when chromatographed in the t-butanol-water-picric acid system. The Rf averaged 0.76 and the position constant (relative to H_3PO_4) averaged 1.12. The Rf and the position constant in a similar solvent system (80 ml of t-butanol + 20 ml of H_2O + 2 g of picric acid) have been reported as 0.68 and 1.06 respectively (24). In three other solvent systems, namely the phenol-water, n-butanol-propionic acid-water, and n-butanol-ethyl

acetate-formic acid systems, a hydrolysate of P-glycolic acid, to which had been added glycolate-^{14}C,[1] showed a leading spot that had essentially the same Rf as commercial glycolic acid. The hydrolysate also showed trailing spots that had essentially the same Rf values in all three systems as commercial phosphoric and unhydrolyzed P-glycolic acid. No other spots were detected. The developed X-ray films corresponding to the chromatograms from the three solvent systems each showed a single spot which exactly matched the leading hydrolysate spots. The concentration of the labeled glycolate was 1/2500 of the total concentration of the P_i and P-glycolate in the hydrolysate, and it was demonstrated that the yellow color in the leading hydrolysate spots could not have come from the radioactive glycolate.

An aliquot of the P-glycolate was exhaustively hydrolyzed using a 14-fold purified phosphatase preparation, and the P_i and glycolate were measured colorimetrically. The glycolate/P_i ratio after hydrolysis was 0.9.

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[1] 150 µl of the acid (after Dowex 50 H^+ treatment), at about 0.1M and pH 1, was sealed by flame in a piece of thoroughly cleaned and dried glass tubing. This sealed sample was then kept at 100° for 12 hours. After cooling, the seal was broken and 15 µl of 2-^{14}C glycolic acid, 10 µc/ml, was added and the sample was mixed. The glycolate 2-^{14}C (4 mc/mmole) had been obtained as the calcium salt from Nuclear Research Chemicals, Inc., Orlando, Fla. It was converted to the free acid with Dowex-50 (H^+) and diluted to 10 µc/ml.

When the P-glycolate at 10^{-3}M was refluxed in 1.0N HCl at 100°, only 1.6% of the phosphate was released by hydrolysis per 7 minutes (Figure 7).[1]

When 5.00 μmoles of NaOH were added to 2.50 μmoles of Dowex-50 H^+ treated P-glycolic acid, the pH was about 4.5. Addition of 2.50 more μmoles of NaOH raised the pH to about 7.5. If the phosphate were joined to the glycolate by an anhydride and an ester bond to form a cyclic compound, 2.50 μmoles of NaOH would have raised the pH above neutrality, while if it were joined by an acid anhydride bond only, then 5.00 μmoles of NaOH would have raised the pH above neutrality. That 7.50 μmoles of NaOH were required to raise the pH above neutrality is consistent with the accepted formula for P-glycolic acid in which the phosphate moiety is joined to the glycolate moiety by an ester bond only.

It is concluded that the P-glycolate was in fact P-glycolate and that it was chromatographically pure.

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[1]It has been reported that the half life of P-glycolic acid in 1N HCl at 100° is 13 hours, and that under these conditions, the hydrolysis of the ester follows first order kinetics (49). From the half life of 13 hours and the relationship $dc/dt = -kc$, the predicted percent of hydrolysis of P-glycolic acid in 7 minutes under these conditions is 0.6%.

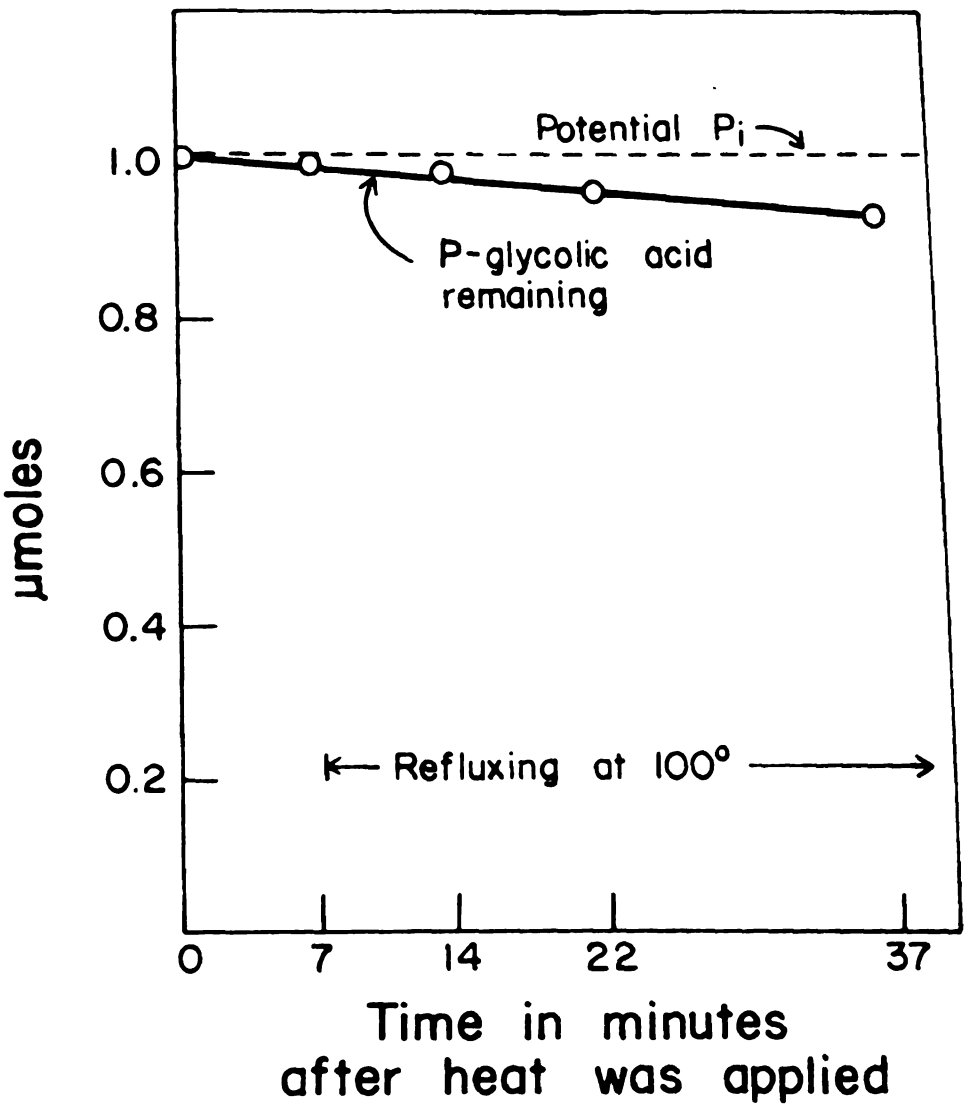
Figure 7

Stability of the Phosphate-Glycolate Bond of P-glycolic Acid

One hundred µl of 0.1M pH 1 Dowex-50 H^+ treated P-glycolic acid was mixed with 9.9 ml of 1.0N HCl so that each ml of the mixture contained 10 µl of the original P-glycolic acid solution. The total phosphorous in 10.0 µl of the original P-glycolic acid solution was determined by digestion and hydrolysis as outlined under "Determination of Total Phosphorous." A 1.0 ml aliquot was removed from the 1.0N HCl:P-glycolic acid mixture and the remainder was refluxed at atmospheric pressure (100-101°). As soon as the mixture started to boil, a second 1.0 ml aliquot was removed. Third, fourth, and fifth 1.0 ml aliquots were removed at 7, 15, and 30 minutes after the start of boiling. As each aliquot was removed from the reflux pot (which was otherwise kept stoppered except for the connection to the condenser), it was cooled to 0° and neutralized with a predetermined volume of concentrated NaOH (0.12 ml). The remainder of each P_i determination was as described by the modified method of Fiske and Subbarow for the determination of P_i.

- - - Potential P_i per ml of the 1.0N HCl:P-glycolic acid mixture.

o——o Potential P_i minus the P_i released by hydrolysis in 1.0N HCl, per ml.


Potential P_i
P-glycolic acid remaining
1.0
0.8
0.6
0.4
0.2
µmoles
← Refluxing at 100° →
0
7
14
22
37
Time in minutes after heat was applied

RESULTS AND DISCUSSION

The Adequacy of P-glycolate Phosphatase

For the summers of 1964 through 1967, the specific activity of P-glycolate phosphatase in extracts from tobacco leaves, which had been harvested from plants grown in the field, varied from 0.036 to 0.810. During the same periods, the units per gram fresh weight of blades from these tobacco leaves varied from 2.7 to 16.5. The average was about 9.6, which is equivalent to 580 μmoles of P-glycolate hydrolyzed per hour per gram fresh weight.

Although no exact quantitative comparison can be made between P-glycolate phosphatase and other leaf phosphatases, sap from green wheat leaves hydrolyzed P-glycolate 5 times faster than PGA at pH 6.3 (265). P-glycolate phosphatase activity in the crude sap from green wheat leaves was 280 μmoles of P-glycolate hydrolyzed per hour per gram fresh weight. Peterkofsky and Racker determined CO_2 fixation rates of 32 μmoles per hour per mg of chlorophyll for spinach leaves, and 1 g wet weight of wheat leaves contains 0.5 to 1 mg of chlorophyll (265). These comparisons suggest that the P-glycolate phosphatase activity in the leaves of higher plants is greatly in excess

of the *in vivo* CO_2 fixation rates. However, P-glycolate phosphatase activity is measured at 30° and the other conditions during the measurement of activity are optimized. The *in vivo* CO_2 fixation rate reported by Peterkofsky and Racker (190) was at 20° and the other conditions may not have been optimal. In contrast to the results reported by Peterkofsky and Racker, the average *in vivo* CO_2 fixation rate for 17 plants, reported by Rabinowitch (195), was 348 µmoles of CO_2 per hour per gram fresh weight. The values ranged from 200 to 524. The CO_2 fixation rates reported by Rabinowitch were also at 20°, but both the light intensity and the CO_2 concentration were optimized for maximum CO_2 fixation. 348 µmoles of CO_2 could give 174 µmoles of P-glycolate. Although no literature value for the µmoles of CO_2 fixed per hour per gram fresh weight was found for tobacco, and no such measurement has been reported for a batch of leaves from which P-glycolate phosphatase activity was also measured, the available evidence suggests that the phosphatase activity in tobacco leaves, and in the leaves of other plants, is significant in comparison to the CO_2 fixation rates by these leaves.

Influence of Leaf Size and Position on P-glycolate Phosphatase Activity

Small and medium sized leaves had the greatest phosphatase activity per gram of leaf and the greatest specific activity, both values being about twice those

found for the large leaves (Table 1). From the data, it is suggested that the variation in activity per gram fresh weight was not predominantly caused by the variation in the percent of water content of the leaves. Two other experiments (data not shown) with field grown tobacco, one performed 8-14-1964 and one performed 9-10-1965, gave essentially the same results. One quantitative difference in the two other experiments was that the small leaves had relatively less activity and specific activity. Furthermore, in the experiment of 9-10-1965, it was found that old leaves even lower than the fully mature large leaves had considerably less activity and specific activity than the mature large leaves. These findings seem to be correlated with evidence that glycolate accumulation occurs in particularly large amounts with young tissue (233).

Effect of the Homogenization Method on P-glycolate Phosphatase

The phosphatase extracted by mortar and pestle was unstable toward dilution at 30°, while the enzyme extracted by Waring blendor was stable (Figure 8). The activity per gram of leaf was about 20% less when the extract was made by Waring blendor than when it was made by mortar and pestle.

The phosphatase in extracts prepared by a Hobart meat grinder was also unstable toward dilution at 30°.

Table 1

Influence of Leaf Size and Position on P-glycolate Phosphatase Activity

The tobacco leaves were harvested and the experiment was completed on 7-13-1966. Fifteen small tobacco leaves were harvested one leaf per plant. The leaf blades were homogenized in a Waring blendor with two weights of cold water for 1½ minutes. Phosphatase and protein assays were run on the extract. The procedure was repeated with fifteen medium sized leaves and then with fifteen large leaves. The small leaves were harvested about 11:20 A.M., the medium leaves about 3:15 P.M., and the large leaves about 5:30 P.M.

	Leaf Size		
Property	Small	Medium	Large
Position of leaf on the plant	Top	Top half	Bottom half
Length of leaf, cm.	14.3 ± 1.9	27.0 ± 5.7	46.3 ± 6.4
Weight*	1.51	5.14	20.0
Activity**	12.0	12.7	5.6
Specific activity	0.47	0.50	0.28

*Average fresh weight in grams of one deribbed leaf.

**Units of phosphatase activity per gram fresh weight of deribbed leaf.

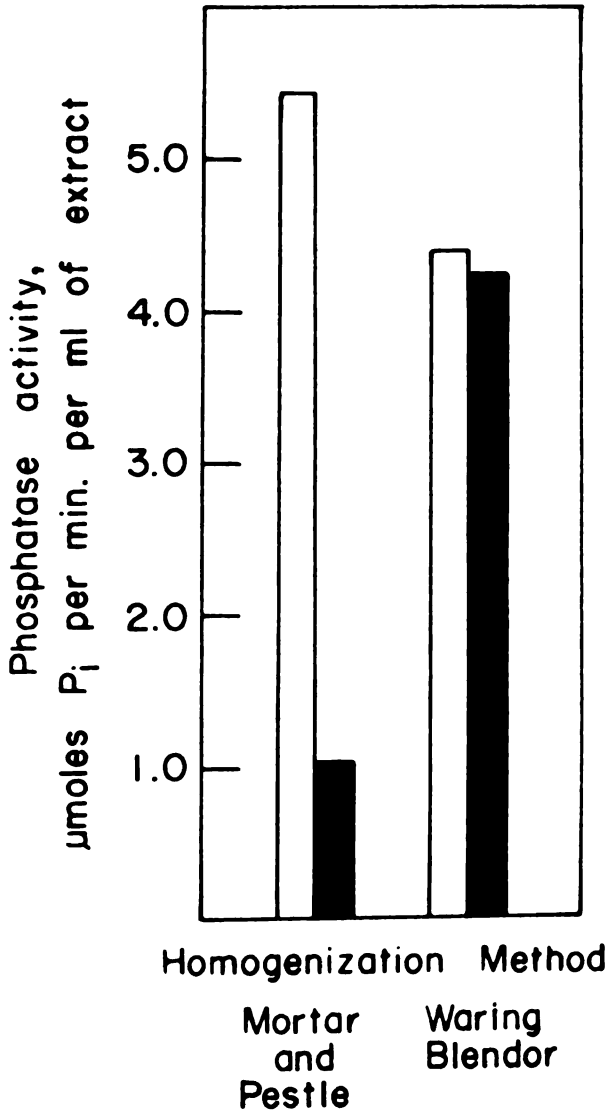
Figure 8

Effect of the Homogenization Method on the Stability Toward Dilution at 30° of P-glycolate Phosphatase

The tobacco leaves were harvested and the experiment was completed on 9-1-1965. Seven medium sized leaves were harvested from two adjacent tobacco plants. A representative portion of leaf tissue was homogenized in a mortar and pestle with sand (3 minutes) and the remainder was homogenized in a Waring blendor (2 minutes). Both homogenizations were made with two weights of cold water per fresh weight of tissue. Assays were run on the two extracts, which had been centrifuged at 20,000 g for 10 minutes.

Open bars: controls.

Closed bars: activity after 20-fold dilution at 30°.


Phosphatase activity,
μmoles P_i per min. per ml of extract
5.0
4.0
3.0
2.0
1.0
Homogenization Method
Mortar and Pestle
Waring Blendor

In this respect, the enzyme in extracts prepared by the meat grinder was similar to the enzyme in extracts prepared by mortar and pestle.

Dilution at 0° for an hour also resulted in considerably more inactivation of the phosphatase than did keeping the undiluted extract at 30° for an hour. However, dilution at 30° for an hour resulted in considerably more inactivation than did dilution at 0° for an hour (Table 2). This pattern was found to be true for extracts prepared by the meat grinder as well as by mortar and pestle.

Since the phosphatase in extracts which are prepared by mortar and pestle or by the meat grinder is unstable toward dilution even at 0°, it is desirable to keep the volume of the extraction fluid to a minimum when using these two methods for the extraction of this enzyme.

Stability of P-glycolate Phosphatase as a Function of pH

The phosphatase was most stable at about pH 6 (Figure 9). It was much less stable on the acid side of pH 6 than on the alkaline side. Since the results shown in Figure 9 were obtained after the designated aliquots were readjusted to pH 6.3 and held at pH 6.3 for 6 hours (see the legend of Figure 9), it is suggested that the losses in activity at the designated pH values

Table 2

The Effect of Dilution at 0° and 30°

The leaf blades, from 3 medium sized tobacco leaves harvested on 8-4-1967, were homogenized in a mortar and pestle with sand with 2 weights of cold H_2O.

Pretreatment	Activity (%)
None	100
Dilution* at 30°, 1 hr.	4
30° without dilution, 1 hr.	90
Dilution* at 0°, 1 hr.	42**

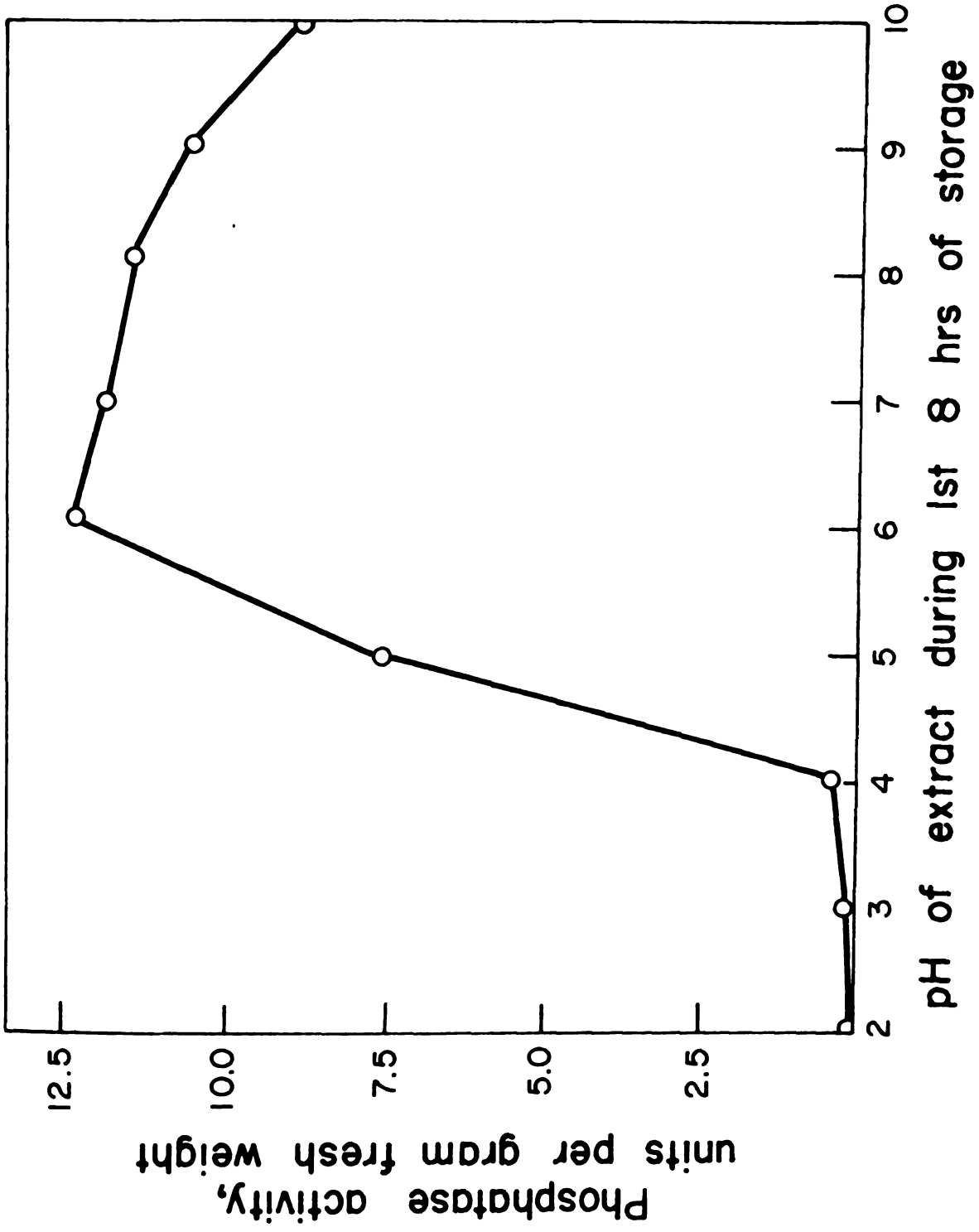
*The extract was diluted 33.3-fold during the 1 hour of dilution.

**The reaction was initiated with substrate which was at 30°. The complete reaction mixture was then immersed in the 30° bath for the 10 minute reaction. This assay needed a separate control because for the first part of the reaction, the temperature in the reaction mixture was less than 30°.

Figure 9

Stability of P-glycolate Phosphatase in Extracts as a Function of pH

Leaf blades, from 20 medium sized tobacco leaves harvested on 7-22-1966, were passed through a Hobart meat grinder with no water added. The pH of the extract was 6.08. 10.0 ml aliquots of the extract were adjusted to the designated pH values with KOH or HCl. The volumes of all aliquots were then adjusted to 15.0 ml with cold water. After 8 hours, all aliquots were adjusted to pH 6.3 and the volumes then were adjusted to 20.0 ml with cold water. After another 6 hours at pH 6.3 with occasional stirring, phosphatase activity in each aliquot was measured.


Phosphatase activity,
units per gram fresh weight
12.5
10.0
7.5
5.0
2.5
2
3
4
5
6
7
8
9
10
pH of extract during 1st 8 hrs of storage

were irreversible.

Over a two year period, the pH of tobacco leaf extracts, which were made with water as the extraction fluid, varied from as low as 5.3 to as high as 6.4. pH values below 5.5 were rare. The pH of extracts showed a diurnal variation of about 0.4 pH unit (Figure 20). The extract pH was also a function of the leaf position. Extracts from lower larger leaves had a lower pH than extracts from upper medium sized leaves. pH also seemed to depend on the availability of ions, since fertilized plants gave extracts of a higher pH than did unfertilized plants. Finally, extract pH also seemed to depend on temperature, with leaves harvested during cold periods giving extracts with lower pH values than leaves harvested in warm periods.

The pH range of 5.5 to 6.4, which was obtained in the tobacco leaf extracts when water was used as the extraction fluid, is about right for the stability of the phosphatase (Figure 9). Therefore, buffers were not necessary for the extraction of the phosphatase. The lower ionic strength in the extracts from the use of water as the extraction fluid may have been beneficial in the subsequent acetone fractionations (67).

The Purification of P-glycolate Phosphatase and the Stability of the Phosphatase at the Different Stages of Purification

A summary of the procedure, including the stabilities

of the enzyme at the different stages of purification, is given in Table 3. The acetone which was used was reagent grade.

Extract

Five batches of leaves were harvested from 9-13-1966 through 9-16-1966 during daylight hours. Five separate extracts were prepared as described in the methods section.[1] Each batch of leaf tissue was separately passed through a Hobart meat grinder. To each of these 5 preparations was added one weight of cold H_2O. After each slurry was allowed to stand for 10-15 minutes, it was squeezed through a double layer of cheesecloth and then centrifuged. The pH of the extracts varied from 5.5 to 6.0. No pH adjustments were made. The 5 extracts were assayed separately for protein and for phosphatase activity. The value for the units of phosphatase per ml which was used, and the specific activity of 0.431 (Table 3), are weighted averages. The total fresh weight of the leaf tissue from which the 5 extracts were made was 6.94 kg. The total volume of the 5 extracts was 10,270 ml. The weighted average of the units of phosphatase activity per gram fresh weight of leaf tissue was 8.25.

[1]Because of the size of the batches of leaves, somewhat more than 15 minutes elapsed between the time of harvest and the time of homogenization. However, the harvesting and the preparation of the leaves were done without delay.

First Acetone Fractionation

Pilot fractionations were first made to accurately determine the acetone concentrations necessary to precipitate the phosphatase. Each of the 5 extracts was separately fractionated with acetone. The total volume of the 5 extracts which was fractionated by acetone was 9,985 ml. A volume of 0° acetone equal to 35 to 39% of the volume of an extract (the extracts varied from 11 to 22 mg of protein per ml) was added simultaneously through five 0.7 mm I.D. teflon or four 1.14 mm I.D. polyethylene tubes over a period of 15-20 minutes while the mixture (held in an ice bath) was continuously stirred by a magnetic stirrer. The mixture was centrifuged at 15,000 x g for 15 minutes and the precipitate was discarded. A volume of acetone equal to 20% of the volume of the original extract was added as before to precipitate the enzyme. This precipitate was removed by centrifugation at 15,000 x g for 8 minutes and the supernatant solution was drained and rinsed as completely as possible from the centrifuge cups. The precipitate from each of the five fractionations was taken up in a volume of cold 0.02M cacodylate, pH 6.3, equal to one-eighth of the volume of the original extract. Each suspension was recentrifuged at 15,000 x g for 8 minutes and the precipitate was discarded. After assay, the 5 supernatant solutions were combined to give 1332 ml of 22.7-fold purified phosphatase. The value for the

units of phosphatase per ml which was used, and the specific activity of 9.80 (Table 3), are weighted averages. It was found that phosphatase preparations made as described here lost no activity at 0-4° in 4 days, which was the time required to accumulate the enzyme which had been fractionated once by acetone.

Second Acetone Fractionation

A pilot fractionation was first run. 197 ml of 0° acetone (15.25 ml per 100 ml of aqueous solution) was added as before to 1290 ml of the aqueous solution from the first acetone fractionation. The mixture was centrifuged at about 6000 x g for 8 minutes and the precipitate was discarded. Then 323 ml of 0° acetone was added as before (25 ml per 100 ml of aqueous solution) and the mixture was centrifuged at 6000 x g for 8 minutes to remove the enzyme. After careful removal of as much of the acetone solution as possible, the precipitate was converted to a powder, as described later, and the 13.56 grams of powder was stored in a dessicator at 0-4° over anhydrous $CaSO_4$.

Third Acetone Fractionation

In this and subsequent steps, the stability at 45° and the stability toward dilution at 30° of the enzyme were measured. After 3 months of storage, 6.5 grams of

the dry powder from the previous step was broken up by mortar and pestle with 248 ml of cold 0.02M cacodylate, pH 6.3 (6.5 g/13.56 g of the 1290 ml of the aqueous solution from the first fractionation x 40%). After 10 hours, the mixture was centrifuged at about 6000 x g for 8 minutes to remove an inactive precipitate. The supernatant solution contained 1.3 mg of protein per ml and the phosphatase in the solution was 52.6-fold purified. No loss in activity was observed during the 3 months of storage of the dry powder from the previous step.

After a pilot fractionation at pH 5.7, the pH of the remaining aqueous solution was adjusted from 6.6 to 5.8 with 0.1N HCl. The volume was then 250 ml. 71 ml of 0° acetone (28.3% of 250 ml) was added as before through four 0.7 mm I.D. teflon tubes. The precipitate was discarded by centrifugation at about 6000 x g for 8 minutes. Then 50 ml of 0° acetone (20% of 250 ml) was added as before to precipitate the enzyme. The precipitate was collected by centrifuging at about 6000 x g for 8 minutes. The supernatant solution was drained and rinsed as completely as possible from the centrifuge cups with cold water and then the precipitate was taken up with 60 ml of 0.02M cacodylate, pH 6.3. The mixture was recentrifuged at about 6000 x g for 8 minutes to remove insoluble material. The final solution contained 73-fold purified phosphatase and 4.8 mg of protein/ml in a volume of 69 ml.

DEAE-Cellulose Chromatography

DEAE-cellulose was pretreated as recommended by Peterson and Sober (192). It was adjusted to pH 6.3 and equilibrated with 0.02M cacodylate, pH 6.3, in a column 2.5 cm in diameter and 11.2 cm high. Thirty-six ml of the enzyme from the previous step was added to the column and eluted with 200 ml of the same buffer containing NaCl in a linear gradient from 0 to 0.50M. Enzyme recovery was 94%. The most active fractions 67 through 91, eluted at a NaCl concentration of 0.12M, contained 196-fold purified phosphatase and 0.86 mg protein per ml in a volume of 49.5 ml.

Concentration of the Pooled Fractions

1.6 cm diameter dialysis tubing was washed with H_2O. 49.0 ml of the pooled fractions from the DEAE-cellulose chromatography was sealed in the dialysis tubing and the whole was buried in 75 g of dry Sephadex G-25 at 0-4°. The removal of water was allowed to proceed for 4½ days, with occasional replacement of damp Sephadex G-25 with dry material. Eighty-one percent of the activity survived in the final volume of 6.50 ml.[1] The dialysis removed

[1]Through this stage of purification, those P-glycolate phosphatase preparations which were tested were found to be stable at 45° for at least an hour (Table 3). These preparations were at pH 6.3. The stability of the partially purified enzyme at pH 6.3 is consistent with the evidence that the enzyme in tobacco extracts is most stable

about 87% of the water and 46% of the NaCl. The final NaCl concentration was 0.50M and the final protein concentration was 5.7 mg/ml.

Bio-Gel P-60 Chromatography

This step was for the further purification of P-glycolate phosphatase and the resolution of the enzyme into an unstable phosphatase fraction and a stabilizing fraction. A Bio-Gel P-60 (50-150 mesh Calbiochem) column 2.5 cm in diameter x 35.2 cm high was prepared. Gel which had been hydrated in 0.02M cacodylate, pH 6.3, for several days was used, and the entire column was equilibrated with the same buffer. 6.35 ml of the enzyme from the pooled and concentrated fractions from the DEAE fractionation was added to the column and eluted with 0.02M cacodylate, pH 6.3. The recovery of activity was 108%. The most active fractions, 13 through 16, contained 372-fold purified phosphatase and 1.18 mg of protein per ml in a volume of 11.0 ml (Table 3). In contrast to the stability of the phosphatase added to the Bio-Gel P-60 column, the stability at 45° of the phosphatase from the column was only 16%. A comparison of the stability at 45° and the protein concentration after

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at a pH of about 6 (Figure 9). Therefore, by the criterion of enzyme stability, conducting the purification of the enzyme at pH 6.3 seems advantageous. A second advantage in having the enzyme preparations at pH 6.3 is that the assays for P-glycolate phosphatase activity are made at this pH. In any assays which might require a relatively large volume of enzyme, the need for buffering capacity in the reaction mixtures is thus reduced.

Table 3

Purification and Stability of P-glycolate Phosphatase

	Activity*	Yield*	Specific activity	Purification	Stability at 45°**		Stability toward dilution at 30°***	
	units	%	units/mg prot.	-fold		%		%
Extract****	12,620	100	0.431	1		(21.4)	4	(100)
First acetone	10,730	85	9.80	23				
Second acetone****	6,880	54	22.8	53		(2.1)	72	(250)
Third acetone	5,240	41	31.6	73	99	(4.8)	44	(1000)
DEAE-cellulose	3,520	28	84.7	196	100	(0.9)	44	(500)
DEAE concentrate					107	(5.7)	56	(2500)
Bio-Gel P-60	2,090	17	161	372	16	(0.6)		

*Corrected for the portion of each preparation not further purified.

**The numbers in parentheses are protein concentrations in mg/ml during the determinations of the stability of P-glycolate phosphatase at 45°.

***The numbers in parentheses are the numbers of fold dilution during the determinations of the stability of P-glycolate phosphatase toward dilution at 30°.

****These values in the "Stability at 45°" and "Stability toward dilution at 30°" columns were not determined for the preparations of this purification sequence. They are values from other preparations which were made in essentially the same way. In particular they are from extracts made with the meat grinder, and the second acetone precipitate was converted to a powder.

DEAE-cellulose chromatography (100% stable at 45° and 0.9 mg/ml) with the relevant values after Bio-Gel P-60 chromatography (16% stable at 45° and 0.6 mg/ml) (Table 3) suggests that any reduction in the stability at 45° which was because of dilution was minor. Further investigation indicated that a large part of the lability was caused by the separation from the phosphatase, during the gel filtration chromatography, of a later fraction which when recombined with the phosphatase, restabilized the enzyme (Figure 10).[1] The elution volumes to the center of the phosphatase peak and to the most active stabilizing fraction were 60 ml and 150 ml respectively. When a portion of the stabilizing fraction at pH 6.3 was kept in a boiling water bath for one hour, it lost no stabilizing activity. Thus the stability factor, which followed the phosphatase through three acetone fractionations and a DEAE-cellulose chromatography step, is small compared to the phosphatase and it was stable for at least an hour at 100° at pH 6.3.

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[1]When a phosphatase preparation which was purified only by three acetone fractionations was submitted to Sephadex G-100 gel filtration, the results were qualitatively the same as shown in Figure 10. The maximum percent stability conferred by adding back the stabilizing factor was then 87%. The enzyme had come from an extract which had been prepared by Waring blendor, and after one fractionation by acetone, it had been stored as a dry powder. The stability assay for the G-100 fractions was different from the stability assay for the Bio-Gel P-60 fractions. The unstable enzyme with and without stabilizing fractions was held at 0-4° for 40 hours. Without the endogenous stabilizing factor, most of the phosphatase activity was destroyed. The most active factor fraction saved 87% of the activity which was otherwise lost.

Figure 10

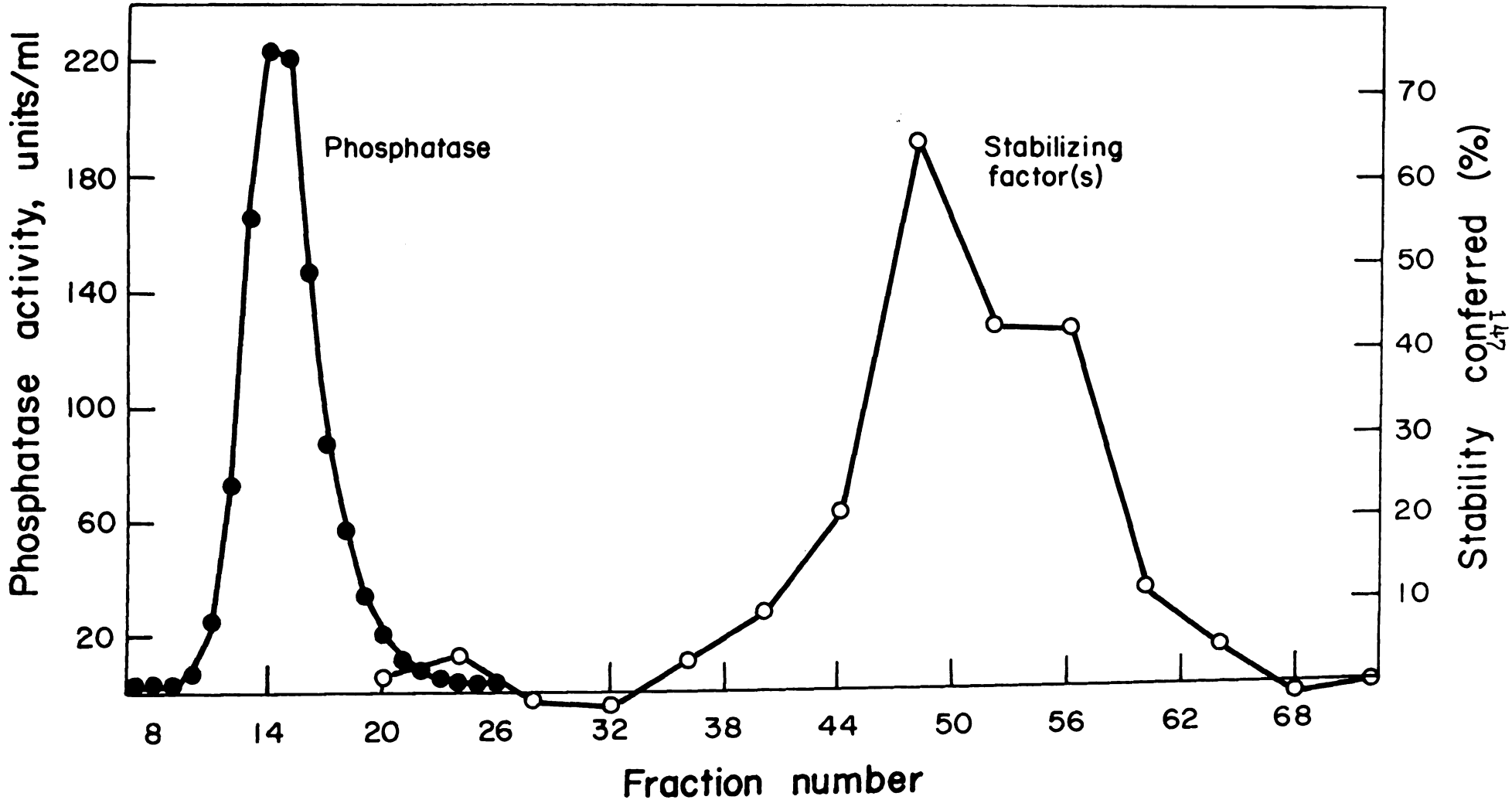
Resolution of P-glycolate Phosphatase into an Unstable Phosphatase Fraction and a Stabilizing Fraction during Bio-Gel P-60 Chromatography

Fractions were first assayed for P-glycolate phosphatase activity, then fractions 13 through 16 were pooled.[1] The assay for the stability factor was as follows: 100 µl aliquots of the designated fractions from 20 to 72 were added to 6 x 50 mm test tubes, followed by 100 µl of the pooled phosphatase. These 14 preparations, together with a control containing 100 µl of 0.02M cacodylate, pH 6.3, and 100 µl of the pooled phosphatase, were tested for stability at 45°. A 0° control also contained 100 µl of 0.02M cacodylate, pH 6.3, and 100 µl of the pooled phosphatase. The activity in the 0° control is defined as 100%. The stability at 45° of the control made up with cacodylate buffer was 16%.

●——● Units of P-glycolate phosphatase per ml.

o——o Stability conferred = $\frac{\text{stability at } 45^{\circ} - 16\%}{84\%} \times 100\%$.

[1]These pooled fractions constituted the Bio-Gel P-60 preparation described in Table 3.


Phosphatase activity, units/ml
220
180
140
100
60
20
Phosphatase
Stabilizing factor(s)
70
60
50
40
30
20
10
Stability conferred (%)
24 hr
8
14
20
26
32
38
44
50
56
62
68
Fraction number

The Best Single Purification Sequence for P-glycolate Phosphatase

A summary of the procedure is given in Table 4. The acetone which was used was reagent grade.

Extract

About 85 medium sized tobacco leaves were harvested at 4:00 P.M. on 8-4-1966. The 721 grams fresh weight of leaf blades were passed through a Hobart meat grinder followed by 1442 ml of cold water. The slurry was allowed to stand about 5 minutes before it was strained through a double layer of cheesecloth. After centrifugation, the extract was adjusted from its pH of 6.0 to 5.7 with 0.1N HCl. The final volume of the extract was 1800 ml.

First Acetone Fractionation

720 ml of 0° acetone was added to the 1800 ml of extract (which contained 11.7 mg of protein per ml) through one 0.70 mm I.D. teflon tube over a period of $3\frac{1}{2}$ hours while the mixture (held in an ice bath) was continuously stirred by a magnetic stirrer. The mixture was centrifuged at 15,000 x g for 15 minutes and the precipitate was discarded. 360 ml of cold acetone was added as before to precipitate the enzyme. This precipitate was removed by centrifugation at 15,000 x g for 8 minutes and the supernatant solution was drained and rinsed as completely as possible from the centrifuge cups. The pre-

cipitate was taken up in 225 ml of 0.02M cacodylate, pH 6.3, and recentrifuged at 15,000 x g for 8 minutes. The 235 ml of supernatant solution from this last centrifugation, which contained 20-fold purified phosphatase, was saved and the precipitate was discarded.

Second Acetone Fractionation

Fifty-one ml of 0° acetone was added as before to 203 ml of the aqueous solution from the first acetone fractionation (which contained 4.7 mg of protein/ml). The mixture was centrifuged at about 6000 x g for 8 minutes and the precipitate was discarded. Then 41 ml of cold acetone was added as before and the mixture was centrifuged at about 6,000 x g for 8 minutes to remove the enzyme. After careful removal of as much of the acetone solution as possible, the precipitate was dissolved in 81 ml of cold 0.02M cacodylate, pH 6.3. The mixture was recentrifuged at about 6,000 x g for 8 minutes to remove insoluble protein. The volume of the active supernatant solution, which contained 59-fold purified phosphatase, was 85 ml.

Third Acetone Fractionation

A pilot fractionation at pH 5.8 was first made. Then 74 ml of the aqueous solution from the second acetone fractionation was adjusted from pH 6.4 to 5.7 with 0.1N

HCl. The volume after the pH adjustment was 80 ml.

Twenty-seven ml of 0° acetone (34% of the adjusted volume of the aqueous solution) was added as before to the 80 ml of aqueous solution (which contained 2.5 mg protein/ml). The precipitate was discarded by centrifugation at 5,900 x g for 8 minutes. Then 16 ml of 0° acetone (20% of the adjusted volume of the aqueous solution) was added as before to precipitate the enzyme. After centrifugation as before, the supernatant was drained and rinsed from the centrifuge cups. The precipitate was dissolved in 18 ml (25% of the 74 ml) of 0.02M cacodylate, pH 6.3. Recentrifugation was not necessary. The final solution contained 99-fold purified phosphatase and 5.5 mg of protein per ml in a volume of 20 ml.

DEAE-Cellulose Chromatography

One hundred ten days elapsed between the third acetone fractionation and the DEAE chromatography during which the enzyme was held at 0-4°. Before the enzyme was added to the DEAE column, an inactive precipitate was removed by centrifugation at 15,000 x g for 10 minutes. 49.5% of the activity was lost in the 110 days of storage, while the specific activity remained almost constant. The loss in activity during storage is not charged against the purification.

DEAE-cellulose (Sigma, 0.80 milliequivalents per

gram, medium mesh) was pretreated as recommended by Peterson and Sober (192). 0.1M EDTA was included in the early NaOH washings. The DEAE slurry was adjusted to pH 6.3 and equilibrated with 0.02M cacodylate, pH 6.3, in a column 1.5 cm in diameter and 6.0 cm high. 6.5 ml of the enzyme containing 3 mg of protein/ml was added to the column and eluted with 200 ml of the same buffer containing NaCl in a linear gradient from 0 to 0.25M. The active fractions, 78 through 84, eluted at a NaCl concentration of 0.13M, contained 450-fold purified phosphatase and 0.09 mg protein/ml in a volume of 17.3 ml.

Bio-Gel P-60 Chromatography

A Bio-Gel P-60 (50-150 mesh Calbiochem) column 1.5 cm in diameter x 17.5 cm high was prepared using gel which had been hydrated in 0.02M buffer, pH 6.3, for 2 days. A 1.2 cm thick layer of DEAE-cellulose, prepared as described in the previous step, was layered over the gel and the entire column was washed at pH 6.3. 12.5 ml of the enzyme from the previous step, which was 0.13M in NaCl, was diluted 4-fold with 0.02M cacodylate, pH 6.3, which also contained 10^{-3}M citrate and 10^{-3}M $MgSO_4$. The diluted enzyme preparation was slowly fed onto the top of the column over a period of about 10 hours. The column and enzyme were first washed with about 30 ml of the buffer containing 0.02M cacodylate, pH 6.3, 10^{-3}M citrate, and 10^{-3}M $MgSO_4$,

which effectively removed the NaCl from the column. The enzyme was then eluted with 10 ml of the same buffer which contained, in addition, 0.25M NaCl, and was then further eluted with buffer without NaCl. The most active fractions, 9 through 16, which were shown to be free of NaCl, contained 1060-fold purified phosphatase and 0.023 mg of protein per ml in a volume of 7.45 ml.

The method which was used for the concentration of the enzyme seems like it should be generally applicable to situations in which gel filtration chromatography is to follow ion exchange chromatography, providing the enzyme is stable to the dilution which is necessary to decrease the salt concentration of the preparation from the ion exchange column. The volume of the ion exchange pad which is required for the concentration of the enzyme is small in comparison to the volume of the column of gel, and the amount of enzyme which the pad will retain should be independent of the volume in which this enzyme is contained. In the procedure described here, the enzyme, which had originally been contained in 12.5 ml containing 0.13M NaCl, had been applied to the column in a volume of 50 ml containing 0.033M NaCl. Even though dilution of the enzyme would be expected to occur during the gel filtration chromatography, 85% of the phosphatase which was recovered from the column was in the 7.45 ml from the pooled fractions. Thus, the method which was used for

concentrating the enzyme was quite effective. If care is taken in the preparation of the column, the overlying relatively thin ion exchange pad, and in particular the interface between the pad and the gel, it should be possible to elute the enzyme in a relatively sharp horizontal band ideal for further development by gel filtration. As was demonstrated, the procedure is also capable of completely removing all the salt which is present in the enzyme preparation from the ion exchange chromatography step, as well as all the salt used to elute the enzyme from the ion exchange pad. Finally, although the requirement for sharp elution is somewhat restrictive, further purification of the enzyme on the pad itself is possible.

Rechromatography on DEAE-Cellulose

The previous preparation was held 3 days at 0-4°, during which time 20% of the activity was lost and the specific activity dropped from 333 to 266. DEAE-cellulose, prepared as described above, was equilibrated with a buffer of 0.02M cacodylate, pH 6.3, 10^{-3}M citrate, and 10^{-3}M $MgSO_4$ in a column 0.7 cm in diameter and 1.0 cm high. 6.3 ml of the NaCl free enzyme preparation from the previous step was slowly added over a period of ½ hour to the column, and then eluted with 20 ml of the same buffer-citrate-$MgSO_4$ solution containing NaCl in a linear gradient from 0 to 0.5M. Only one major protein peak,

which contained the phosphatase, was eluted (Figure 11). In the most active fractions 10 and 11, eluted at a NaCl concentration of 0.12M, containing 0.043 mg protein per ml in a volume of 2.2 ml, the specific activity of the phosphatase was 214.

Discussion of the Purification Procedures

The purification of the phosphatase which was described on pages 137 to 148 was part of experiments which were conducted to test the hypothesis that P-glycolate phosphatase from tobacco leaves contains an endogenous stabilizing factor. The three acetone fractionations may have been especially beneficial in removing other small molecules from the phosphatase-endogenous stabilizing factor complex. In particular, care had to be taken not to add compounds either for stabilization or as specific chromatographic eluents. Attempts to further improve the purification procedure for the phosphatase per se should not be subject to these restrictions.

The specific activity of 333 for the purest preparation (Table 4) is considerably greater than the previously reported high of 1.6[1] (202). Part of the increase is because the starting specific activity in my procedure (0.314 (Table 4)) was about 10 times greater than the

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[1] 480/309.75 = 1.6 (see p. 109 for a discussion of this calculation).

Figure 11

Final DEAE-Cellulose Chromatography of P-glycolate Phosphatase

●——● Units of P-glycolate phosphatase per ml.

O---O μg protein per ml.

Phosphatase activity, units/ml
Protein, µg/ml
Fraction number
2
4
6
8
10
12
14
16
18
20
22
10
20
30
40
50

Table 4

Purification of P-glycolate Phosphatase

	Activity*	Yield*	Specific activity	Purification
	units	%	units/mg prot.	-fold
Extract	575	100	0.314	1
First acetone	603	105	6.30	20
Second acetone	430	75	18.5	59
Third acetone	407	71	31.1	99
DEAE-cellulose	163	28	141	450
Bio-Gel P-60	57	10	333	1060
DEAE-cellulose			214	

*Corrected for the portion of each preparation not further purified, including the portion of activity which was lost during 110 days of storage after the 3rd acetone fractionation.

starting specific activity in the procedure used by Richardson and Tolbert,[1] perhaps partly because advantage was taken of the study conducted on the influence of leaf size and position on the specific activity of P-glcyolate phosphatase from tobacco (Table 1). Another part of the increase was because of the use of cacodylate instead of Tris-acetate, with consequent improved buffering and rate increase. Finally, some of the increase was because of the higher fold purification. The final DEAE-cellulose chromatography of the purest preparation (Figure 11) failed to show significant protein contamination of the phosphatase. However, the enzyme cannot be considered homogeneous on the basis of one criterion of purity.

Further Data and Observations on the Purification of P-glycolate Phosphatase

Preparation of Acetone Powders

When it was desired to store the phosphatase for long periods of time, the acetone precipitates from the first or second fractionations were converted to a dry powder. The acetone precipitates, without first rinsing the excess acetone from the centrifuge tubes with water, were dried in a dessicator for 15 hours at an ambient temperature of 20-25°. The first 12 hours of the drying

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[1]The specific activity of the crude tobacco sap after the first centrifugation was 9.5/309.75 = 0.031 (202).

period were under a vacuum, from a water pump, of about 15 mm Hg, and the final 3 hours were under a vacuum, from a Cenco vacuum pump, of 1 to 2 mm Hg.[1] It was found that rapid drying of the precipitates under a vacuum of less than 1 mm Hg caused the destruction of much of the phosphatase activity. As discussed elsewhere, the phosphatase at this stage of purification was found to be unstable to freezing. Inactivation by freezing may explain the low recoveries which were obtained when the acetone precipitates were dried rapidly.

When making powders of first acetone precipitates, it was profitable with respect to time and yield to centri-

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[1]The procedure as described was used for acetone precipitates which had come from extracts which had been prepared by Waring blendor. Although only precipitates from the first acetone fractionation of these extracts were converted to a powder, there is no evidence that precipitates from the second or third acetone fractionations from such extracts could not successfully be converted to dry acetone powders.

When the extracts were made by the meat grinder, a modified procedure was used for the preparation of the acetone powders. The phosphatase in such extracts was not as stable as the enzyme in extracts prepared by Waring blendor (p. 129). As discussed in a later section, the unstable enzyme was stabilized by acetone precipitation, but the stabilization was not as great as the stabilization by Waring blendor (compare Figures 24 and 42, with Figures 8 and 21). The modifications included the following. The acetone was first rinsed from the centrifuge tubes with cold water. The initial drying at 15 mm Hg was for 3 to 4 hours, with the ambient temperature at 0-4°, until the smell of acetone had vanished from the precipitates. The next 12 hours of the drying period at 15 mm Hg and a final 12 hours at 1 to 2 mm Hg were also at an ambient temperature of 20-25°. Only precipitates from the second acetone fractionation of extracts which had been made by the meat grinder were converted to a powder.

fuge the active precipitates in centrifuge tubes containing precipitates from previous centrifugations until each tube contained precipitates from three centrifugations.

After the precipitates were dry, static electricity made it difficult to remove the powder from the polyethylene centrifuge tubes. This difficulty was overcome with the aid of a polyethylene rod charged by rubbing with a clean cloth.

Little or no activity was sacrificed in the drying of theprecipitates.[1] When the phosphatase in the dry acetone powder was stored at 0-4° in a dessicator over anhydrous $CaSO_4$, it retained all its activity for at least $5\frac{1}{2}$ months. When the powder was stored in a polyethylene bottle at room temperature, the enzyme retained all its activity for at least one week.

When it was desired to redissolve the dry acetone powder, the same volume of cold 0.02M cacodylate, pH 6.3, was used as would have been used had the precipitate not been dried, and insoluble material was removed by centrifugation at 6,000 x g for 8 minutes.

Observations on the Acetone Fractionations

The arrangement used for making large scale acetone

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[1]No activity was lost when precipitates from extracts which had been made by Waring blendor were converted to a powder. A relatively small percent of the activity was lost when precipitates from extracts which had been made by a meat grinder were converted to a powder by the modified method described in the previous footnote, page 159. However, even more activity was lost in the latter case when the unmodified procedure was used.

fractionations is shown in Figure 12. The height of the ice bucket containing the acetone was easily adjusted so as to control the rate of the addition of acetone.

Some of the following observations are from a large number of experiments designed for other purposes, and therefore were not controlled with respect to each observation.

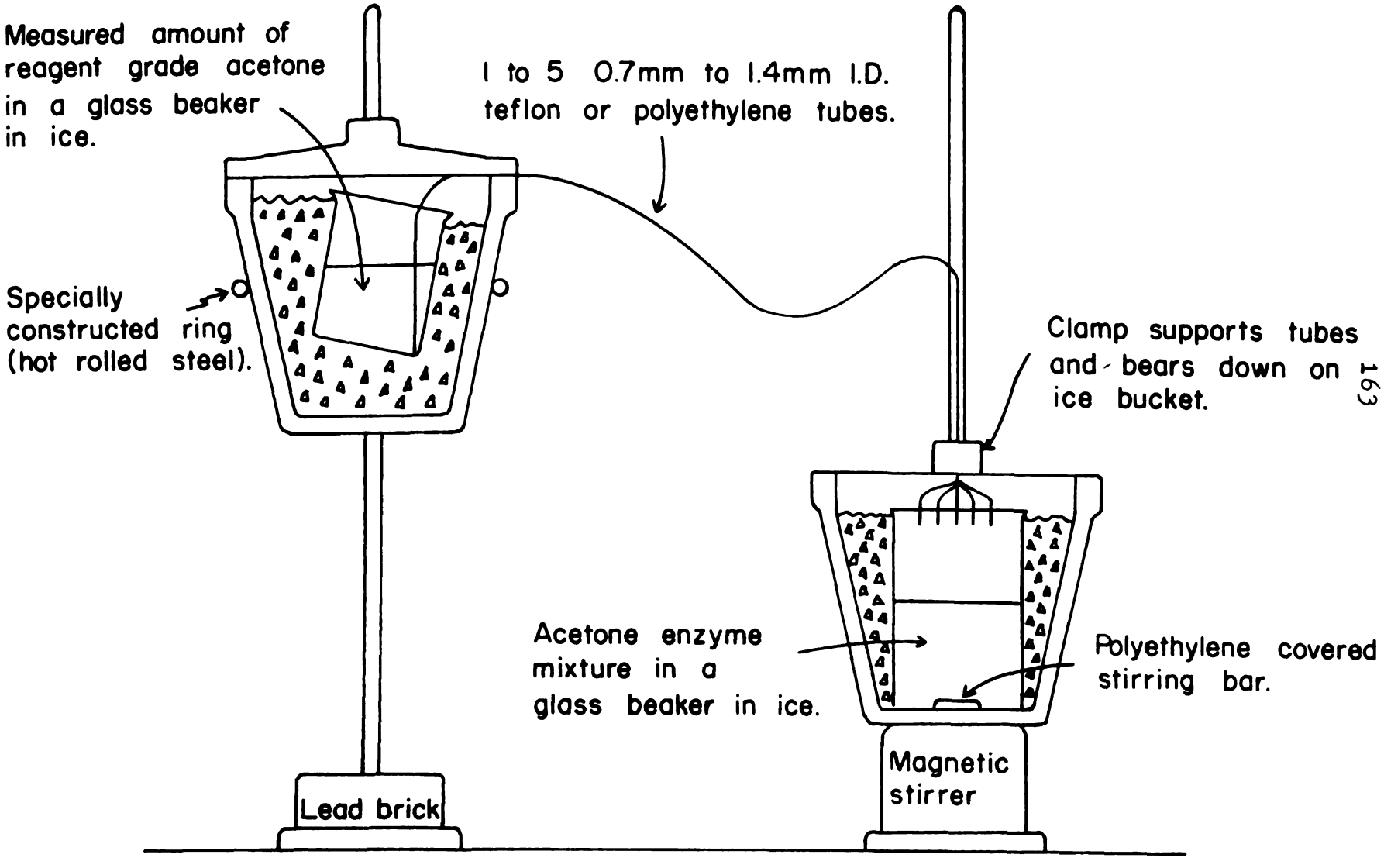
Effect of the Homogenization Method and the Amount of H_2O Used

The phosphatase from extracts prepared by meat grinder or by mortar and pestle was about 2 times more highly purified by the first acetone fractionation than from extracts prepared by Waring blendor. Part of this advantage was lost during the two subsequent acetone fractionations.

The increment of acetone needed to precipitate the phosphatase during the first fractionation depended on the amount of water used in preparing the leaf extract. When an extract was prepared by the meat grinder without the addition of water, the activity was precipitated over a broad range of acetone concentration. The addition of one weight of water per fresh weight of leaf tissue in the preparation of an extract resulted in virtually all of the activity being precipitated by an increment of acetone that was equal to only 15% of the volume of the extract. Addition of twice as much water did not seem

Figure 12

The Arrangement Used for Making Large Scale Acetone Fractionations


Measured amount of reagent grade acetone in a glass beaker in ice.
1 to 5 0.7mm to 1.4mm I.D. teflon or polyethylene tubes.
Specially constructed ring (hot rolled steel).
Clamp supports tubes and bears down on ice bucket.
Acetone enzyme mixture in a glass beaker in ice.
Polyethylene covered stirring bar.
Lead brick
Magnetic stirrer

to contribute further to this pronounced decrease in the range of acetone concentration within which virtually all of the enzyme was precipitated.

Post Homogenization Time

Since the phosphatase in extracts prepared by a meat grinder or mortar and pestle slowly changed from an enzyme unstable to dilution at 30° to an enzyme stable to these conditions (Figure 19), the effect of post homogenization time on the concentration of acetone required to precipitate thephosphatase[1] from an extract, which had been prepared by meat grinder, was tested (Table 5). Post homogenization time had no significant effect on the concentration of acetone required to precipitate the enzyme.[1]

Table 5

The Acetone Concentration Required to Precipitate P-glycolate Phosphatase as a Function of Post Homogenization Time

Post homogenization time	Maximum activity precipitated
hours	ml acetone/10 ml of extract
1½	4.8
6	4.8
34	4.7

[1]The concentration of acetone required to precipitate the phosphatase is defined to include half of the range of acetone concentration within which virtually all of the phosphatase was precipitated.

Effect of pH and Unknown Factors

Over a 2 year period, a considerable variation was found in the acetone concentration needed to precipitate the phosphatase. One of the smallest acetone concentrations needed to reach the maximum activity which was precipitated was 4.5 ml of acetone per 10 ml of extract, while the greatest was 6.5. Both of these extracts were prepared by mortar and pestle using two weights of water per fresh weight of leaf. In the former case, the enzyme was from a pH 5.3 extract, and in the latter the enzyme came from a pH 6.4 extract. Both pH values were those naturally occuring in the extracts.

An extract which had been prepared by mortar and pestle and in which the pH was 5.9, was divided into 3 aliquots, which were then adjusted to pH 5.3, 5.8 and 6.3. These three aliquots were then individually fractionated with acetone (Table 6). The relationship between the pH of the extract and the concentration of acetone required to precipitate the phosphatase (Table 6) was opposite to the relationship which had been found in extracts (pH 5.3 and 6.4) which had originated from two different batches of leaves harvested months apart. Factors other than pH must be more important than pH in determining the concentration of acetone required to precipitate the phosphatase.

Table 6

The Acetone Concentration Required to Precipitate P-glycolate Phosphatase as a Function of pH

pH	Maximum activity precipitated
	ml acetone/10 ml of extract
5.3	4.6
5.8	4.3
6.3	4.1

Purification did not seem to be better at pH 5.7 than at unadjusted pH values in the range of 5.5 to 6.4. Because of this and because pH control alone did not insure reproducibility in the concentration of acetone required to precipitate the phosphatase, adjustment of the extract pH before fractionation, as was done in the purification sequence shown in Table 4, is generally not warranted.

The main reason for the success of the first acetone fractionation seems to be that most of the protein is irreversibly denatured by the treatment. Thus, a 7-fold purification of the wheat phosphatase was obtained simply by precipitating a Waring blendor extract with 3 volumes of acetone (265). The additional purification is obtained from the fractionation of the remaining proteins at the different acetone concentrations. When the phosphatase in a fresh tobacco leaf extract made by mortar

and pestle was precipitated between 3 ml and 8 ml of acetone per 10 ml of extract, the purification was 14-fold. This 5 ml increment of acetone was wide enough to include the considerable variation which was found, over the 2 year period, for the acetone concentration needed to precipitate the enzyme. The additional purification possible by the first acetone fractionation requires at least an occasional pilot fractionation.

Effect of Time and Temperature

Although the slow addition of acetone through a single 0.7 mm I.D. teflon tube over a period of 3 to 4 hours resulted in excellent enzyme yields, it was found that adding the acetone simultaneously through five 0.7 mm I.D. teflon tubes or four 1.14 mm I.D. polyethylene tubes over only 15-20 minutes also resulted in excellent yields, even though the temperature during the mixing rose to as high as 8°. Even for these short times, the flow of acetone through any one tube was not allowed to be quite rapid enough to give a continuous stream.

When the final centrifugation of an acetone mixture at 0-4° was delayed for 20 hours, the yield was down to 40% and the fold purification was also low.

Acetone precipitation of the phosphatase at room temperature was not as effective as acetone precipitation of the enzyme with the temperature held between 0 and 8°.

Activation of P-glycolate Phosphatase by Acetone Precipitation

It was not unusual to obtain recoveries in excess of 100% from the first acetone fractionation. Furthermore, it was sometimes noted that the activity in the aqueous solutions from the acetone precipitates increased for a time.

Substrate Specificity

Enzyme specificity for P-glycolate was greatly increased by the first acetone fractionation which was designed to save the P-glycolate phosphatase activity (Table 7).

Table 7

Improvement in Substrate Specificity by the First Acetone Fractionation

	Relative rate of P_i release	
Substrate	Extract	Acetone preparation
P-glycolate	1.000	1.000
3-P-glycerate	0.248	0.046
Phenolphthalein-di-phosphate	0.481	0.083

Purification by Aging of Extracts

When extracts were held overnight, a large precipitate developed. Removal of this precipitate by centrifuga-

tion gave about a 1.7-fold purification. Little activity was lost by this procedure. When the recentrifuged extract was further aged, little if any precipitate developed. That the purification from aging was at least partly additive with the purification from the first acetone fractionation is suggested by the turbidity which often gradually developed in once acetone purified enzyme prepared from fresh extracts. Three times acetone purified enzyme stayed clear for long periods of time. Thus it may be that part of the success of the acetone fractionations was due to the elapsed time required for all three fractionations.

Optimum pH for DEAE-Cellulose Chromatography

Using pH as the variable, an attempt was made to increase the specific activity of the enzyme by causing a shift in the eluted peak of phosphatase activity relative to the protein elution pattern. The following pH values and buffers were used: 5.0 (acetate); 5.5, 6.3, and 7.0 (cacodylate); 8.0 and 9.0 (glycyl-glycine).

A separate aliquot of DEAE-cellulose was used at each pH. For each chromatographic run, the cellulose, pretreated as recommended by Peterson and Sober (192), was equilibrated with 0.02M buffer in a column 0.7 cm in diameter and 2-3 cm high. 0.10 ml of 3 times acetone purified enzyme was added to the column and then eluted

with 20 ml of the same buffer, which was used in the preparation of the column, containing NaCl in a linear gradient from 0 to 0.5M. All eluents were monitored at 254 mμ.

Except for the chromatographic run at pH 5.0, the positions of the phosphatase peaks relative to the A_{254} patterns were relatively constant, and the NaCl concentrations at the maximums of the phosphatase peaks varied only between 0.12M and 0.16M, so that the shifts were not large enough to take advantage of. At pH 5.0, the phosphatase was eluted at a NaCl concentration of 0.07M. However, the recovery of activity was too low to take advantage of this shift. It was demonstrated that pH and not acetate was responsible for this shift. At pH 6.3, 0.02M acetate did not decrease the NaCl concentration (0.15M) necessary to elute the phosphatase. The highest recoveries (as high as 90%) were obtained with the pH 6.3 cacodylate system. It was concluded that no apparent advantage was to be gained by changing from pH 6.3 for the DEAE-cellulose chromatography.

Suspected Inactivation of P-glycolate Phosphatase by Metal

The following circumstantial observations suggest that contact between the partially purified phosphatase and metal containers, plumbing, and utensils should be completely and carefully avoided.

1. Higher yields were obtained with reagent grade

acetone from glass bottles than with reagent grade acetone from cans.

2. During DEAE-cellulose chromatography, higher yields were obtained when the use of all metal was avoided in the plumbing than when the plumbing contained some metal. This includes the plumbing ahead of the column, including the gradient device, as well as after the column.

3. When a syringe (metal needle) was repeatedly used to remove enzyme from a 3 times acetone purified preparation, there was a concomitant increase in the rate of phosphatase inactivation.

The Effect of Preincubation with Mn^{++}, Fe^{++}, or Fe^{+++}

Preincubation with Mn^{++} had little effect on the activity or the stability toward dilution at 30° of the once acetone fractionated phosphatase. Preincubation with Fe^{++} had a significant effect on the activity and some effect on the stability toward dilution at 30° of the phosphatase in the same preparation. Preincubation with Fe^{+++} had little effect on the activity but had about the same effect as Fe^{++} on the stability toward dilution at 30° of the once acetone fractionated phosphatase. Preincubation with Fe^{++} had little effect on the activity or stability toward dilution at 30° of the phosphatase in a buzzed extract (Table 8).

Table 8

The Effect of Preincubation with Mn^{++}, Fe^{++}, or Fe^{+++} on the Activity and Stability of P-glycolate Phosphatase

The extract was prepared by mortar and pestle and was buzzed in air. The acetone fractionated phosphatase originated as an extract made by Waring blendor and was fractionated once; the active precipitate was then converted to an acetone powder. The Fe^{++} preincubation mixture with the buzzed extract consisted of 400 µl of sap, 50 µl of 10^{-2}M Fe^{++}, and 50 µl of H_2O. The preincubation mixtures with the acetone fractionated phosphatase consisted of 50 µl of enzyme, 25 µl of 4 x 10^{-3}M Fe^{++} or 25 µl of 4 x 10^{-3}M Mn^{++} + 25 µl of 0.02M cacodylate, pH 6.3, or 50 µl of 2 x 10^{-3}M Fe^{+++}. All preincubations were at 0°.

Enzyme description	Metal	Concentration of metal: Preincubation	Concentration of metal: Phosphatase reaction	Preincubation time: Activity	Preincubation time: Stability*	Activity	Stability*
Buzzed	None	--	--	--	--	100%	67%
extract	Fe^{++}	10^{-3}M	3.3 x 10^{-5}M	75 min.	15 min.	97%	69%
Acetone	None	--	--	--	--	100%	86%
fractionated	Mn^{++}	10^{-3}M	1.3 x 10^{-5}M	84 min.	20 min.	102%	87%
phosphatase	Fe^{++}	10^{-3}M	1.3 x 10^{-5}M	?	20 min.	78%	76%
	Fe^{+++}	10^{-3}M	1.3 x 10^{-5}M	76 min.	20 min.	97%	75%

*Toward dilution at 30°.

Richardson and Tolbert (202) found that an endogenous metal accompanied P-glycolate phosphatase during the purification of the enzyme. Arguments have been presented which suggest that the endogenous metal was Mg^{++}, but the evidence did not exclude Fe^{++} as the metal (literature review, p. 40). The relatively significant inactivation rate of P-glycolate phosphatase at pH 6.3 and at 0°, in the presence of 10^{-3}M Fe^{++} (Table 8), suggests that the endogenous metal which accompanied the phosphatase (202) was not Fe^{++}. Furthermore, kinetic evidence excludes Fe^{+++} as the endogenous metal for the enzyme (202).

Gel Filtration Chromatography

The phosphatase Rf (effluent volume/void volume) on Sephadex G-25 was 1.0, on Sephadex G-100 it was 1.6, and on Sephadex G-200 the Rf varied from 2.3 to 3.0. The Rf values on G-100 and G-200 suggest that the enzyme is relatively small.

The phosphatase preparations were highly colored (orange-brown) through the 3 acetone steps. This color could not be removed by dialysis. DEAE removed much of the color. Gel filtration chromatography gave a colorless phosphatase, as the color emerged in a peak far behind the phosphatase but ahead of the salts.

Whenever the phosphatase activity was assayed in

all the fractions which were eluted, including those later fractions which would normally be expected to contain only very small molecules, no large second phosphatase peak was ever found. However, a very minor phosphatase peak, containing 1 to 2% of the activity, usually was found, and it coincided with or slightly followed the color peak.

Protein Determinations

Determinations on colored preparations by the 260/280 method gave very inconsistent results. The 260/280 method was therefore not used. During acetone purification, the A_{280} method gave results 3 to 4 times higher than Lowry's method, although both showed about the same fold purification over the 3 acetone purification steps. When the color was removed during gel filtration chromatography, the fold purification based on the A_{280} method was as much as 5 to 6 times greater than when based on Lowry's method. The colored peak absorbed very strongly at 280, 260, and 254 mμ. This UV absorption peak was not matched by a major protein peak, as measured by Lowry's method, although a small amount of protein did coincide with the color. Apparently, the colored material was responsible for undependable protein determinations by either the 260/280 method or the A_{280} method and the related A_{254} method. Under the conditions that prevailed, the Lowry's modified Folin-Ciocalteau method[1] for the

[1]For a good analysis of Lowry's method and other methods for measuring protein, see the article by Ennis Layne (142).

determination of protein seemed the most conservative and dependable.

Stability of P-glycolate Phosphatase Toward Freezing and Thawing

Freezing and thawing of enzyme which had undergone one or three acetone fractionations resulted in the destruction of 1/4 to 1/3 of the phosphatase activity.[1] When these same preparations were refrozen and rethawed, 1/4 to 1/3 of the remaining activity was destroyed. By contrast, slow or quick freezing of the Bio-Gel P-60 preparation, which is described in Table 3, caused the destruction of only about 4% of the phosphatase activity. When neutralized citrate was added to the latter preparation so that it contained 10^{-3}M citrate, the phosphatase in the preparation was still stable to freezing and thawing.[2]

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[1]The enzyme had come from extracts prepared by Waring blendor and had not been powdered.

[2]The enzyme in the Bio-Gel P-60 preparation was different in at least three ways from the enzyme in those preparations in which it was unstable to freezing and thawing. The enzyme in the Bio-Gel P-60 preparation had come from an extract which had been prepared by meat grinder, it had been made into an acetone powder, and it had been purified beyond the acetone fractionations by DEAE-cellulose and Bio-Gel P-60 chromatography. Without further experiments, it is not possible to know the reason for the stability toward freezing of this enzyme. However, the data suggest that at early stages of purification, the phosphatase may be associated with something other than citrate which makes it unstable to freezing.

Stability of P-glycolate Phosphatase Toward Dilution at 30° and Toward Dialysis

The test for the stability of the phosphatase toward dilution at 30° could cause greater inactivation of the enzyme than the test for the stability of the enzyme at 45°. For example, the enzyme in the third acetone, the DEAE-cellulose, and the DEAE concentrate preparations which are described in Table 3, was completely stable at 45° but was only 45-55% stable toward dilution at 30°. The enzyme in these preparations had been extracted by meat grinder and had been converted to an acetone powder.

Enzyme which had come from an extract prepared by Waring blendor, and which had been purified by two acetone fractionations, but had not been converted to an acetone powder, was 100% stable toward dilution at 30°. This preparation was then dialyzed overnight against 175 volumes of 0.02M cacodylate, pH 6.3, in a 4° room while being stirred by magnetic stirrer. The dialyzed enzyme retained 90% of its activity compared to the nondialyzed control. The remaining activity in the dialyzed preparation was 90% stable toward dilution at 30°.

Results obtained with preparations which had been converted to acetone powders suggest that conversion to a powder rendered the enzyme in the preparations less stable toward dilution at 30°.

Storage Characteristics of P-glycolate Phosphatase

During the time the phosphatase was stored as an acetone powder, no loss in activity was ever detected (see p. 160). When acetone precipitates or acetone powders at any of the 3 stages of purification were dissolved in 0.02M cacodylate, pH 6.3, the phosphatase retained all or most of its activity at 0-4° for at least 2 weeks.

The phosphatase in those preparations from DEAE-cellulose chromatography in which the enzyme was most stable, lost only a little activity in 2 weeks at 0-4°.

Following one or more purification steps that included at least one acetone fractionation, enzyme which was further purified by Sephadex G-100, Sephadex G-200, or Bio-Gel P-60 gel filtration chromatography lost activity relatively rapidly at 0-4°. This loss occured whether or not the enzyme, before addition to the gel filtration column, had been converted to an acetone powder. Furthermore, the loss in activity at 0-4° occured with enzyme which had come from extracts made by Waring blendor, meat grinder, or mortar and pestle. Most of the loss in activity could be prevented by including 10^{-3}M citrate, isocitrate, or cis-aconitate in the preparations from gel filtration chromatography (Figure 15).

The enzyme in the preparation described as "Bio-Gel P-60" in Table 3 was stable at -20° for at least 2 weeks.

Purification by Other Methods

Ammonium Sulfate

The phosphatase in extracts from tobacco leaves, which had been prepared by Waring blendor, was purified by the first acetone fractionation. The acetone precipitated enzyme, after it had been taken up in 0.02M cacodylate, pH 6.3, was further purified by an $(NH_4)_2SO_4$ fractionation. The phosphatase was precipitated between 3.5 grams and 5.0 grams of solid $(NH_4)_2SO_4$ per 10 ml of acetone purified enzyme, and was redissolved in 0.02M cacodylate, pH 6.3. The yield in the active fraction was 64-74% of the activity from the acetone step. The $(NH_4)_2SO_4$ step gave a moderate additional purification and removed much of the color.

The phosphatase in the $(NH_4)_2SO_4$ preparations was more stable toward freezing and thawing than the acetone precipitated enzyme. The phosphatase in an $(NH_4)_2SO_4$ preparation was 90% stable toward dilution at 30°, and the enzyme retained most of its activity at 0-4° for at least two weeks. Dialyzing this preparation overnight in a 4° room resulted in 50% inactivation of the phosphatase.

The $(NH_4)_2SO_4$ fractionated phosphatase was completely unstable to further fractionation with acetone.

The phosphatase from plants other than tobacco

was unstable to $(NH_4)_2SO_4$ fractionation (265), and even in tobacco, the recoveries were only moderate (202, 265). The instability of the $(NH_4)_2SO_4$ fractionated enzyme from tobacco leaves toward acetone fractionation or dialysis is consistent with these earlier findings.

Ultracentrifugation

When an extract from tobacco leaves which had been prepared by Waring blendor[1] was recentrifuged at 151,000 g for 90 minutes, only 5% of the phosphatase was found in the small pellet while 95% was in the supernatant solution. Part of the activity in the pellet would be due to trapped solution. The specific activity in the solution increased 1.3-fold.

When 3 times acetone purified phosphatase (the enzyme had come from an extract prepared by Waring blendor and had been powdered after the first fractionation, or had come from an extract prepared by the meat grinder and had not been powdered) was centrifuged at 122,000 g for 10 to 12 hours, 90% of the activity which was recovered (81 to 91%) was found in the bottom 6 to 10% of the volume. However, not more than 4% was found in the tiny pellets. The specific activity increased from the top to the bottom of the centrifuge tubes, but not enough to be profitable

[1]The extract had been centrifuged at 37,000 g for 5 minutes.

for purification.

When precipitated enzyme from a 3rd acetone fractionation (the enzyme had come from an extract prepared by Waring blendor, and had been powdered after the first fractionation) was taken up in a salt solution with a density of 1.21, and the solution was centrifuged at 122,000 g for 24 hours, the same results were obtained. Most of the phosphatase was concentrated near the bottom of the centrifuge tube. The method has been used to float lipoprotein[1] with a density less than 1.21 (113).

Ion Exchange Chromatography Other Than DEAE

All the following results were obtained with 3 times acetone purified enzyme which had come from an extract prepared by the meat grinder and which had not been converted to an acetone powder.

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[1]Freezing and thawing of enzyme which had been purified by 3 acetone fractionations resulted in partial inactivation of the phosphatase and the formation of a thin, oily, upper phase. Freezing and thawing has been used as a method for the dissociation of lipoproteins (166).

It has been determined that the relative effectiveness of organic solvents for dissociating lipoprotein complexes in aqueous media and for releasing undenatured protein in general may be expressed as follows: (1) most effective: n and iso-butanols; (2) partially effective: sec-butanol, cyclohexanol, and tert-amyl alcohol; (3) ineffective: all other solvents tested, including chloroform, carbon tetrachloride, toluene, ether, acetone, etc. (166).

Chromatography on TEAE-cellulose with the same NaCl gradient at pH 6.3 as used for DEAE-cellulose chromatography gave results very similar to those obtained with DEAE-cellulose.

When the enzyme was chromatographed on cellulose-phosphate at pH 6.3 or CM-cellulose at pH 5.7 or 6.3, most of the protein emerged as the first peak. This peak contained all of the phosphatase, which was purified no more than 1.5-fold, in 0.02M cacodylate, pH 6.3, which was free of NaCl. The results were not significantly altered on CM-cellulose at pH 6.3 when the eluting cacodylate was reduced from 0.02M to 0.001M.

Identification of the Phosphatase Stabilizing Factors

Purification

Fractions 44 through 58 (Figure 10) were pooled to give a 43.0 ml stabilizing fraction. 11.0 ml of this stabilizing fraction was acidified with 10N H_2SO_4 to a pH of about 1 (by calculation and pH paper). The acidified fraction was then continuously extracted for 3 days in a soxhlet apparatus with anhydrous diethyl ether (Fisher reagent grade). After the ether and water phases in the soxhlet apparatus were separated by separatory funnel, the combined ether portions were evaporated in a flash evaporator at room temperature to a volume of about

0.5 ml. The clear colorless concentrated ether extracted fraction was transferred to a pointed test tube, and the flash evaporator flask was carefully rinsed with two 0.2 ml aliquots of water which were then individually transferred to the pointed test tube. The ether extracted fraction and the remaining aqueous fraction (ether washed fraction) were then exposed to a vacuum of 15 mm Hg until the bubbling ceased and the smell of ether had vanished. The volumes were then 0.8 ml and 8.0 ml for the ether extracted and ether washed fractions respectively. Initial paper chromatography of the ether extracted fraction was with the preparation described, but the final paper chromatographic determinations were run after the 0.8 ml ether extracted fraction was diluted 4-fold with water.

The most active factor fractions which had been separated from the phosphatase by Sephadex G-100 chromatography, after the phosphatase had been purified by three acetone fractionations, were also pooled and then continuously extracted with anhydrous diethyl ether in a soxhlet apparatus using a procedure essentially the same as that described above. 14.8 ml of the pooled stabilizing fraction gave a clear colorless 0.6 ml ether extracted fraction. The ether extracted and ether washed fractions of this extraction were assayed for the presence of the phosphatase stabilizing factor(s). Twenty-fold diluted

ether extracted fraction was more effective in conferring stability than was the ether washed fraction (Table 9). The two fractions together seemed to confer stability additively. The pretreatment with either fraction also gave some activation, and the pretreatment with the two fractions together seemed to confer activation additively.

Although the stabilizing factor peak in both gel filtration chromatography runs was behind the color peak, it overlapped with it, so that both pooled factor fractions were colored. In both ether extractions, all of the color remained behind in the ether washed fraction.

Identification of Tricarboxylic Acids

Citric, isocitric, and cis-aconitic acids were identified by paper chromatography of the purified (ether extracted) phosphatase stabilizing fraction (Table 10). The only other acidic spot which was evident on the chromatograms was sulfuric acid. Only one unknown organic acid spot showed up in the ether-acetic acid-water system. It could have been citric, isocitric, or cis-aconitic acid or a mixture of all three (Table 10). The presence of cis-aconitic acid was evident with the other 3 solvent systems. The n-butanol-ethyl acetate-formic acid and phenol-water systems did not distinguish between citric and isocitric acids. The major unknown spot in both these systems could have been citric or isocitric acid or both.

Table 9

Phosphatase Stabilizing Factor(s) in the Ether Extracted Fraction

After extraction of the pooled factor fractions from Sephadex G-100 chromatography, 30 µl of the ether washed fraction was mixed with 2 µl of 1N KOH in a small test tube. 1.5 µl of the ether extracted fraction was mixed with 22.5 µl of 0.02M cacodylate, pH 6.3, plus 6 µl of 0.01 N KOH in another small test tube. The pH values of the two aliquots were then about 6.5 to 7.0 (pH paper).

The four pretreatment mixtures each contained 10 µl of unstable phosphatase. The phosphatase, described in Figure 10, had been held at -20° for about two weeks and was thawed just before use. In addition, they contained the following: 1) 10 µl of 0.02M cacodylate, pH 6.3, 2) 10 µl of the neutralized ether washed fraction, 3) 10 µl of the neutralized and diluted ether extracted fraction, and 4) 5 µl of the neutralized ether washed fraction + 5 µl of the neutralized and diluted ether extracted fraction. All four preparations were then tested for stability at 45°. The activities are averages of two determinations; the values in each pair were in close agreement.

$$\text{Activation} = \frac{\text{Activity of } 0^\circ \text{ control in units/ml} \times 100\%}{141.5} - 100\%.$$

$$\text{Stability conferred} = \frac{\text{Stability at } 45^\circ - 32.8\%}{67.2\%} \times 100\%.$$

Fraction added	Pretreatment of enzyme with fraction	Activity*	Activation	Stability at 45°	Stability conferred
			%	%	%
1) None	Held at 0°	141.5	0		
	Heated at 45°, 1 hour	46.5		32.8	0.0
2) Aqueous	Held at 0°	151.5	7		
	Heated at 45°, 1 hour	69.0		45.6	19.0
3) Ether	Held at 0°	158.8	12		
	Heated at 45°, 1 hour	112.7		71.0	56.9
4) Aqueous plus ether	Held at 0°	173.6	23		
	Heated at 45°, 1 hour	139.0		80.0	70.2

*In units/ml of the phosphatase described in Figure 10.

Table 10

Identification of Tricarboxylic Acids

The unknown, citric acid, isocitric acid, and cis-aconitic acid solutions were the same as those described in Figure 13. Although sulfuric acid also was evident, its Rf is not included.

Solvent system	Rf, paper chromatography					
	Commercial acids			Lagging unknown	Middle unknown	Leading unknown
	Citric	Isocitric	Cis-aconitic			
n-Butanol-EtOAc-HCOOH	0.28	0.26	0.41	0.26		0.40
Phenol-H_2O	0.15	0.13	0.23	0.14		*
Ether-CH_3COOH-H_2O	0.15	0.18	0.13	0.17		
n-Butanol-Propionic acid-H_2O	0.41	0.42	0.60 & 0.45**	0.39	0.45	0.59

*The unknown gave an intense yellow spot (Rf = 0.14) and a smaller less intense leading shadow. The position of the leading shadow compared well with that of commercial cis-aconitic acid.

**Commercial cis-aconitic acid gave two spots, a larger spot with an Rf of 0.60 and a smaller spot with an Rf of 0.45. Trans-aconitic acid (Calbiochem A grade) also had an Rf of 0.60 in this system.

When the unknowns were mixed with commercial citric, isocitric, cis-aconitic, or sulfuric acids, the identification of the acids in the purified phosphatase stabilizing fraction was further extended (Figure 13). In every case, the intensity, but not the shape, of a spot was increased by the corresponding acid.

The only system which distinguished between citric and isocitric acid was the n-butanol-propionic acid-water system. With this system, the unknown mixture was shown to contain both of these acids (Figure 14). The lagging spot was citric acid. Commercial cis-aconitic acid gave two spots in this system, with the leading spot being the larger (Table 10). The Rf of the middle spot (Figure 14) was such that it could have been the lagging cis-aconitic acid spot or isocitric acid. But the ratio of spot sizes for the middle and leading spots (Figure 14) was opposite to that for cis-aconitic acid. The pattern suggests that the middle spot was predominantly isocitric acid.

The spot sizes suggest that the concentrations of citric, isocitric, and cis-aconitic acids in the phosphatase stabilizing fraction may have been citric > isocitric > cis-aconitic acid (Figures 13 and 14). Spot areas are a function of the logarithm of the concentration of the material in each spot (31).

Figure 13

Identification of the Tricarboxylic Acids in the n-Butanol-Ethyl Acetate-Formic Acid System

The four separate paper chromatograms were developed simultaneously by descending chromatography for 8 3/4 hours. The solvent just ran off the chromatograms, the ends of which were notched. The chromatograms were air dried for 15 minutes, autoclaved, sprayed with bromocresol green and then 3 x 10^{-3}M Na_2CO_3 where necessary. The well defined dark yellow spots (on a dark blue background) were immediately outlined and later traced. The figure is a photograph of the tracings.

U stands for unknowns in the ether extract of the pooled fractions 44 through 58 described in Figure 10. The 0.8 ml extract was diluted 4-fold with H_2O before chromatography. C is for a citric acid standard containing 10 µg or about 1/20 µmole per µl (Nutritional Biochemicals Corp.). Iso is for an isocitric acid standard containing about 10 µg or about 1/20 µmole per µl (Calbiochem C grade, dl + allo, trisodium salt converted to the acid with Dowex H^+). Cis is for a cis-aconitic acid standard containing 10 µg or about 1/17 µmole per µl (Calbiochem A grade).

Chromatogram I: C = 4 µl of citric acid standard. U = 8 µl of unknown. C + U = 6 µl of a mixture 1:2 (v/v) of citric acid standard and unknown.

Chromatogram II: Iso = 6 µl of isocitric acid standard. U = 8 µl of unknown. Iso+U = 7 µl of a mixture 3:4 (v/v) of isocitric acid standard and unknown.

Chromatogram III: Cis = 6 µl of cis-aconitic acid standard. U = 64 µl of unknown. Cis + U = 70 µl of a mixture 6:64 (v/v) of cis-aconitic acid standard and unknown.

Chromatogram IV: H_2SO_4 spotted with 3 µl of 0.1N sulfuric acid. U = 8 µl of unknown. H_2SO_4 + U = 11 µl of a mixture 3:8 (v/v) of 0.1N sulfuric acid and unknown.

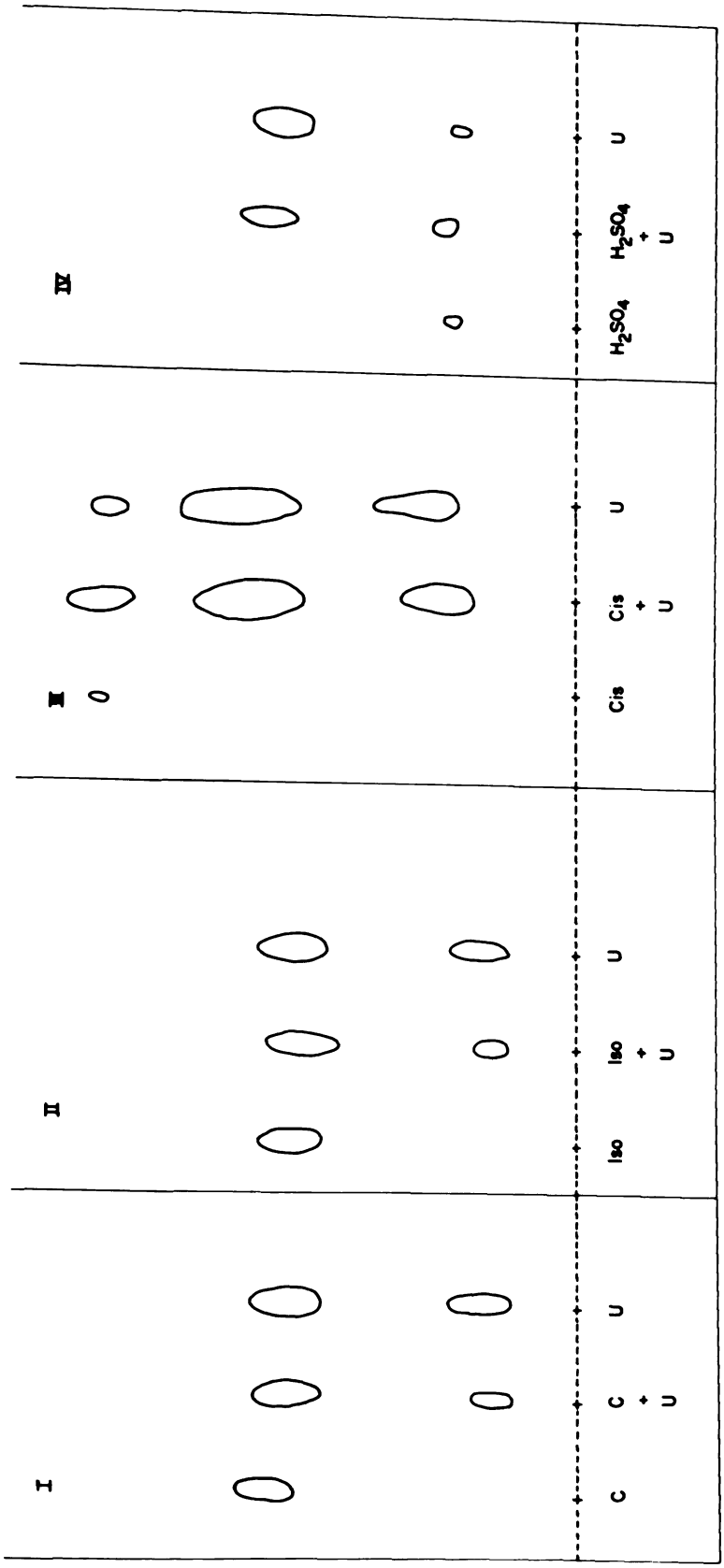
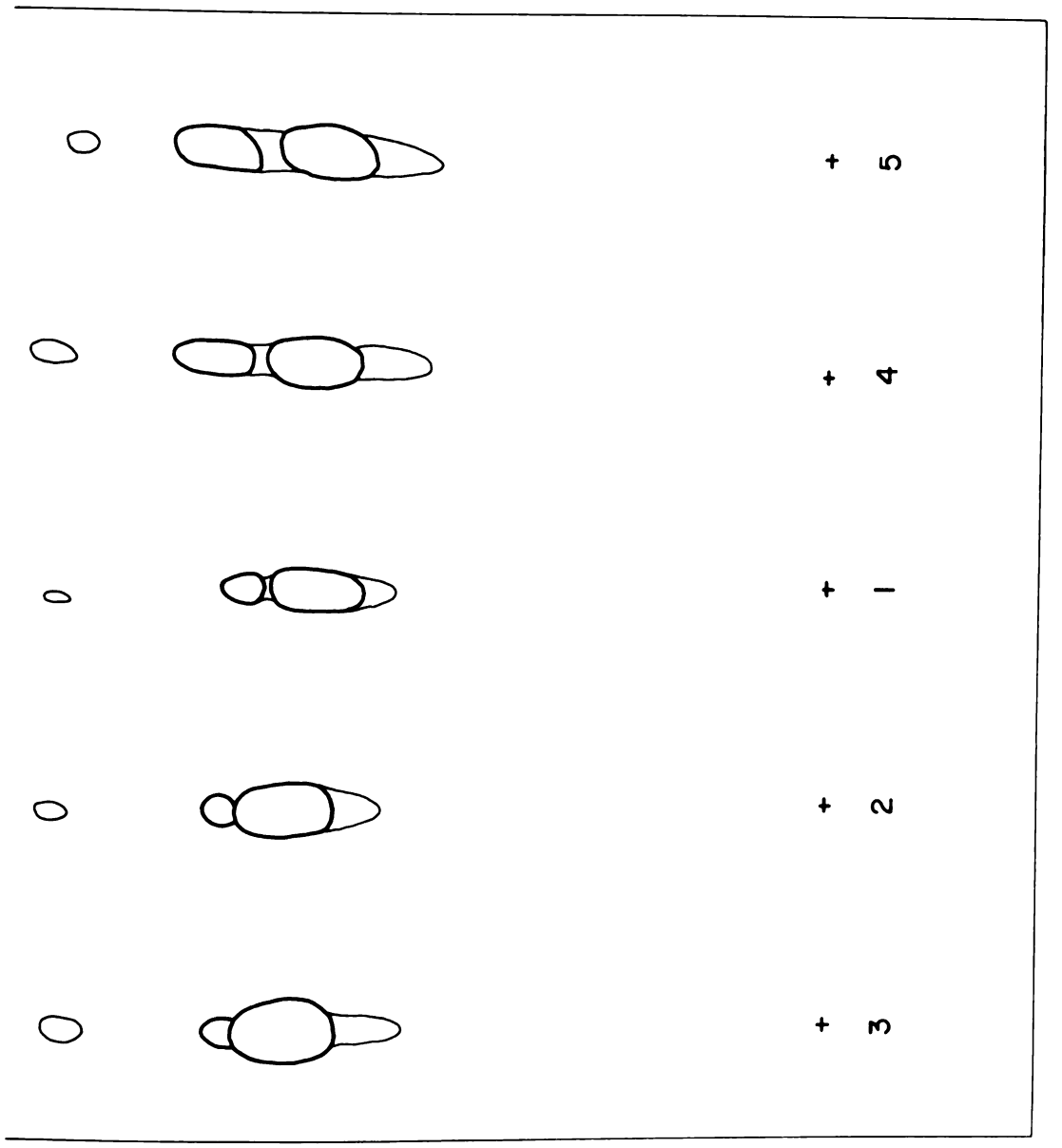

IV
III
II
I
U
H_2SO_4 + U
H_2SO_4
U
Cis + U
Cis
U
Iso + U
Iso
U
C + U
C

Figure 14

Identification of Citric and Isocitric Acids

The paper chromatogram was developed by descending chromatography in the n-butanol-propionic acid-water system. Running time was about 20 hours. The procedures for handling the developed chromatograms and obtaining the figure were as described for Figure 13. The unknown and standard solutions were also as described for Figure 13.

1. 48 µl of unknown. 2. 48 µl of unknown + 32 µl of citric acid standard. 3. 48 µl of unknown + 64 µl of citric acid standard. 4. 48 µl of unknown + 32 µl of isocitric acid standard. 5. 48 µl of unknown + 64 µl of isocitric acid standard.


+ 5
+ 4
+ 1
+ 2
+ 3

Stabilization of the Phosphatase by the Tricarboxylic Acids

At 10^{-3}M, citrate, isocitrate, cis-aconitate, and trans-aconitate stabilized the phosphatase (Figure 15). Although all four acids were about equally effective, the data suggest that cis-aconitate was somewhat more effective than isocitrate which was somewhat more effective than citrate. Trans-aconitate was about as effective as citrate. For the other 9 acids which were tested, the phosphatase activity decreased about the same extent as for a water control.

When the test solutions were made 10^{-3}M in $MgSO_4$, the overall stabilizing pattern was essentially the same as shown in Figure 15. The tricarboxylic acids plus $MgSO_4$ stabilized the phosphatase while the other 9 acid plus $MgSO_4$ combinations seemed essentially ineffective (data not shown). The $MgSO_4$ + citrate and $MgSO_4$ + isocitrate combinations gave somewhat more stabilization than these acids without $MgSO_4$, while the reverse was true for cis-aconitate. The $MgSO_4$ + trans-aconitate combination was also somewhat more effective than trans-aconitate alone. $MgSO_4$ alone at 10^{-3}M had no effect on the stability of the phosphatase (Table 11).

Figure 15

Stabilization of Phosphatase by Tricarboxylic Acids

Stock solutions contained the designated commercial acids at pH 6.3 and 10^{-2}M. To each of 14 individual 6 x 50 mm test tubes were added 25 µl of H_2O and then 25 µl of a stock acid solution. Another 25 µl of H_2O was added to the H_2O control.

Three times acetone purified phosphatase, which had originated as an extract prepared by Waring blendor and which had been converted to an acetone powder, was further purified by Sephadex G-100 chromatography using 0.02M cacodylate at pH 6.3 as the eluting buffer. The fractions were assayed for phosphatase activity as they accumulated and the most active fractions were pooled.

At zero time, 200 µl of the pooled phosphatase was added to each of the test tubes, they were corked and the contents mixed. The concentration of the acid in each tube was then 10^{-3}M. Next, the phosphatase in the pooled fractions was immediately assayed for activity to give the 100% value (4.32 units per ml - - -).[1] After 4 days at 0-4°, the contents of the 14 test tubes were assayed for the phosphatase activity which remained. The H_2O control retained 34% of the original activity (1.47 units per ml ———).[1]

Stability conferred =

$$\frac{\text{Activity remaining, units per ml}^1 - 1.47}{4.32 - 1.47} \times 100\%.$$

[1]

Phosphatase activity, units per ml
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Stability conferred (%)
100
90
80
70
60
50
40
30
20
10
0
Citrate
Isocitrate
Cisaconitate
Transaconitate
Succinate
Fumarate
Maleate
Acetate
Glycolate
Iodoacetate
Glycine
Serine
Oxalate

Table 11

Effect of $MgSO_4$ on the Stability of the Phosphatase

The conditions were as described in Figure 15 except that, where indicated, 25 µl of 10^{-2}M $MgSO_4$ was added to the preincubation mixtures, rather than 25 µl of H_2O. Thus the concentration of $MgSO_4$ during the 4 days that the enzyme was held at 0-4° was 10^{-3}M.

Acid	Stability conferred	
	- $MgSO_4$	+ $MgSO_4$
Citrate	57%	71%
Isocitrate	68%	85%
Cis-aconitate	76%	65%
Trans-aconitate	55%	79%
None	0	0

Coincidence of the Tricarboxylic Acids with the Phosphatase in Fractions from DEAE-Cellulose

A DEAE-cellulose column was prepared as described on p. 142. The remaining enzyme from the 3rd acetone fractionation (described on pp. 140-141), which had been held 34 days at 0-4°, was centrifuged at 5900 g for 8 minutes to remove an inactive precipitate. Eighty percent of the phosphatase activity survived the 34 days of storage. Thirty-three ml of the enzyme was added to the column and eluted with 400 ml of 0.02M cacodylate, pH 6.3, containing NaCl in a linear gradient from 0 to 0.5M. The eluent was continuously monitored at 253.7 mµ. The phosphatase recovery was 90%.

A paper chromatographic assay for citric and/or isocitric acid and the enzymatic assay for isocitrate revealed a correspondence between isocitrate (and/or citrate) and the phosphatase in the fractions from the DEAE-cellulose chromatographic fractionation (Figures 16 and 17).[1] The results of the paper chromatographic assay were completely consistent with the results of the enzymatic assay for isocitrate (Figure 17).[2]

Assuming that the phosphatase with a specific activity of 333 (Table 4, Figure 11) was homogeneous, 1 mole of isocitrate per 10 to 15 moles of amino acid residue in the phosphatase fractionated with the phosphatase in fraction no. 22. There were 55.3 units of phosphatase/ml in fraction no. 22 which calculates to be 55.3/333 units x mg^{-1} or 0.166 mg of phosphatase/ml in fraction 22. Using 100 mg/millimole for an average amino acid residue, there were 0.166 x 10^{-2} millimole or 1.66 μmoles of phosphatase amino acid reside/ml in fraction 22. In

---

[1]The heating and recooling, and the filtering methods which were used in the preparation of the fractions for the enzymatic determination of isocitrate (Figure 16) did not affect the recovery of isocitrate (Figure 17).

[2]The n-butanol-ethyl acetate-formic acid system did not distinguish between citric and isocitric acids (Table 10 and Figure 13). Since both citric and isocitric acids were identified in the purified ether extracted stabilizing fraction (Figure 14) which had originated from the same batch of 3 times acetone purified enzyme from which the enzyme for the experiment of Figure 17 had originated, the citric and/or isocitric acid spots (Figure 17) probably contained both acids.

Figure 16

Isocitrate and the Phosphatase in Fractions from DEAE-Cellulose

○—○ P-glycolate phosphatase. △—△ Isocitrate. -···- Absorbance at 253.7 mμ.

The DEAE-cellulose chromatography of the phosphatase was as described in the text. After the phosphatase assays were made, the stoppered fractions were held at -20°. The fractions were thawed and then placed in a boiling water bath, with glass marbles over the test tubes, for 30 minutes, rapidly recooled in an ice bath, then filtered through individual Whatman No. 1 filter papers. Enzymatic determinations of isocitrate were as described in methods.

Isocitrate recovery from a fraction was tested as follows: To 100 μl of fraction no. 22, before it was heated to 100°, cooled, and filtered, was added 100 μl of the stock isocitrate solution. The mixture was kept at 0° for more than 1 hour. Then an enzymatic determination of isocitrate was made on 90 μl of the stock isocitrate ($\triangle A = 0.0526$), 90 μl of fraction no. 22 ($\triangle A = 0.0594$), and 180 μl of the mixture ($\triangle A = 0.1160$). The △ absorbance for the mixture was 3% greater than the sum of the other two △ A values ($\Sigma = 0.1120$), i.e. the isocitrate which was added to fraction no. 22 was quantitatively recovered.

Cis-aconitate was used to test for aconitase activity, which was absent in the heated, cooled, and filtered fractions. The aconitase assay which was used is described in methods. The blank absorbance values which were recorded during the enzymatic determinations of isocitrate did not increase, which indicates that there was no endogenous isocitrate dehydrogenase activity in these same fractions.

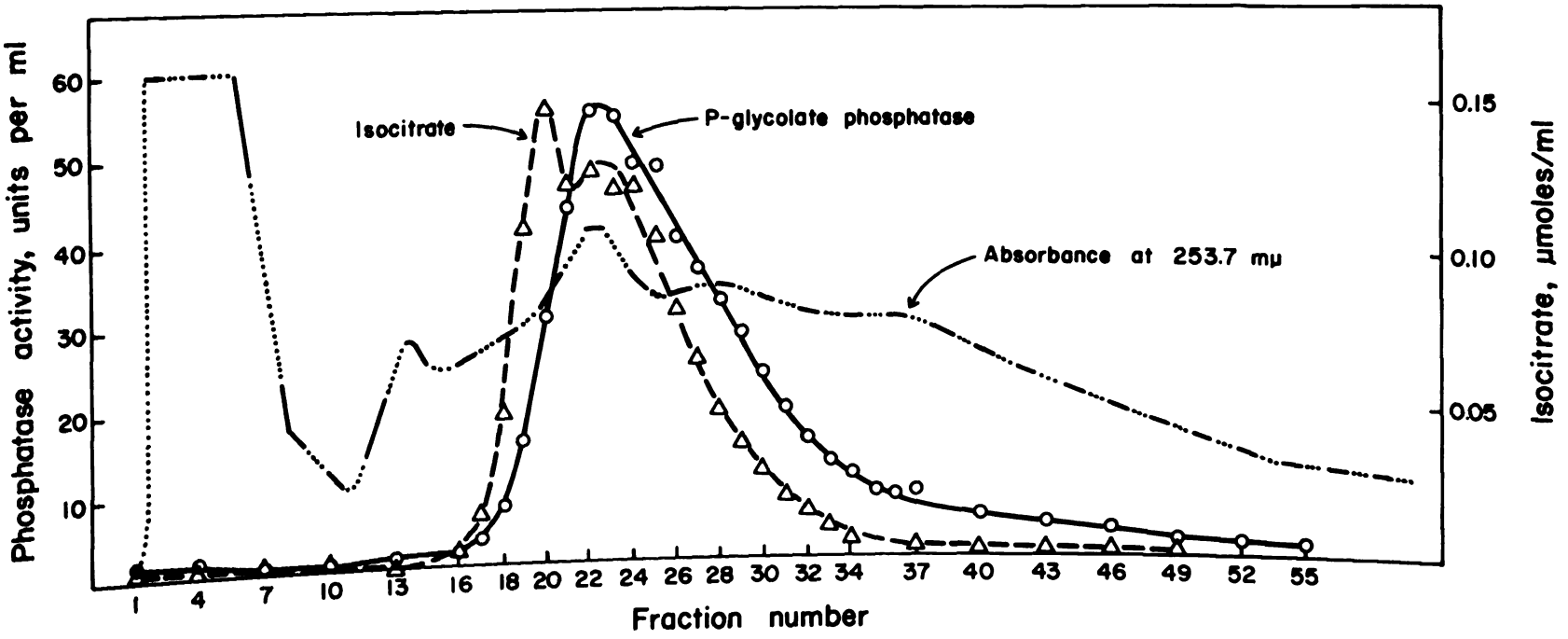

Phosphatase activity, units per ml
60
50
40
30
20
10
Isocitrate
P-glycolate phosphatase
Absorbance at 253.7 mμ
0.15
0.10
0.05
Isocitrate, μmoles/ml
1 4 7 10 13 16 18 20 22 24 26 28 30 32 34 37 40 43 46 49 52 55
Fraction number

Figure 17

A Magnified Plot of Figure 16

○——○ P-glycolate phosphatase.

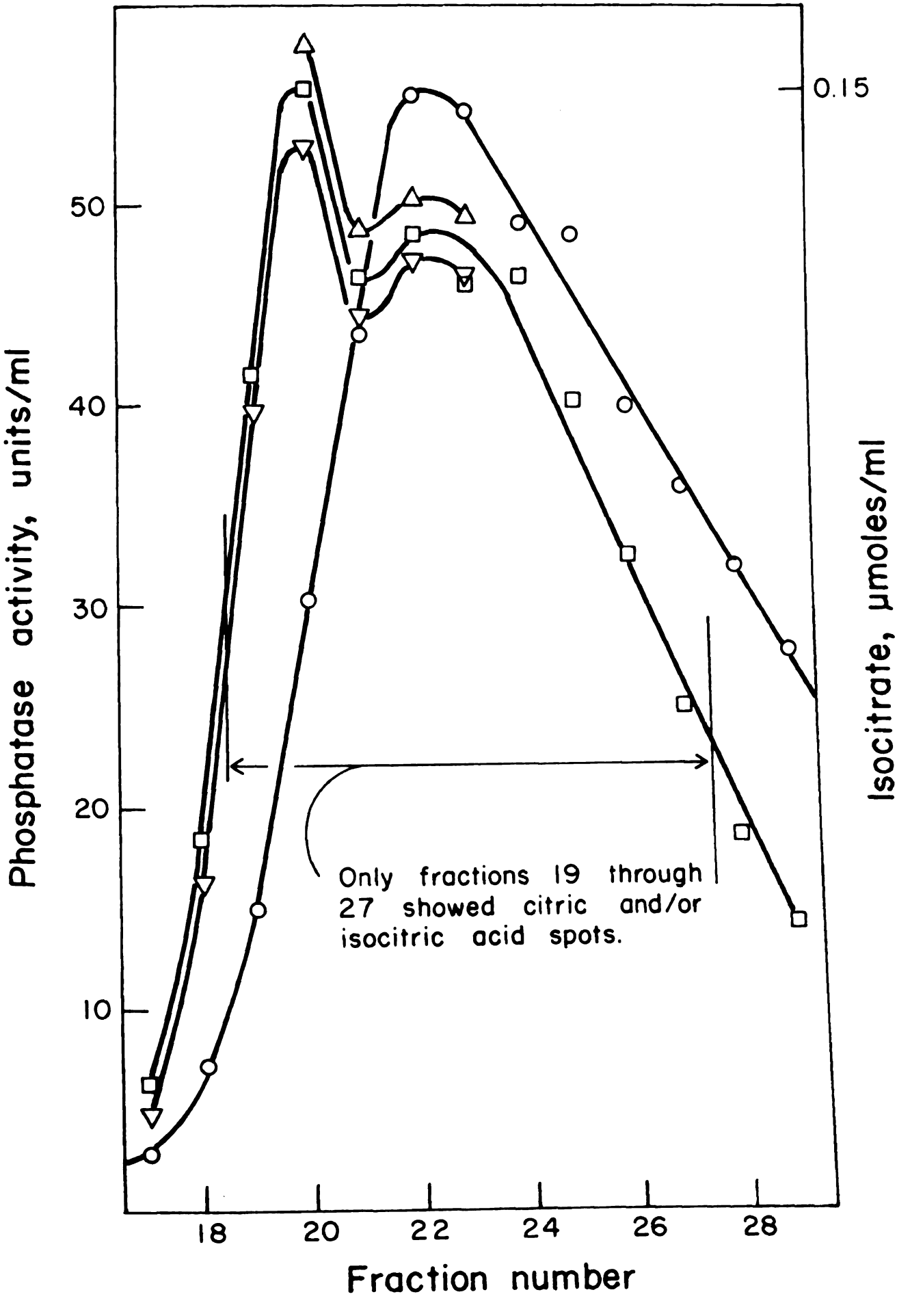
△——△ Isocitrate. The fractions had been heated and recooled, but not filtered.

□——□ Isocitrate. The fractions had been heated and recooled, then filtered.

▽——▽ Isocitrate. The fractions had not been heated.

The plots of the phosphatase and of the isocitrate concentrations after the fractions had been heated, recooled, and filtered are taken from Figure 16. The isocitrate concentrations were determined before heating, and after heating and recooling but before filtering, in the fractions indicated, by using the same procedure described for the fractions after they had been heated, recooled, and filtered.

The determination of citrate and/or isocitrate by paper chromatography was performed before the experiment of Figure 16, i.e. before any of the fractions were heated, cooled, and filtered. 0.50 ml aliquots from the 33 fractions which were assayed for phosphatase activity were pipetted into individual pointed test tubes. The aliquots were acidified with 10N H_2SO_4 (10 to 30 µl) to a pH of 1-2 (pH paper). The acidified aliquots were placed under 15 mm Hg at room temperature overnight. 50 µl of H_2O was then added to the dry contents of each tube, the contents were mixed, and the tubes were centrifuged. 12 µl from each tube was spotted on Whatman no. 1 paper as described in the methods section, except 50° air was used to dry the spots. Commercial citric acid was similarly spotted. The chromatograms were run in the n-butanol-ethyl acetate-formic acid system, air dried, autoclaved, sprayed with bromocresol green and then 3×10^{-3}M Na_2CO_3 where necessary (see methods). Only the fractions indicated showed spots which corresponded in position to the commercial citric acid.


Phosphatase activity, units/ml
Isocitrate, µmoles/ml
Fraction number
50
40
30
20
10
0.15
18
20
22
24
26
28
Only fractions 19 through 27 showed citric and/or isocitric acid spots.

addition, there was 0.131 μmole/ml of isocitrate in fraction no. 22 (Figure 16). In fraction no. 22, the isocitrate molecules/amino acid residue of the phosphatase calculates to be 0.131/1.61 or 1 isocitrate/12.5 amino acid residues. If the phosphatase with a specific activity of 333 were not pure, the actual ratio of isocitrate molecules/phosphatase amino acid residue was even greater.[1]

Furthermore, the chromatographic evidence suggests that more citrate than isocitrate was separated from the phosphatase (Figure 14), and that, in addition, a relatively small amount of cis-aconitate was also separated from the enzyme (Figure 13). Thus, even after 3 acetone fractionations, at least one tricarboxylic acid per 5 amino acid residues of the phosphatase could have been present in fraction no. 22 from the DEAE-cellulose chromatography.

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[1]Other than the assumption concerning the purity of the most pure phosphatase preparation, three other assumptions are implied in the above calculations.

a. The average molecular weight of the amino acid residues in the phosphatase is approximately 100.

b. Equal weights of the phosphatase and of bovine serum albumin would give reasonably comparable results with the Lowry's modified Folin-Ciocalteu method for measuring protein, which was the method used when the specific activity of 333 was measured.

c. The number of substrate molecules transformed per minute per molecule of enzyme was about the same when the specific activity of 333 was measured as when the activity of the phosphatase in fraction no. 22 was measured. In both cases, the phosphatase activity was measured using the standard P-glycolate phosphatase assay.

Competitive Inhibition of the Phosphatase by Cis-aconitate

P-glycolate phosphatase which had been precipitated by acetone was essentially inactive without added metal (footnote, p. 106).

When an activator, such as Mg^{+2}, is required for a reaction, inhibition data must be interpreted with care, since the site of inhibition could be at the activator rather than on the enzyme (252). With the concentration of Mg^{+2} equal to 2×10^{-3}M, kinetic data suggest that cis-aconitate at 10^{-2}M is a competitive inhibitor of the P-glycolate phosphatase reaction (data not shown).[1]

---

[1]The enzyme had been fractionated once by acetone and then once by ammonium sulfate. Residual ammonium sulfate was not removed from the enzyme. Kinetic experiments conducted with this enzyme preparation, when it was fresh, suggest that cis-aconitate could be both an activator and a competitive inhibitor of the phosphatase. After this enzyme preparation had aged, the enzyme was no longer activated by cis-aconitate, but it continued to be inhibited competitively by the acid.

Because cis-aconitate is an inhibitor of the phosphatase competitive with respect to the substrate, activation by cis-aconitate in the reaction mixtures was noted only at high substrate concentrations. The value of the abscissa (Figure 18) corresponding to the concentration of substrate which is used in the standard P-glycolate phosphatase assay is 0.03. It is apparent that at this concentration of substrate, the inhibition by cis-aconitate is almost completely reversed.

It should be noted that when pretreatment with the ether washed and ether extracted factor fractions activated the phosphatase (Table 9), the enzyme was relatively fresh and the factor fractions contained SO_4^{-2} from the H_2SO_4 which was added before the extraction with ether. In this activation, tricarboxylic acids were not added to the phosphatase reaction mixtures. Therefore, any activation by

Under these conditions, it is possible that the competitive inhibition might be by the chelation of Mg^{+2} by cis-aconitate.

This objection was overcome by including an excess of Mg^{+2} in the reaction mixtures. With the concentrations of cis-aconitate and Mg^{+2} which were used in the experiment of Figure 18, excess Mg^{+2} available for the phosphatase reaction, which was not chelated by cis-aconitate, should have been in excess of 10^{-2}M. Variation of the concentration of Mg^{+2} over a range from 10^{-3}M to 10^{-1}M did not significantly affect the reaction rate (Table 12).

The evidence suggests that cis-aconitate is a competitive inhibitor of P-glycolate phosphatase, that the inhibition is with respect to the substrate, and that the site of inhibition is not at the Mg^{+2} (Figure 18). When the concentration of Mg^{+2} during the reactions was 2 x 10^{-2}M, the apparent K_m for P-glycolate was 7.5 x 10^{-5}M and

---

the tricarboxylic acids probably resulted from the pretreatment with the acids, which were also in the factor fractions.

The possible activation of the phosphatase by cis-aconitate under certain conditions suggests that the enzyme may possess a binding site(s) for the acid which is different from the site of competitive inhibition. It is of interest that fumarase, an enzyme thought to be similar to aconitase (164, 179), is activated (158) and inhibited competitively (159) by citrate, and that the site of the activation of fumarase by anions is thought to be different from the active site (158).

Further work is required to determine under what conditions the phosphatase may be activated by the tricarboxylic acids.

Figure 18

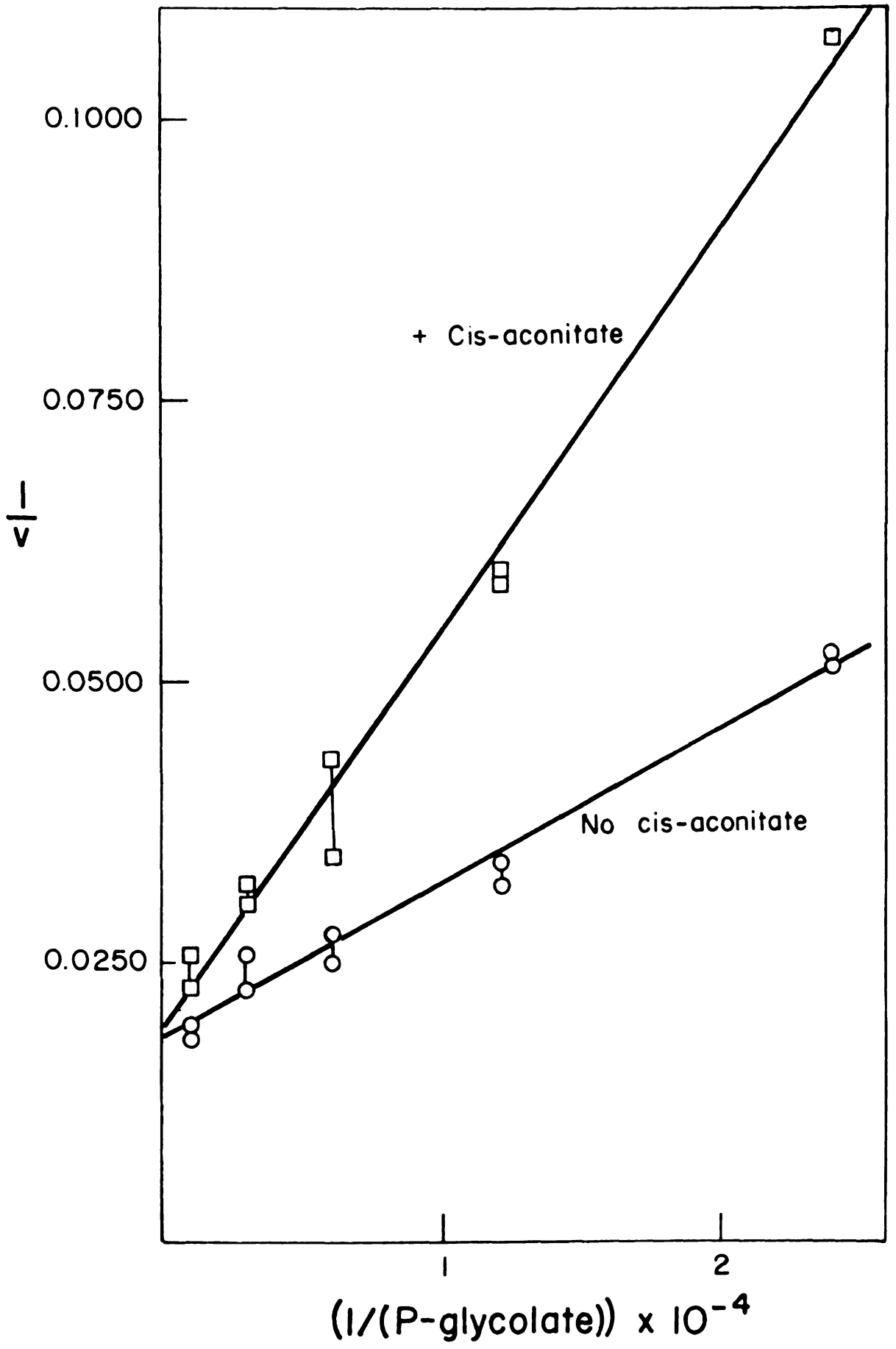
Competitive Inhibition of the Phosphatase by Cis-aconitate

O——O No cis-aconitate.

□——□ Plus cis-aconitate.

v = velocity in mμmoles of P-glycolate hydrolyzed per 30 seconds per 3 ml reaction mixture.

All reaction mixtures contained 0.50 ml of 0.20M Na cacodylate, pH 6.3, and 0.30 ml of 0.20M $MgSO_4$ (reaction concentration of Mg^{++} was 2.0×10^{-2}M). The tubes with cis-aconitate contained 0.10 ml of 0.30M Na cis-aconitate, pH 6.3, (reaction concentration was 1.0×10^{-2}M). The amounts of substrate used were 0.30 ml, 0.10 ml, 0.050 ml, 0.025 ml, and 0.0125 ml of Na P-glycolate, pH 6.3, 10.0 μmoles/ml. Water was added to make the final reaction volume 3.00 ml. The reaction mixtures were brought to 30.0° in a water bath and the reactions were initiated with 2.50 μl of the Bio-Gel P-60 preparation described in Table 3. (The enzyme had been held at -20° for 6½ months and had been thawed and refrozen several times.) After 30.0 seconds, the reactions were terminated with 0.75 ml of the acid molybdate solution which was used for the isobutanol benzene extraction method. P_i was determined on a 2.50 ml aliquot (containing 2.0 ml of the reaction mixture + 0.50 ml of the acid molybdate solution) by the iso-butanol benzene extraction method. For every reaction, a zero time control was run by adding the enzyme after the acid molybdate solution had been added. After one of the values, which was obviously in error, was discarded, the straight lines were fit by the method of least squares.


0.1000
0.0750
0.0500
0.0250
1/v
+ Cis-aconitate
No cis-aconitate
1
2
(1/(P-glycolate)) x 10⁻⁴

the apparent K_I for cis-aconitate was 6.5×10^{-3}M. When the concentration of Mg^{+2} during the reactions was 2×10^{-3}M, the apparent K_m for P-glycolate was 6.7×10^{-5}M and the apparent K_I for cis-aconitate was 5.0×10^{-3}M.

Table 12

Effect of High Concentrations of $MgSO_4$ on the Phosphatase

Standard P-glycolate phosphatase reaction mixtures were used except that the $MgSO_4$ concentrations were varied. The enzyme (same preparation as the one described in Figure 18) was saturated with substrate during the 10 minute reactions at 30°. Each mixture held 1.0 µl of enzyme.

($MgSO_4$)	Activity*
M	
10^{-3}	0.555
10^{-2}	0.591
10^{-1}	0.520

*µmoles P-glycolate hydrolyzed per 10 minutes per 0.75 ml reaction mixture.

Discussion of the P-glycolate Phosphatase, Tricarboxylic Acid Relationship

The evidence concerning the separation of endogenous stabilizing factors from the phosphatase, after preliminary purification by three acetone fractionations and DEAE-cellulose chromatography (Figure 10), and the identification of the stabilizing factors as citrate, isocitrate, and cis-aconitate (Table 10, Figures 13 and 14), suggest

that these acids may somehow be associated with the phosphatase *in vivo*. The correspondence between large quantities of the tricarboxylic acids and the phosphatase in fractions from a DEAE-cellulose chromatographic fractionation, after 3 preliminary acetone fractionations (Figures 16 and 17), could have been a coincidence, but consideration of the stabilization of the phosphatase by the tricarboxylic acids (Figure 15) and the competitive inhibition of the phosphatase reaction by cis-aconitate (Figure 18) makes it seem that the correspondence between the phosphatase and the tricarboxylic acids was more than a coincidence.

The above observations, together with knowledge that aconitase is stabilized by its substrates, that any one substrate is a competitive inhibitor of the interconversion of the other two tricarboxylic acids by aconitase, and suggestive evidence that relatively large quantities of the tricarboxylic acids may bind to aconitase from plant and animal tissues (literature review), led to the hypothesis that in tobacco leaves, aconitase activity may be associated with P-glycolate phosphatase. Experiments which tested this hypothesis are described in a later section.

Investigations on the Stability Toward Dilution at 30° of P-glycolate Phosphatase in Fresh Extracts from Tobacco Leaves

Unless noted otherwise, each experiment was initiated with the harvest of 2 to 4 medium sized tobacco leaves (see Table 1), and the leaf blades were homogenized in a mortar and pestle with sand with two weights of water.

Stabilization by Time After Homogenization

When extracted from tobacco leaves by mortar and pestle, the phosphatase in the extract remained active if stored at 0°. However, the enzyme was initially unstable toward dilution at 30°, but with time it became stable to this dilution (Figure 19). The enzyme in the extract from the greenhouse grown tobacco leaf harvested in the winter was initially more stable and became stabilized many times faster than the phosphatase in the extract from the field grown tobacco leaves harvested in the summer.[1] During the summer, the greatest initial stability toward dilution at 30° was about 50%, while during the winter, the smallest

[1]When water rather than 0.1M cacodylate, pH 7.5, was used as the extraction fluid, the initial stability toward dilution at 30° of the phosphatase in an extract during the winter was 18% and the stability after only four hours post homogenization time was 93%. Thus, the increased initial stability and the increased rate of stabilization of the phosphatase in the extract during the winter compared to the enzyme in an extract during the summer (Figure 19) were not because of the use of pH 7.5 cacodylate as the extraction fluid.

Figure 19

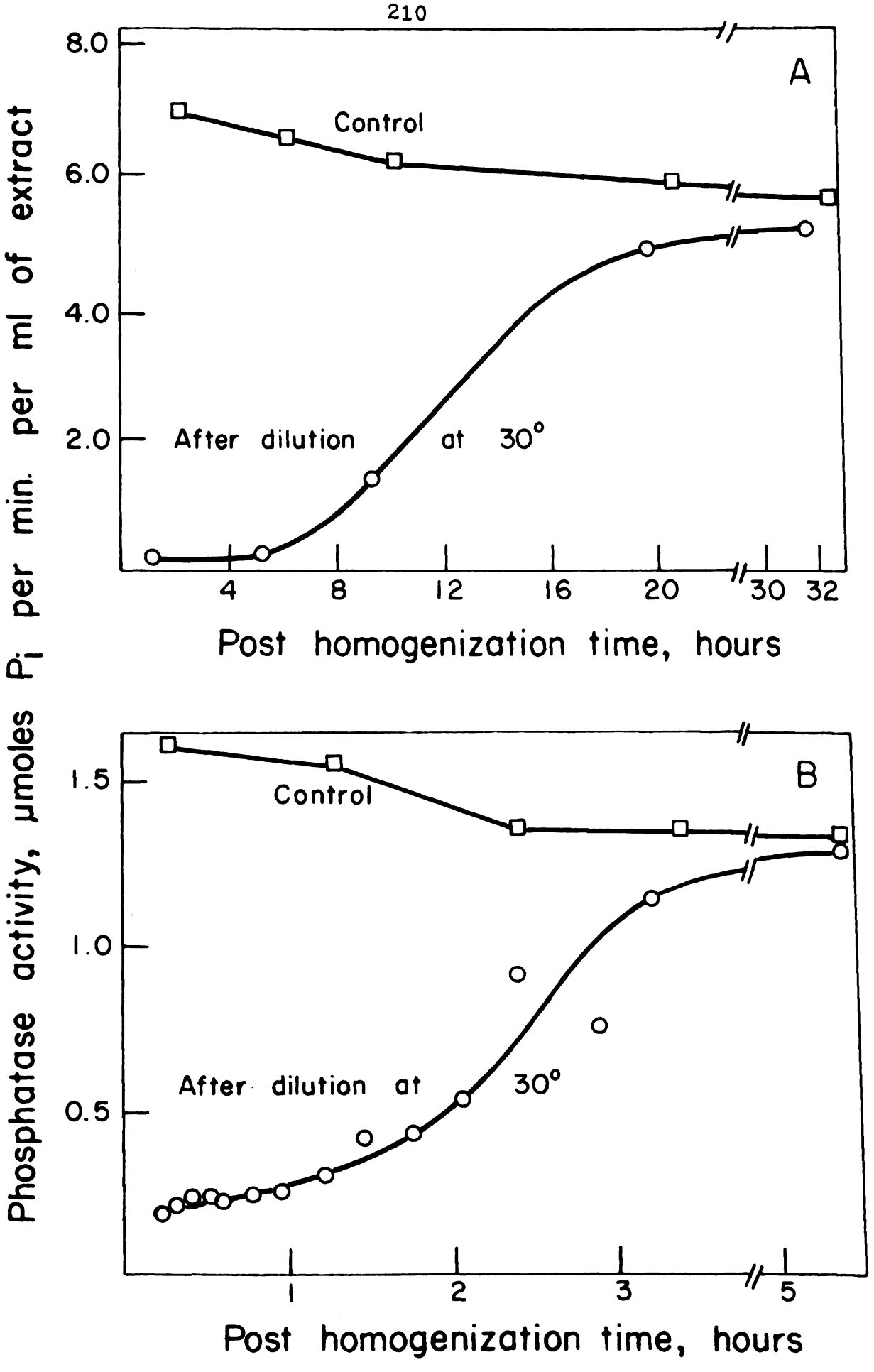
Stabilization by Time After Homogenization

□——□ Controls.

○——○ Activity after 33.3-fold (Figure A) or 10-fold (Figure B) dilution at 30°.

Figure A: The leaves were harvested from the field at 3:30 P.M. on 8-3-1967 (light intensity = 4000 ft-c). The extract was held near 0° in a 100 ml glass beaker open to the air during the time of this experiment. Its depth in the beaker was 1.8 cm, and it was stirred gently about once an hour.

Figure B: One leaf was harvested at 5:00 P.M. on 12-30-1964 from the second growth of a plant which had been grown in the field and transplanted back to the greenhouse about Oct. 1. The leaf blades were homogenized in a mortar and pestle with sand and two weights of 0.1M cacodylate, pH 7.5. The extract, which had been centrifuged at 35,000 g for 5 minutes, was held under air near 0° in a glass beaker during the time of this experiment.


A
Control
After dilution at 30°
8.0
6.0
4.0
2.0
4
8
12
16
20
30
32
Post homogenization time, hours
B
Control
After dilution at 30°
1.5
1.0
0.5
1
2
3
5
Post homogenization time, hours
Phosphatase activity, µmoles Pi per min. per ml of extract

initial stability toward dilution at 30° was about 13%. However, the initial stability toward dilution at 30° of the phosphatase during the summer was usually less than 10% and was sometimes 0%. In extracts from tobacco leaves harvested in the winter from senescent plants grown in the greenhouse, the initial stability toward dilution at 30° of the phosphatase was as great as 95%. The data suggest that the more vigorous the leaves, the less the initial stability toward dilution at 30° of the phosphatase and the more the post homogenization time which is required to stabilize it.

Stabilization as a Diurnal Function

The phosphatase in extracts from tobacco leaves harvested at night did not become more stable with time. In contrast, the enzyme did become more stable with time in extracts from leaves harvested during daylight hours (Figure 20). Even though in the experiment of Figure 20 there was considerably more cloud cover in the afternoon hours than in the morning hours, the optimum hour of harvest for the maximum stabilization rate of the phosphatase in the extracts was about 3 or 4 P.M. Thus it seems that the stabilization rate of the phosphatase in the extracts was a function not only of the light intensity before harvest but also of the time of exposure to light or darkness before harvest. The 6 to 7 minutes of

Figure 20

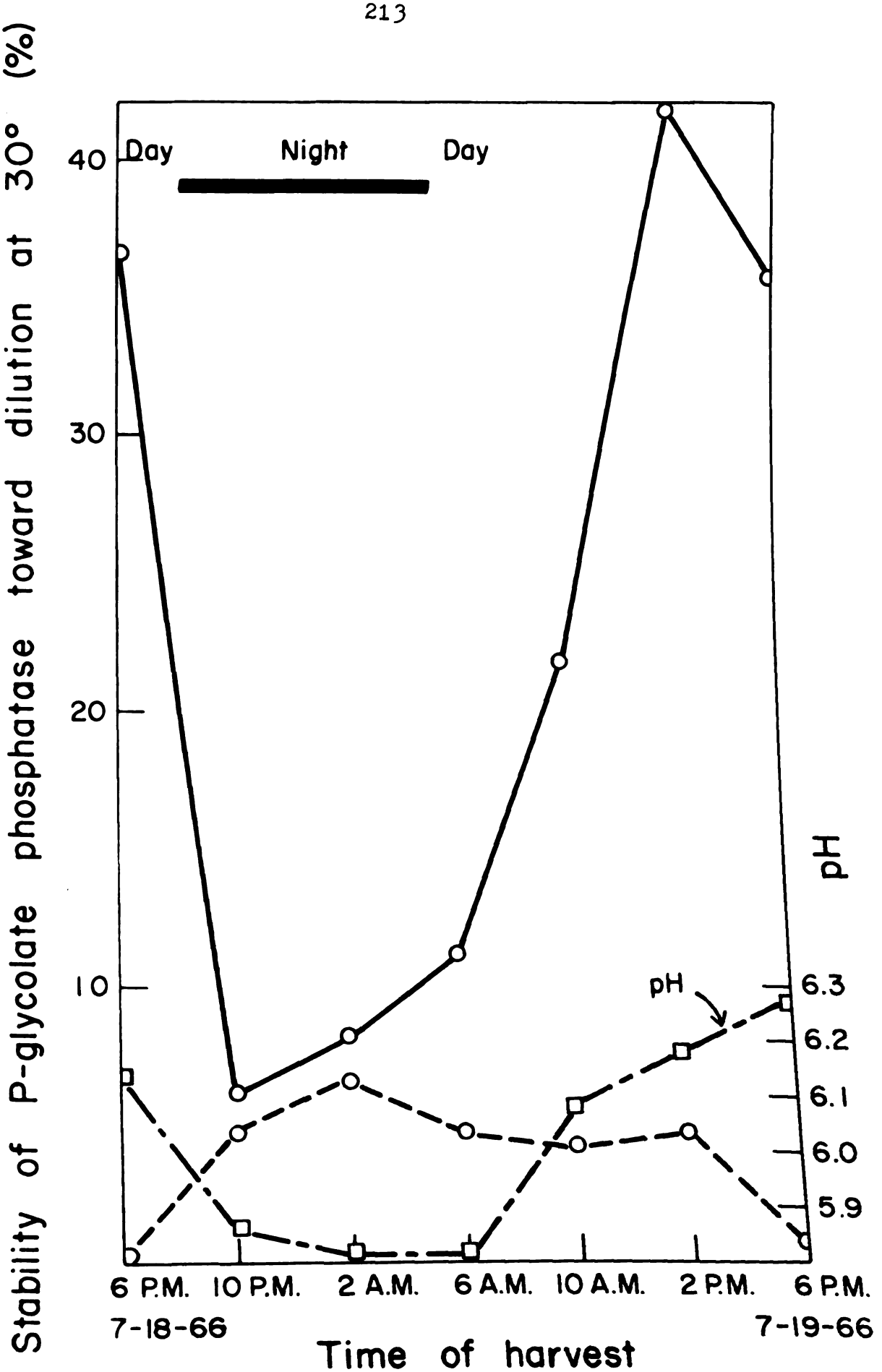
Stabilization as a Diurnal Function

Leaves which were selected at random from similar field grown plants were harvested in pairs at the times indicated. Each extract, which had been centrifuged at 15,000 g for 10 minutes, was held near 0° in a glass beaker open to the air long enough for the completion of the assays of that extract.

□-·-□ pH of the extracts.

O---O The stability toward dilution at 30° of P-glycolate phosphatase in each extract 45 minutes after the homogenization.

O——O The stability toward dilution at 30° of the phosphatase in each extract 12 hours and 45 minutes after the homogenization.


Stability of P-glycolate phosphatase toward dilution at 30° (%)
40
30
20
10
Day
Night
Day
pH
6.3
6.2
6.1
6.0
5.9
6 P.M.
7-18-66
10 P.M.
2 A.M.
6 A.M.
10 A.M.
2 P.M.
6 P.M.
7-19-66
Time of harvest

exposure to artificial light (10-100 ft-c) immediately before and during the homogenization had no apparent effect on the results, which is consistent with the approximately 3 hour lag between the light intensity maximum and the harvest time for the maximum stabilization rate of the phosphatase in the extracts.

Even when extracts from field grown tobacco leaves which were harvested at night were held for longer periods of time, the phosphatase did not become stable toward dilution at 30° (Figure 26). When leaves were harvested during daylight hours but the light intensity was very low (rain and fog, for example), the phosphatase in the extracts likewise did not become stable toward dilution at 30° with time (Figure 24). However, in these cases, the phosphatase retained most of its activity in the extracts stored at 0° (Figures 24 and 26), and it lost no more activity in an extract stored at 0° than did the phosphatase in an extract from leaves harvested during a sunny day (Figure 26).

In the experiment of Figure 20, the specific activity of the phosphatase in the 7 extracts varied in a random way. There was no apparent relationship between the time of day of harvest and the specific activity of the enzyme in these extracts (data not shown).

Stabilization by Mixing in a Waring Blendor

The phosphatase extracted from tobacco leaves by mortar and pestle was unstable toward dilution at 30° while the phosphatase extracted by Waring blendor was stable (Figure 8). When an extract which had been prepared by mortar and pestle was mixed in a Waring blendor, the unstable phosphatase was stabilized (Figure 21). Thus, the stabilization (Figure 8) was not dependent on material removed by the cheesecloth filtration or by centrifugation. The results also suggest that the lower activity per gram of leaf for the phosphatase in the extract prepared by Waring blendor (Figure 8) was primarily due to inactivation of extracted enzyme rather than less efficient extraction. The amount of inactivation by Waring blendor (Figure 21) was about equal to the amount of inactivation which accompanied the time dependent stabilization of the phosphatase (Figure 19). The enzyme in extracts prepared by Waring blendor lost no further activity in 24 hours at 0° (data not shown). The data suggest that the time dependent changes in the activity and stability toward dilution at 30° of the phosphatase (Figure 19) also occured during the Waring blendor treatments, which lasted 2 minutes (Figure 21).

Stabilization by Oxygen

When fresh extracts were "buzzed" under N_2 or CO_2,

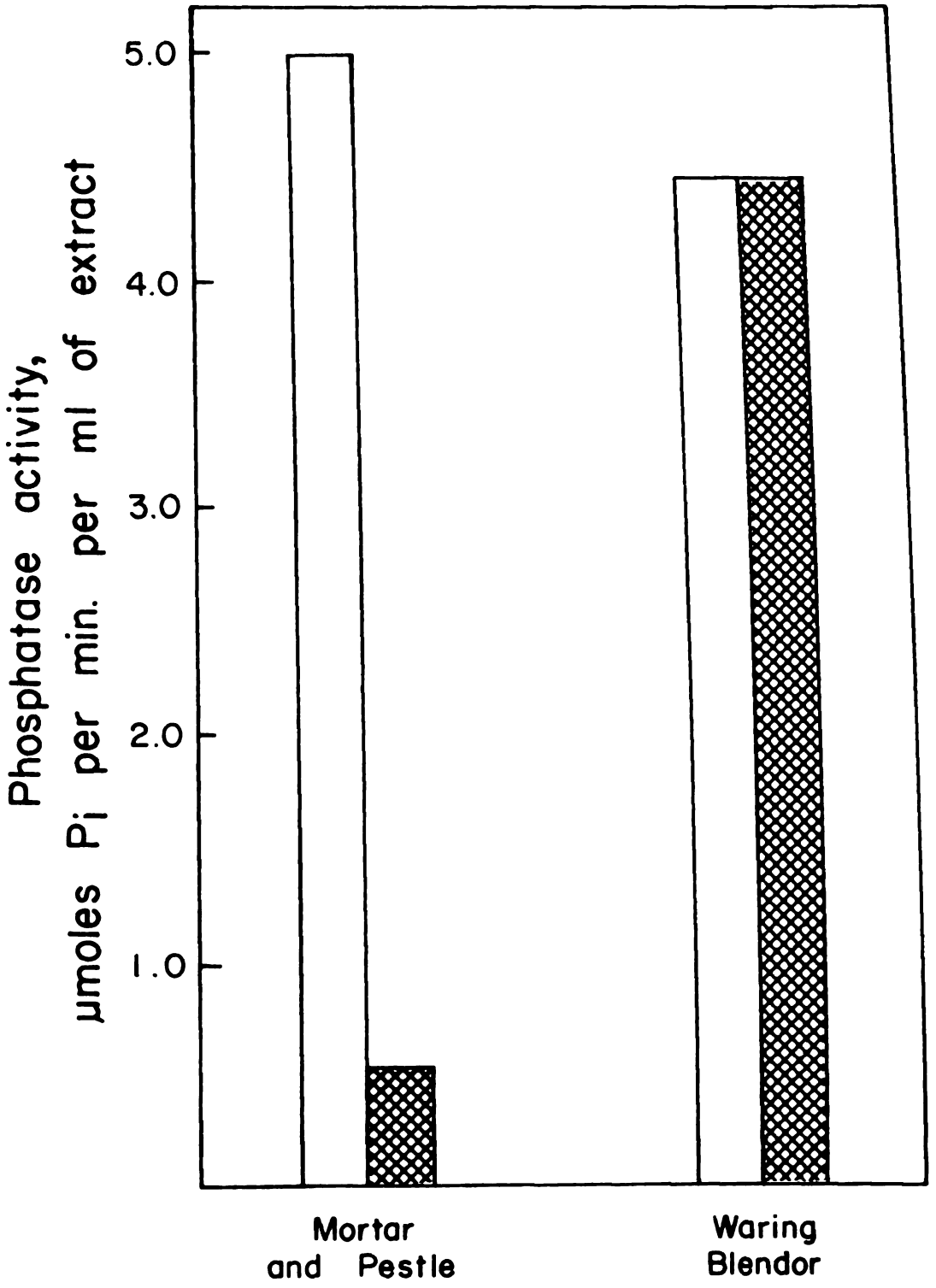
Figure 21

Stabilization by Mixing in a Waring Blendor

Six leaves were harvested from the field at 12:30 P.M. on 9-7-1965. After removal of an aliquot of the extract, which had been centrifuged at 20,000 g for 10 minutes, the remainder of the extract was vigorously mixed in a Waring blendor for 2 minutes. The extract in the Waring blendor was under air during the mixing. The aliquot which was mixed in the Waring blendor was then refiltered through cheesecloth and recentrifuged as before.

Open bars: controls.

Closed bars: activity after 20-fold dilution at 30°.


Phosphatase activity,
μmoles P_i per min. per ml of extract
5.0
4.0
3.0
2.0
1.0
Mortar
and Pestle
Waring
Blendor

the phosphatase remained unstable toward dilution at 30°, but if the extracts were "buzzed" under air or O_2, the phosphatase was stabilized (Figure 22). The data suggest that O_2 is the component in air which stabilizes the phosphatase and that the vigorous mixing is necessary only to increase the oxygen concentration near the phosphatase molecules.

In another experiment, done once, a fresh extract from tobacco leaves was divided into two aliquots. One was gassed with N_2 and the other was left open to the atmosphere. While the phosphatase in the extract exposed to air became more stable with time, the phosphatase in the extract under N_2 did not become more stable with time.

A Diurnal in the Stabilization by Buzzing[1]

In general, the stabilization by buzzing of the phosphatase in fresh extracts followed a diurnal (Table 13) that was qualitatively similar to the diurnal in the stabilization by post homogenization time (Figure 20). The relatively low stability value for the phosphatase in the extract from the leaves harvested at 11:45 A.M. could be explained by the 100% cloud cover and low light intensity at the time of harvest. However, the 67%

[1]The data were collected from 7 different experiments conducted for other purposes in Aug. and Sept. of 1967.

Figure 22

Stabilization by Oxygen

The leaves were harvested from the field at 5:40 P.M. on 7-26-67 (light intensity = 1100 ft-c). Five 3.0 ml aliquots of the extract, which had been centrifuged at 15,000 g for 15 min., were placed in separate side arm test tubes, filling them to a depth of 1.8 cm. The five were treated as described in the following schedule.

1. Left open to the air; not buzzed.
2. Left open to the air; buzzed.
3. Gassed with N_2; buzzed.
4. Gassed with CO_2; buzzed.
5. Gassed with O_2; buzzed.

Open bars: controls.

Closed bars: activity after 33.3-fold dilution at 30°.

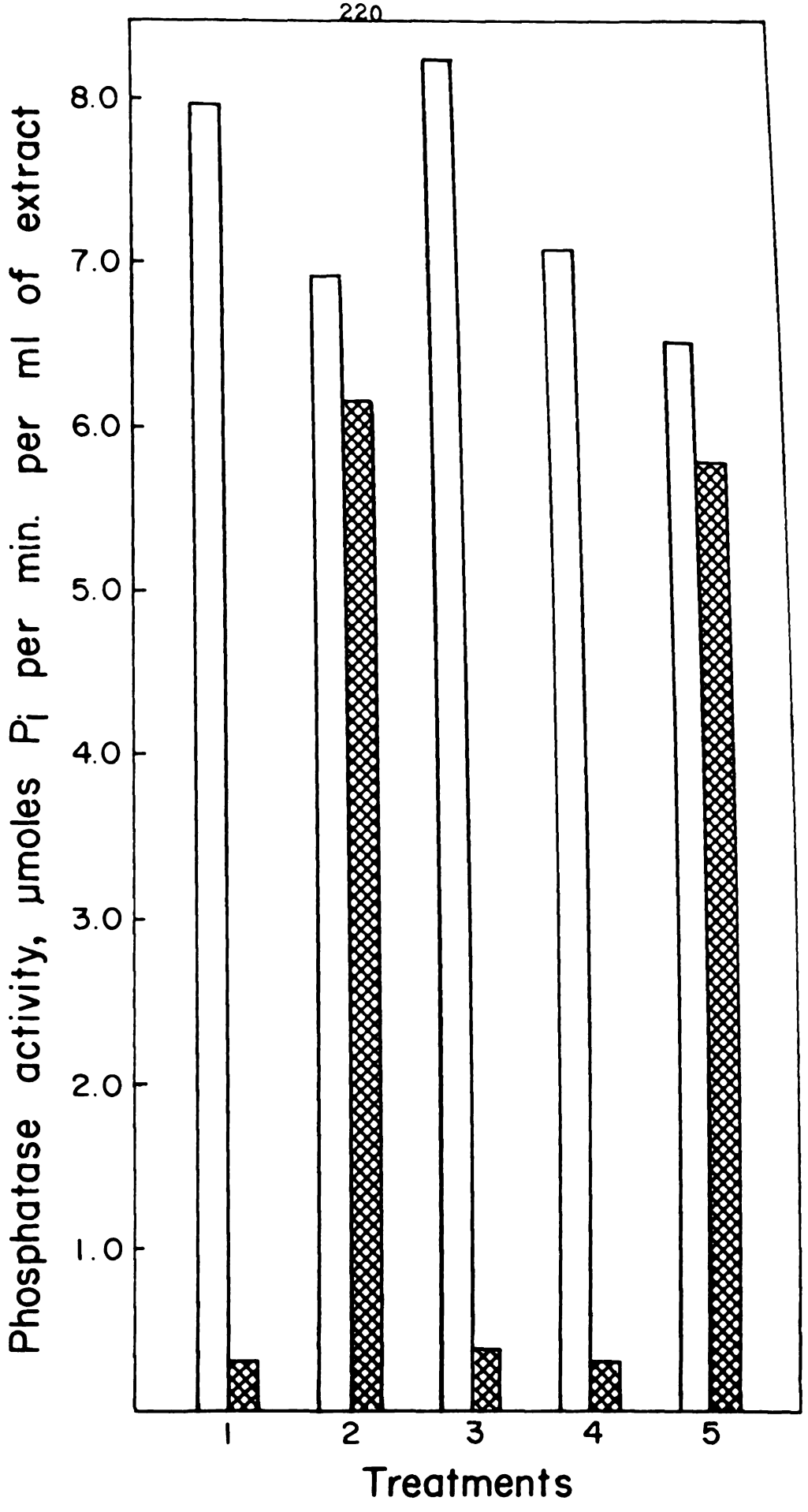

Phosphatase activity, µmoles P_i per min. per ml of extract
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1
2
3
4
5
Treatments

Table 13

A Diurnal in the Stabilization by Buzzing

Date of experiment	Time of harvest	Light conditions*		Stability toward dilution at 30° after buzzing
		Cloud cover	Foot candles	
		%		%
9-16	3:42 A.M.			60
9-5	8:35 A.M.	30	2600	66
9-1	10:45 A.M.	0	10600	82
8-30	11:45 A.M.	100	1700	75
9-7	1:02 P.M.	20	10800	92
8-22	2:31 P.M.	50	12000	67
8-31	5:31 P.M.	10	9200	91

*At the time of harvest.

stability value for the phosphatase in the extract from the leaves harvested at 2:31 P.M. does not fit the pattern.

The two diurnals were quantitatively different. The time dependent stabilization (under aerobic conditions) of the phosphatase in extracts from leaves harvested at night was essentially 0% of the time dependent stabilization of the phosphatase in extracts from leaves harvested during the day. The stabilization by buzzing of the phosphatase in extracts from night harvested leaves was about 2/3 of the maximum stabilization by buzzing of the phosphatase in extracts from day harvested leaves.

Effect of Sephadex G-25 Chromatography

Rapid passage of extracts from tobacco leaves through a Sephadex G-25 column stabilized the phosphatase toward dilution at 30° (Figure 23). The time of the day at which the leaves were harvested had no effect on the results, i.e. the phosphatase in extracts from leaves harvested during daylight hours was also completely stabilized by the same treatment. Furthermore, significant activation was noted when extracts from leaves harvested during the day or during the night were passed through the Sephadex G-25 column (Figure 23).[1]

[1]The actual activation probably was greater than indicated by Figure 23, since in none of the experiments was an effort made to be sure that the fraction from the column which was saved contained all of the phosphatase.

Figure 23

Effect of Sephadex G-25 Chromatography

A Sephadex G-25 (medium grade) column 1.0 cm x 8 cm high was prepared in a 0-4° room using gel which had been hydrated in 0.02M cacodylate, pH 6.3, for 4 hours, and was washed with 100 ml of the same buffer.

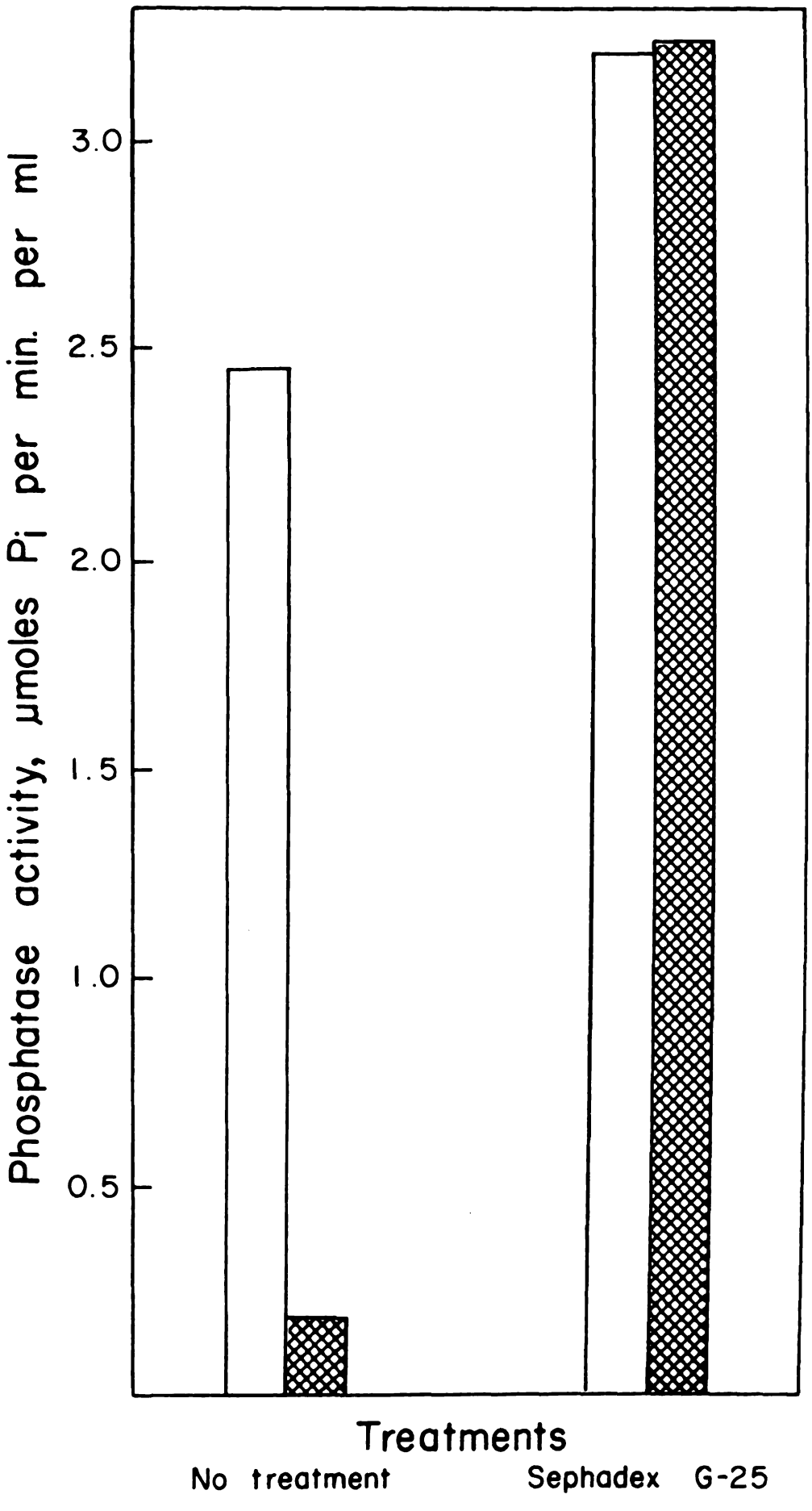
The leaves were harvested from the field at 2:00 A.M. on 8-17-1966. The leaf blades were homogenized in a mortar and pestle with sand with two weights of cold 0.02M cacodylate, pH 6.3. The pH of the extract, which had been centrifuged at 15,000 g for 8 minutes, was 6.3.

0.75 ml of the extract was added to the column and eluted with 0.02M cacodylate, pH 6.3. The elution was started at 2:45 A.M. and was completed in about 10 minutes. The phosphatase emerged with the leading color peak, and 1.5 ml of the peak was collected.

The "no treatment" preparation was made by diluting 0.75 ml of the extract to 1.5 ml with the buffer.

Open bars: controls.

Closed bars: activity after 10-fold dilution at 30°.


Phosphatase activity, µmoles Pi per min. per ml
3.0
2.5
2.0
1.5
1.0
0.5
Treatments
No treatment
Sephadex G-25

Stabilization by Acetone Precipitation

When the phosphatase in a fresh extract was precipitated by acetone, the rate of the stabilization of the phosphatase was increased (Figures 24 and 42). Even from an extract in which the phosphatase did not stabilize with time, the enzyme which had been precipitated by acetone became stable (Figure 24), although not as rapidly as did the acetone precipitated enzyme which came from an extract in which the phosphatase did stabilize with time (Figure 42).

Effect of Dilution

As discussed later, the stabilization of the phosphatase by Sephadex G-25 column chromatography or by precipitation by acetone could have been by decreasing the concentration of endogenous reductants and inhibitors of the oxidation of the enzyme, and by supplying O_2 dissolved in the buffer. For the same reasons, it was anticipated that dilution of extracts with buffer might have had a stabilizing effect on the phosphatase.

About half of the activity of the enzyme was destroyed by the first hour of dilution at 0°. The remaining activity was not stable toward dilution at 30°, for 90% of the remaining activity was destroyed by the next hour at 30° (Table 14). The data suggest that the dilution itself may prevent any possible stabilization to be

Figure 24

Stabilization of the Phosphatase by Acetone Precipitation

The leaves were harvested from the field at 5:20 P.M. on 7-14-66 (drizzling, 100% overcast, low visibility). The pH of the extract, which had been centrifuged at 15,000 g for 10 minutes, was 6.4. A 10.0 ml aliquot of the extract was fractionated by adding ten 1.0 ml aliquots of acetone sequentially. After each addition, the precipitate was removed by centrifugation, rinsed with cold water, and resuspended in 2.0 ml of cold 0.02M cacodylate, pH 6.3. The most active phosphatase fraction was precipitated by the 7th ml of acetone. For the rest of the experiment, the remainder of the extract and the most active phosphatase fraction were held as the extract had been held before the fractionation, near 0° in glass containers open to the air.

□——□ Controls.

○——○ Activity after 40-fold dilution at 30°.

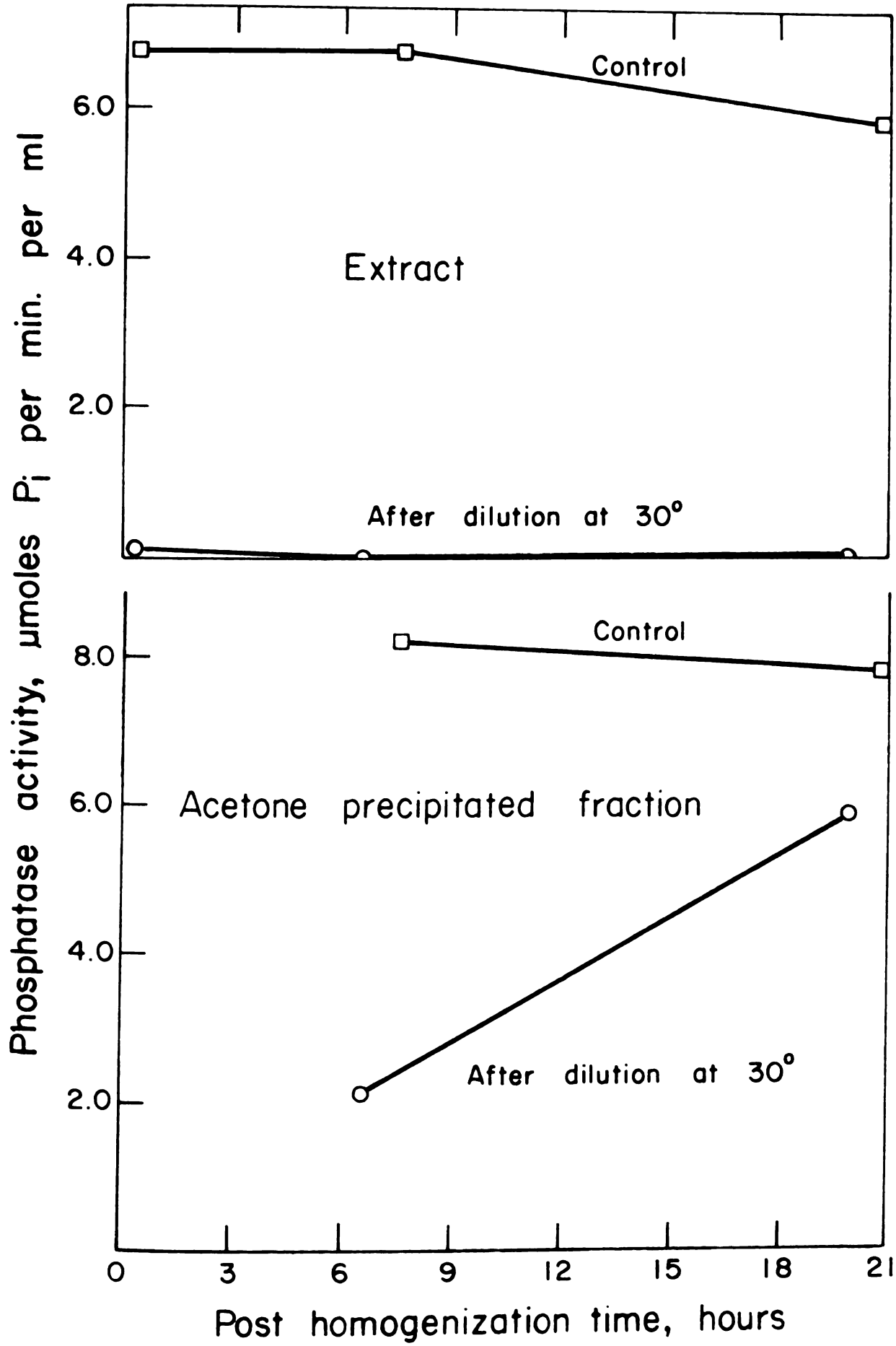

Control
Extract
After dilution at 30°
Control
Acetone precipitated fraction
After dilution at 30°
Phosphatase activity, μmoles P_i per min. per ml
6.0
4.0
2.0
8.0
6.0
4.0
2.0
0
3
6
9
12
15
18
21
Post homogenization time, hours

Table 14

Effect of Dilution on the Stability of the Phosphatase

Activity after dilution, % of controls			Dilution of the extract during the pretreatments
0°, 1 hr	30°, 1 hr	0°, 1 hr; then 30°, 1 hr	
%	%	%	-fold
54	6.1	6.5	50
	5.1	5.3	50
47	4.6	5.8	50
50	6.0	6.3	50
66	2.2	1.9	100

expected from decreasing the concentration of endogenous reductants and inhibitors of oxidation, and from supplying O_2 dissolved in the buffer. Apparently, something which was decreased in concentration by the dilution is needed to permit the stabilization of the phosphatase by O_2.

Effect of Metals

Calcium: In a single experiment, a fresh extract from greenhouse grown tobacco leaves was divided into two aliquots. To one aliquot was added an equal volume of 0.02M $CaCl_2$. The phosphatase became stable toward dilution at 30° much more rapidly in the extract containing $CaCl_2$ than in the extract containing no calcium. The preincubation with $CaCl_2$ also partially inhibited the phosphatase reaction.

Magnesium sulfate (and sodium cacodylate buffer): In the assay for activity after dilution at 30°, the $MgSO_4$ and Na cacodylate concentrations during the 1 hour of dilution were 3×10^{-3}M and 5×10^{-2}M respectively. It was found that at the usual temperature of 30°, the $MgSO_4$ and Na cacodylate did not protect the enzyme compared to a control containing only water and enzyme during the 1 hour of dilution. However, when the dilution was at 0° rather than 30°, only 25% of the activity survived when the dilution mixture included only water besides the enzyme, while 50% of the phosphatase activity

survived when the dilution mixture contained the standard amounts of $MgSO_4$ and cacodylate. It was also found that the $MgSO_4$ alone at 3×10^{-3}M and the buffer alone at 5×10^{-2}M were about equally effective in giving partial protection against inactivation by dilution at 0°, but neither was quite as effective as the two together. The two did not act synergistically in conferring this limited stabilization.

EDTA and orthophenanthroline: Preincubation of extracts with low concentrations of orthophenanthroline made the phosphatase more stable toward dilution at 30°, but the magnitude of the stabilization varied. EDTA was not as effective as orthophenanthroline and EDTA was less effective as the concentration was increased (Table 15).

Endogenous metal(s): The phosphatase in an extract from tobacco leaves had 43% as much activity when no metal was included in the reaction mixture as when the standard amount of $MgSO_4$ was included. When a control included enough EDTA to make the reaction concentration 0.1M, the phosphatase had zero activity, which is consistent with earlier findings (202). The initial stability toward dilution at 30° of the phosphatase in the extract was 6%. When the extract was held near 0° under air for 3 days, the activity with $MgSO_4$ in the reaction mixture had dropped to 82% of its original value. The surviving activity had a stability toward dilution at 30° of 98%,

Table 15

Effect of EDTA or Orthophenanthroline

		Experiment 1		Experiment 2	
EDTA*	Stability toward dilution at 30°	Orthophenanthroline*	Stability toward dilution at 30°	Orthophenanthroline*	Stability toward dilution at 30°
M	%	M	%	M	%
0	0	0	47	0	5
10^{-4}	11	10^{-4}	80	10^{-5}	16
10^{-3}	13	5×10^{-4}	66	10^{-4}	16
10^{-2}	4	2.5×10^{-3}	89	10^{-3}	21

*Concentrations during the 1 hour preincubations.

while the activity with no metal added to the reaction mixture was 47% of the 82% of the activity which survived. Thus, as the stability toward dilution at 30° of the phosphatase changed from 6% to 98%, there was no significant change in the phosphatase activity which depended on endogenous metal. These results suggest that the time dependent change in the stability toward dilution at 30° was not because of a time dependent binding or release of endogenous metal by the active site of the enzyme.

Effect of OHPMS and of Glycolate

A ten minute preincubation with glycolate made the phosphatase a little more stable toward dilution at 30°, but the preincubation with glycolate partially inhibited the stabilization of the enzyme by oxygen (Figure 25). Glycolate also inhibited the stabilization of the phosphatase by post homogenization time (Figure 26). The small decrease in phosphatase activity which always accompanied the stabilization by oxygen was also partially prevented by the ten minute preincubation with glycolate. A ten minute preincubation with OHPMS under aerobic or anaerobic conditions was even more effective in stabilizing the phosphatase than was the oxygen treatment which was used (Figure 25).

When an aliquot of fresh extract was made 10^{-2}M in OHPMS and then buzzed under air, the intense color change

Figure 25

Effect of OHPMS and of Glycolate

The leaves were harvested from the field at 10:50 A.M. on 9-23-67 (light intensity = 10,600 ft-c).

Preincubation mixture	Preincubation conditions		
	Addition	Atmosphere	Buzzing
1	H_2O	aerobic	not buzzed
2	H_2O	aerobic	buzzed
3	Glycolate	aerobic	not buzzed
4	Glycolate	aerobic	buzzed
5	OHPMS	aerobic	not buzzed
6	OHPMS	anaerobic	not buzzed

Each preincubation mixture contained 9 parts by volume of extract and 1 part by volume of additive. The stock glycolate and OHPMS solutions were both 0.10M, pH 7, so that the preincubation concentration for both was 10^{-2}M. The preincubations with glycolate and OHPMS were for 10.0 min. before the start of the dilution at 30°. The buzzing of the glycolate preincubation mixture was for 2 min. starting 2 min. after the addition of the glycolate. The anaerobic preincubation was in a Thunburg tube. After the system was gassed with N_2, the preincubation was started by dumping the OHPMS from the side arm into the extract. The Thunburg was opened just before the start of the dilution at 30°.

Open bars: controls.

Closed bars: activity after 20-fold dilution at 30°.

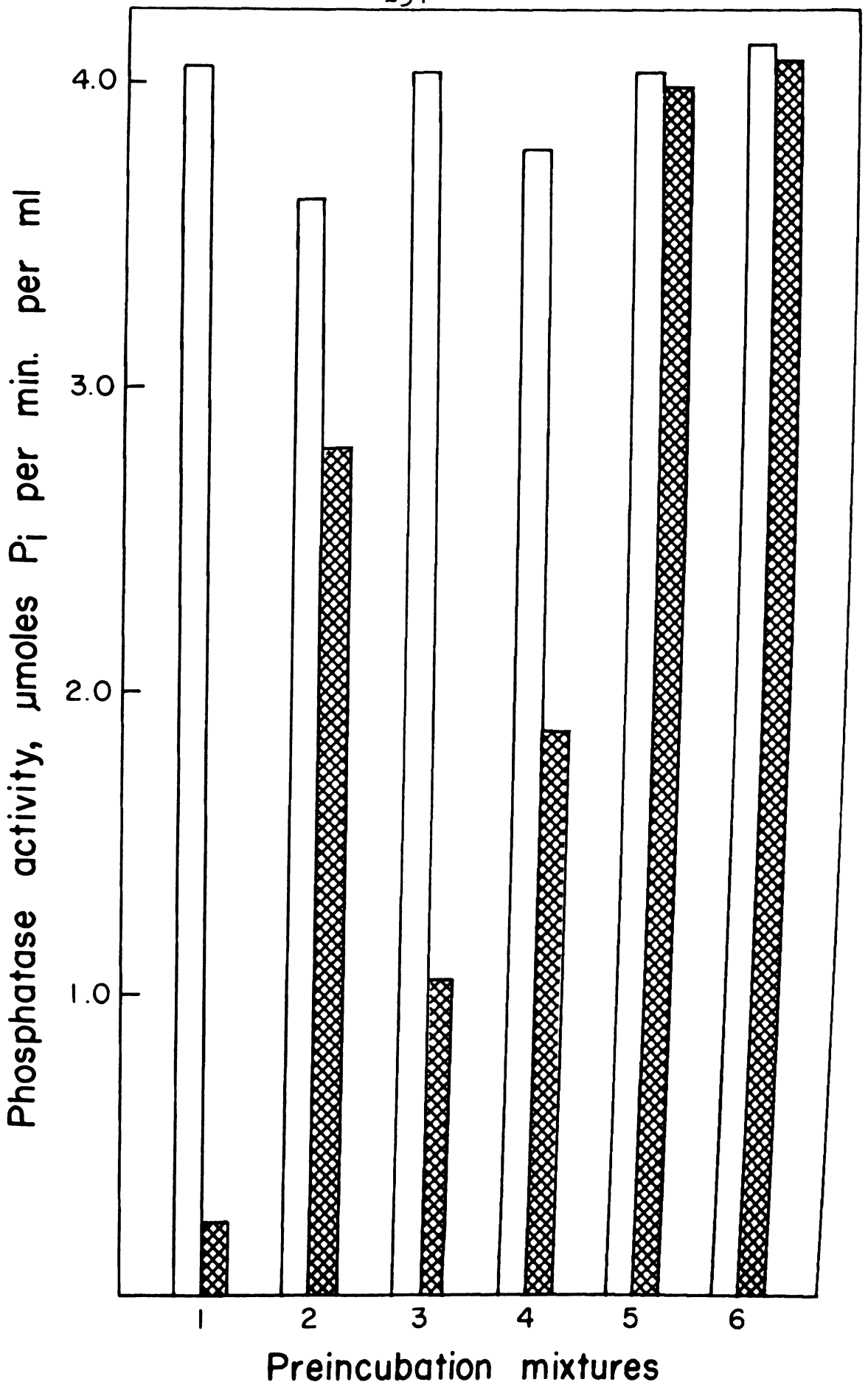

Phosphatase activity, μmoles P_i per min. per ml
4.0
3.0
2.0
1.0
1
2
3
4
5
6
Preincubation mixtures

Figure 26

Effect of Glycolate and Time After Homogenization

The four preincubation mixtures, each containing 3.60 ml of extract, plus either 0.40 ml of H_2O or 0.40 ml of 0.10M glycolate, pH 7.0, were held near 0° in 1.6 cm diam. x 15 cm high test tubes open to the air during the time of this experiment.

Top figure: Leaves harvested from the field at 5:20 P.M., 9-15-1967 (light intensity = 4000 ft-c). The two preincubation mixtures were made up 35 min. after homogenization.

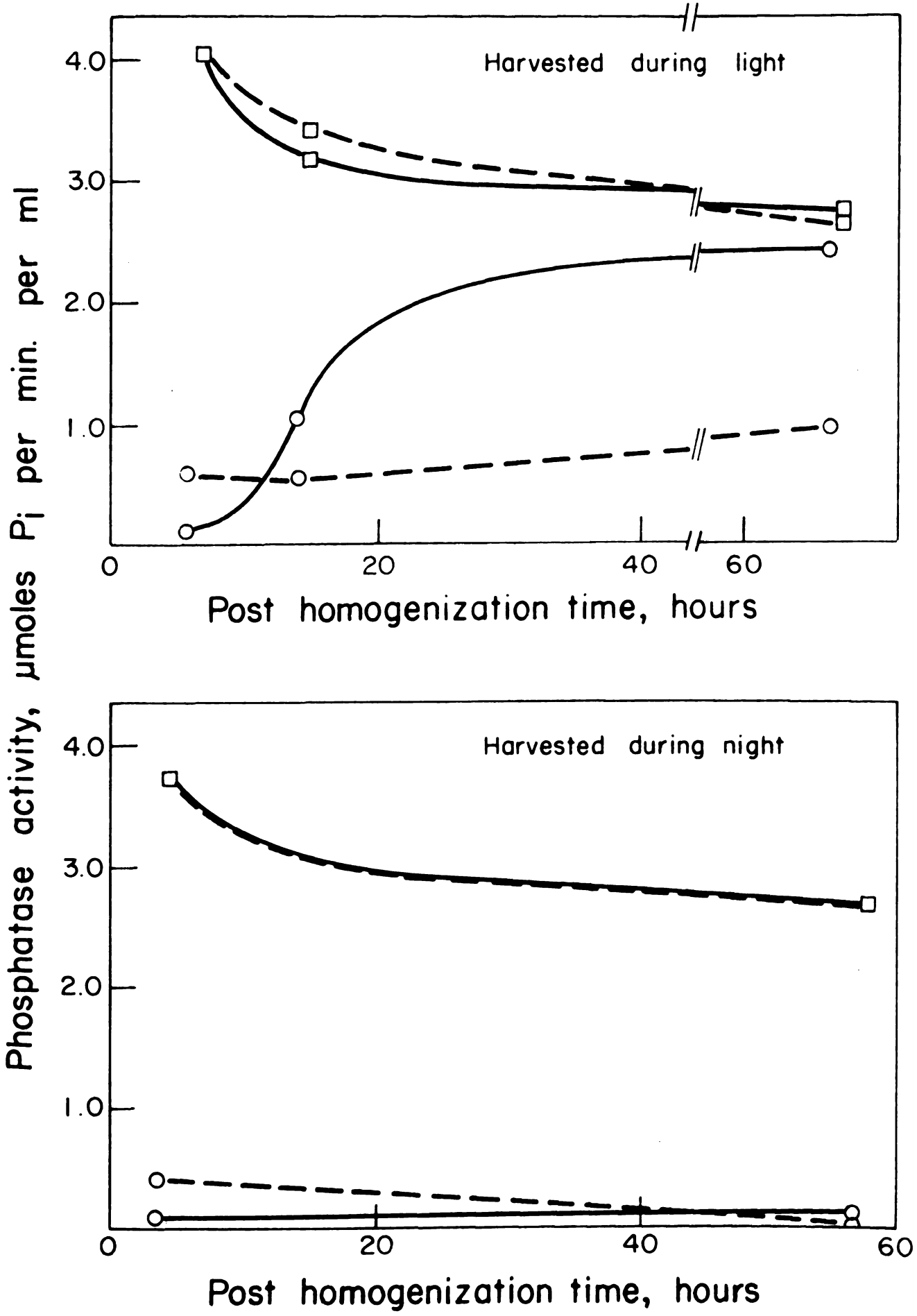
Bottom figure: Leaves harvested from the field at 3:40 A.M., 9-16-1967. These leaves were kept in complete darkness before and during the homogenization. The two preincubation mixtures were made up about 30 min. after homogenization.

□——□ Without glycolate; controls.

□---□ With glycolate; controls.

○——○ Without glycolate; activity after 20-fold dilution at 30°.

○---○ With glycolate; activity after 20-fold dilution at 30°.


Harvested during light
4.0
3.0
2.0
1.0
0
20
40
60
Post homogenization time, hours
Harvested during night
4.0
3.0
2.0
1.0
0
20
40
60
Post homogenization time, hours
Phosphatase activity, μmoles Pi per min. per ml

to dark orange brown, which always accompanied such buzzing, was completely prevented. OHPMS is an inhibitor of glycolate oxidase. Inhibition of the oxidase could have prevented the formation of H_2O_2 during the buzzing of the extracts. In this way, the oxidation of the colorless compounds to their brown form might have been prevented. Furthermore, under certain conditions, which seem to include the presence of light plus FMN or leaf extracts, OHPMS itself seems to be oxidized (personal communication from Dr. N. E. Tolbert). Any oxidation of OHPMS, rather than the colorless compounds in the extract, could also have decreased the oxidation to their brown form.

The prevention of the formation of H_2O_2 would not explain the stabilization of the phosphatase by OHPMS. Likewise, the possibility that OHPMS might serve as a reductant would not explain the stabilization of the enzyme. Both the prevention of the formation of H_2O_2 and the possibility that OHPMS might serve as a reductant would be expected to keep the phosphatase in the reduced, unstable form. An explanation for the stabilization of the phosphatase by OHPMS is proposed in the discussion which follows later.

Protection of the Phosphatase by Cis-aconitate

Cis-aconitate at 6×10^{-3}M gave partial stabilization against inactivation by dilution at 30° (Figure 27).

Figure 27

Protection of the Phosphatase by Cis-aconitate

The extract used for this experiment was the same as the one described in Figure 6.

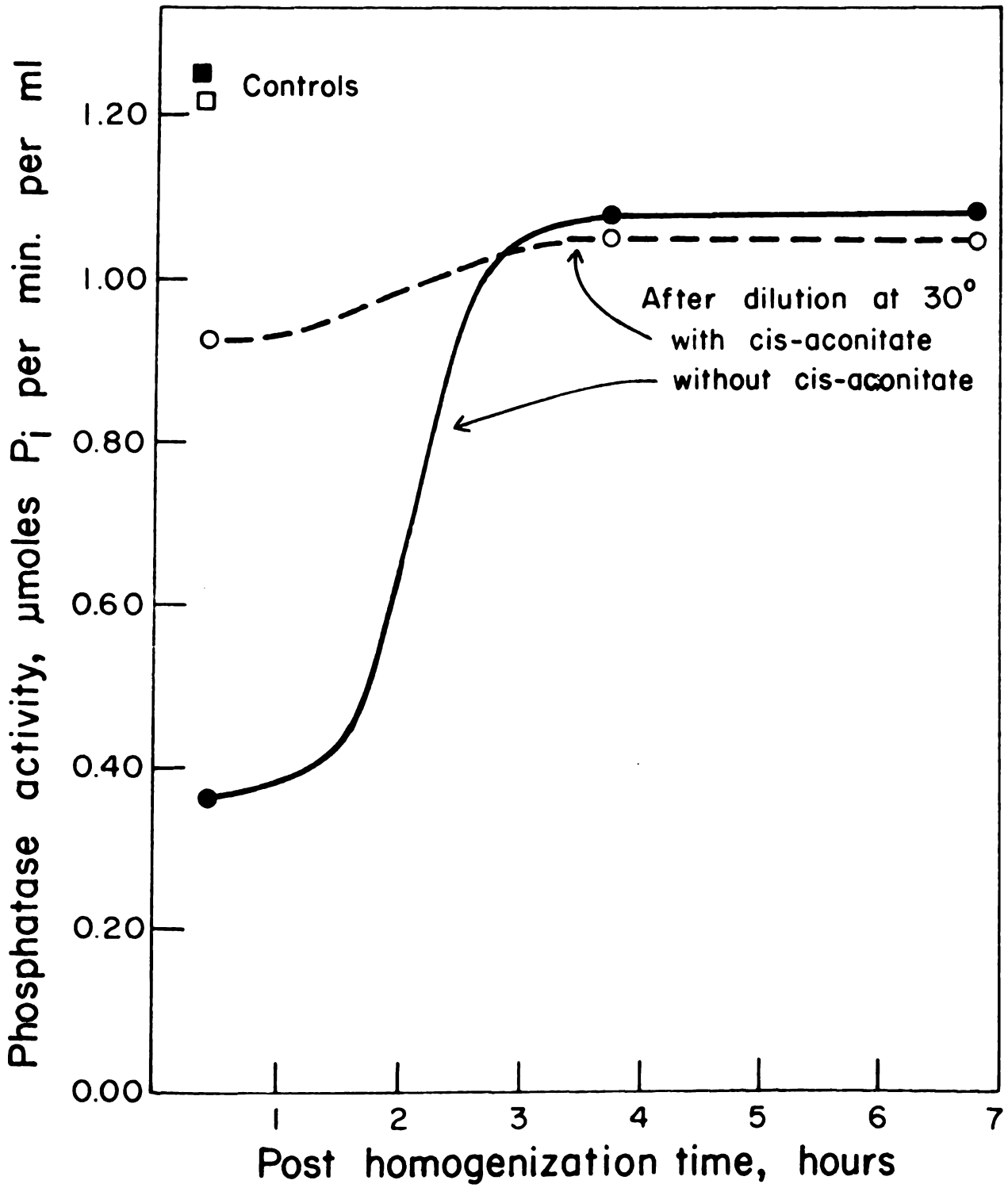
■ Control; no cis-aconitate.

□ Control; plus cis-aconitate.

●——● Activity after 20-fold dilution at 30°; no cis-aconitate.

O---O Activity after 20-fold dilution at 30°; plus cis-aconitate.

Cis-aconitate was at 6×10^{-3}M during the dilution at 30°, and at 4×10^{-3}M during the reactions.


Controls
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Phosphatase activity, μmoles P_i per min. per ml
After dilution at 30°
with cis-aconitate
without cis-aconitate
1
2
3
4
5
6
7
Post homogenization time, hours

The small percent inhibition by the cis-aconitate is consistent with the saturating level of substrate which was used (see footnote, p. 202).

Effect of Arsenite and Cd^{++}

Preincubation with either arsenite or Cd^{++}, even at low concentrations, slightly activated the phosphatase (Table 16). At high concentrations, Cd^{++} caused much precipitation to occur in the preincubation mixtures, suggesting that its inhibition at higher concentrations was nonspecific.

Preincubation with arsenite or Cd^{++} at the concentrations shown in Table 16 did not stabilize the phosphatase toward dilution at 30°. Neither 10^{-3}M arsenite nor 10^{-3}M arsenite + 10^{-3}M BAL had any significant effect (inhibitory of stimulatory) on the stabilization by oxygen of the phosphatase in the preincubation mixtures. The arsenite-BAL combination in the preincubation mixtures also did not stabilize the phosphatase toward dilution at 30°. Preincubation with the arsenite-BAL combination did not give as much activation as preincubation with arsenite alone (data not shown).

P_1, at a concentration of 5×10^{-4}M during the reaction, resulted in a slight (1.5%) inhibition of the phosphatase reaction.

Table 16

Activation of the Phosphatase by Arsenite and Cd^{++}

The leaves were harvested in the afternoon in August, 1967. Preincubation mixtures were 9 parts by volume of fresh extract and one part by volume of additive. After about 1 hour of preincubation at 0°, standard P-glycolate phosphatase assays were run on the preincubation mixtures.

Preincubation		Concentration of additive during assay	Activity				
Additive	Concentration		Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
	M	M	%	%	%	%	%
Water	-	-	100	100	100	100	100
Arsenite	10^{-5}	2×10^{-7}	113	-	-	-	-
	10^{-4}	2×10^{-6}	118	-	103	105	103
	10^{-3}	2×10^{-5}	122	111	-	-	-
	10^{-2}	2×10^{-4}	129	-	106	109	105
$CdCl_2$	10^{-5}	2×10^{-7}	118	-	-	108	-
	10^{-4}	2×10^{-6}	106	-	-	-	-
	10-3	2 x 10-5	57	-	-	-	-
	10^{-2}	2×10^{-4}	15	-	-	-	-

Reversal of the Stabilized Phosphatase to an Unstable Enzyme

Ten minutes of preincubation of buzzed extracts with glycolate partially reversed the oxygen stabilized phosphatase to a less stable but just as active enzyme. A combination of glycolate, and of fresh extract (not buzzed) which had been heated to 100° for 15 min., gave more reversal than either additive alone. No reversal was noted when OHPMS was included with the glycolate and the heated extract during the preincubations. More reversal was obtained under anaerobic than under aerobic preincubation conditions (Figure 28, Table 17). Glycolate was also effective in decreasing the stability toward dilution at 30° of the phosphatase which had been stabilized by post homogenization time under aerobic conditions.

In preliminary experiments, the following compounds, when preincubated at the indicated concentrations with buzzed extracts, were completely ineffective in decreasing the stability toward dilution at 30° of the oxygen stabilized phosphatase: NADPH, NADH, or Fe^{++} at 10^{-3}M, citrate, isocitrate, or cis-aconitate at 10^{-2}M, or a combination of cysteine at 10^{-2}M and Fe^{++} at 10^{-4}M. The effect of preincubation with ascorbate on the activity and stability of P-glycolate phosphatase was tested using an enzyme with the following history: it had originated

Figure 28

Reversal of the Stabilized Phosphatase to an Unstable Enzyme

The leaves were harvested from the field at 11:45 A.M. on 9-14-1967 (light intensity = 11,000 ft-c). The pH of the extract was 5.6. Preincubation mixture 1 contained 0.175 ml of extract (not buzzed) plus 0.075 ml of H_2O. The other six preincubation mixtures contained the following:

Mixture	Extract, buzzed	H_2O	Extract, boiled and clarified	OHPMS	Glycolate
	ml	ml	ml	ml	ml
2	0.175	0.075	-	-	-
3	0.175	0.050	0.025	-	-
4	0.175	0.050	-	-	0.025
5	0.175	0.025	0.025	-	0.025
6	2.800	0.400	0.400	-	0.400
7	0.175	-	0.025	0.025	0.025

Extract, buzzed: 6.0 ml of extract buzzed under air. Extract, boiled and clarified: 3 ml of the extract (not buzzed) was placed in a boiling water bath, with a marble over the test tube, for 15 minutes. The clear supernatant was decanted and held at 0° in a stoppered test tube until needed. OHPMS and glycolate were both 0.10M, pH 7.0. The preincubation concentration for both was 10^{-2}M. The components of the preincubation mixtures were added in the order indicated in the above table, from left to right.

Preincubation no. 6 was made anaerobically. The other six preincubations were aerobic. The aerobic preincubations, at 0°, were in 5 ml test tubes open to the air. The anaerobic preincubation, at 0°, was in a double side arm Warburg flask. The main part of the flask contained the buzzed extract and H_2O. One side arm contained the boiled and clarified extract and the other contained the glycolate. This system was gassed with N_2. The boiled extract was tipped into the buzzed extract just before the glycolate was added. The Warburg flask was opened just before the start of the dilution at 30°.

The addition of glycolate started the 10 minute preincubations, and the start of the dilution at 30° ended them.

During the 1 hour of dilution at 30° and during the 10 minute phosphatase reactions, the assay tubes were open to the air.

Open bars: controls.

Closed bars: activity after 20-fold dilution at 30°.

The stabilities toward dilution at 30° of the phosphatase in preincubation mixtures 1 and 2 were 0.8% and 69.5% respectively. For any of the other preincubation mixtures:

$$\text{Reversal of stability toward dilution at } 30^\circ = \frac{69.5\% - \text{stability of the phosphatase}}{69.5\% - 0.8\%} \times 100\%.$$

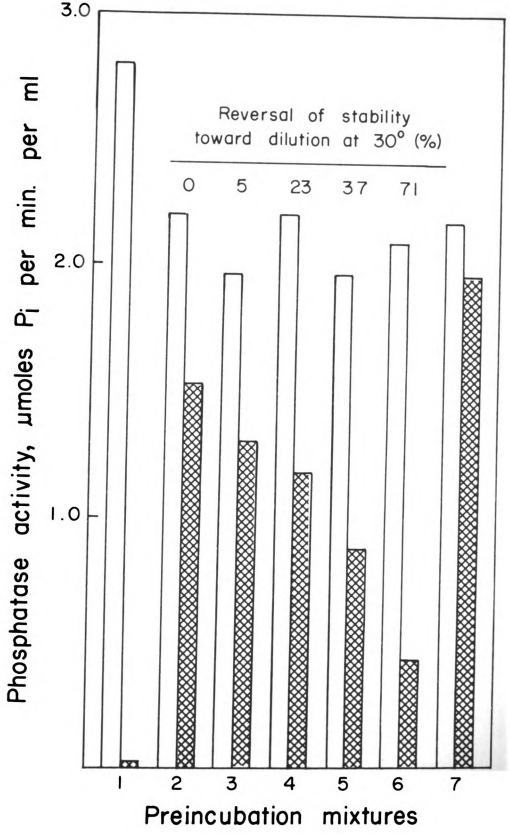

3.0
2.0
1.0
Phosphatase activity, μmoles P_i per min. per ml
Reversal of stability toward dilution at 30° (%)
0
5
23
37
71
1
2
3
4
5
6
7
Preincubation mixtures

Table 17

Correlation Between the Extent of the Reversal of the Stabilized Phosphatase to the Unstable Enzyme, and the Extent of the Stabilization

The nine experiments were conducted from 8-22-1967 to 9-22-1967. Ext. = extract (not buzzed). N_2 - ext. = extract (not buzzed) gassed with N_2. Boiled ext. = the clear supernatant from the extract (not buzzed) after a boiling H_2O bath treatment for 15 min. In experiments 3, 5, and 7, the buzzed extract was 70% by volume of the preincubation mixtures, while in the other experiments, the buzzed extract was 80% by volume of the preincubation mixtures. The anaerobic preincubation mixtures contained glycolate and boiled extract in addition to the buzzed extract. In all of the preincubation mixtures, glycolate, boiled extract, extract, and N_2 - ext. were each 10% by volume of a preincubation mixture. Water was added, where required, to complete the volume of the preincubation mixtures. The glycolate was 0.10M, pH 7.0, so that the preincubation concentration was 10^{-2}M. Preincubations were started with the addition of glycolate and were for 10 or 15 minutes at 0°. Aerobic preincubations were in 5 ml test tubes open to the air. Anaerobic preincubations were as described in Figure 28.

$$\text{Reversal of stability toward dilution at } 30^\circ = \frac{\text{Stability of buzzed extract before reversal} - \text{Stability of buzzed extract after reversal}}{\text{Stability of buzzed extract before reversal} - \text{Stability of extract (not buzzed)}} \times 100\%.$$

In the case of preincubation mixtures which included N_2 - ext. or ext., the stability toward dilution at 30° before reversal was calculated as the weighted average of the stability of the phosphatase in the buzzed extract and the stability of the phosphatase in the ext. or N_2 - ext.

Experiment	Conditions at harvest: Time	Temp.	Stability in the extract*	Stability in the buzzed extract*	Reversal of stability toward dilution at 30°: Aerobic preincubations. Buzzed extract plus: Glycolate	Boiled ext.**	Ext.**	N_2 ext.**	Glycolate plus: Boiled ext.	Ext.	N_2 ext.	Anaerobic preincubations
			%	%	%	%	%	%	%	%	%	%
1	8:35 A	15°	0.0	66							65	
2	2:31 P	23		67	23			1				
3	11:45 A	26	0.8	70	23	5			37			71
4	11:45 A	20	0.0	75				15			57	
5	4:21 P	24	4.4	78	30				40			64
6	10:45 A	21		82	11			-13			37	
7	12:00 P	11	21.2	88	8	-4	-2		15	19		42
8***	5:31 P	19	0.0	91	15			-17			22	
9	1:02 P	28		92						55		

*Toward dilution at 30°.

**The four negative values represent additional stabilization rather than reversal to a less stable phosphatase.

***At the start of experiment 8, the pH of the extract was adjusted to 7.6 with 0.1N KOH. In the other 8 experiments, the pH values of the extracts were left unadjusted. The pH values of such extracts normally varied from about pH 5.5 to about pH 6.

as an extract prepared by Waring blendor, had been fractionated once with acetone, and had been converted to an acetone powder. Eighty minutes of preincubation at 0° with ascorbate at 10^{-2}M had a variable effect on the activity of the phosphatase. In general, however, the activity was greatly decreased. A similar preincubation for 20 min. also had a variable effect on the stability of the phosphatase. In general, however, the stability toward dilution at 30° was also much decreased. Thus, ascorbate may reverse the oxygen stabilized phosphatase to a less stable enzyme, but not without greatly decreasing the activity.

In a single experiment, glyoxylate, at a preincubation concentration of 10^{-2}M, partially reversed the oxygen stabilized phosphatase to a less stable but just as active enzyme, but it was only about half as effective as glycolate at the same preincubation concentration. In another single experiment, when FMN at 10^{-3}M was included with glycolate at 10^{-2}M during the preincubation, the reversal of the stability toward dilution at 30° was not as great as with glycolate alone. In preliminary experiments, when a combination of cysteine at 10^{-2}M and Fe^{++} at 10^{-3}M or 10^{-4}M was included with glycolate at 10^{-2}M during the preincubation, there was no reversal of the stability of the phosphatase.

In other preliminary experiments Fe^{++}, Cu^{++}, or Cu^{+},

plus glycolate, in the preincubation mixtures gave more reversal of the stability of the phosphatase than did glycolate alone, but the magnitude of the enhancement by the metals varied significantly from one experiment to another. Zn^{++} was less effective while Mn^{++} was without significant effect when combined with glycolate.

Although greater reversal of the stability of the phosphatase by glycolate was possible under anaerobic preincubation conditions than under aerobic preincubation conditions, anaerobic conditions for the one hour of dilution at 30° were not more effective than aerobic dilution conditions in further reversing the stability of the enzyme. Using prepurified grade N_2 (purity = 99.997%), or washing the N_2 by passing it through Fieser's solution followed by a $PbAc_2$ solution (82), did not seem to improve the results which were obtained using unwashed high purity grade N_2 (purity = 99.9%).

There seemed to be a negative correlation between the amount of stabilization by oxygen and the amount of reversal of the stability (Table 17). Of the seven reversal columns of Table 17 which have more than one value posted, the amount of reversal decreased as the amount of stabilization by oxygen increased in 6 of the columns while the opposite was true in only one of the columns. The data suggest that the greater the stabilization by oxygen, the less was the reversal to the unstable form of the phosphatase.

Table 17 has other relationships of possible significance. In all five combinations where synergism was tested, the reversal by glycolate plus nonbuzzed extract was significantly greater than the sum of the reversals which each alone contributed. In particular, in the cases where the nonbuzzed extracts alone gave added stability rather than reversal, the reversal by glycolate plus a nonbuzzed extract was still significantly greater than the reversal by glycolate alone. Secondly, three of the four smallest amounts of stabilization by oxygen were from leaves harvested in the morning while three of the four largest amounts of stabilization by oxygen were from leaves harvested in the afternoon. Third, the amount of reversal at pH 7.6 was not greater than at pH 5.5 to 6. Fourth, the greatest initial stability of 21.2% in an extract (not buzzed) was from leaves harvested at the lowest temperature (11°). This is additional evidence that less vigorous growth conditions result in greater initial stability of the phosphatase. Fifth, the 19% reversal value of experiment 7 may be abnormally small and the 55% reversal value of experiment 9 may be abnormally large because of the low harvest temperature of experiment 7 and the high harvest temperature of experiment 9. The temperatures at and before harvest may thus partly explain the single discrepancy in the negative correlation between the amount of stabilization by oxygen and

the amount of reversal of stability. It may also be that more vigorous growth conditions, or at least higher temperatures at and before harvest time, produce a system in which the phosphatase is more easily reversed from an oxygen stabilized enzyme to the unstable enzyme.

Data allowing meaningful comparison between the reversal by extract alone, N_2 gassed extract alone, and boiled extract alone, are scarce. The N_2 gassed extract seemed to give about the same reversal as the extract which was not gassed. The boiled extract in one experiment was more effective than either of the other two, and in another experiment it was less effective. The most consistent observation is that extract (not buzzed), N_2 gassed extract, or boiled extract (not buzzed), each acted synergistically with glycolate in reversing the oxygen stabilized phosphatase to the unstable form.

With glycolate, the shortest preincubation time tested, 15 seconds, was as effective as longer preincubation times for reversing the oxygen stabilized phosphatase to the unstable form. When held for long periods of time under air, the phosphatase, which had been made unstable with glycolate, slowly restabilized (Figure 29).

Discussion of the Stability Toward Dilution at 30° of P-glycolate Phosphatase in Fresh Extracts From Tobacco Leaves

Except for post homogenization time itself, all of the stabilization procedures which were used, such as

Figure 29

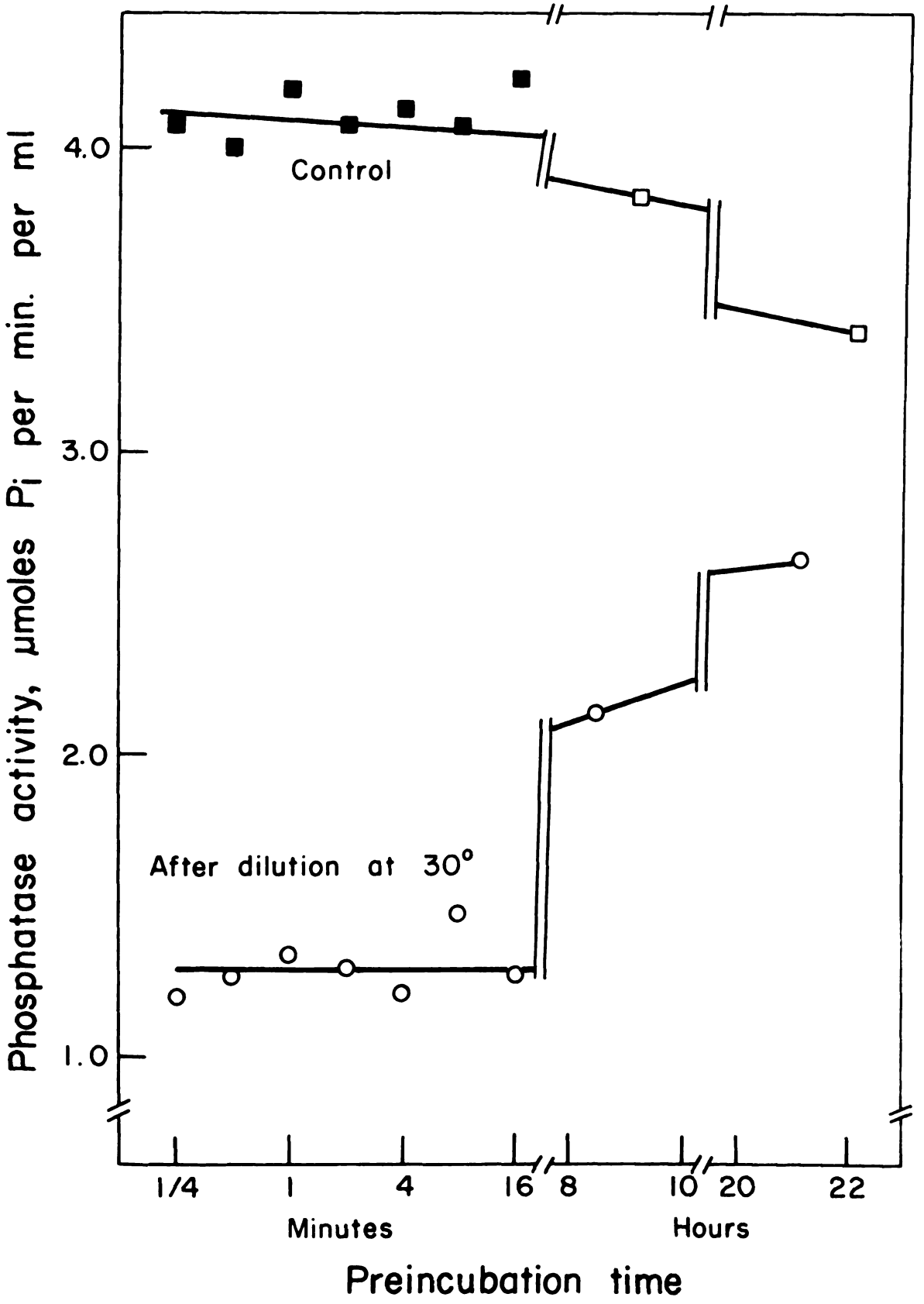
Reversal of the Stabilized Phosphatase to the Unstable Enzyme as a Function of the Preincubation Time with Glycolate, and the Time Dependent Restabilization of the Phosphatase

The leaves were harvested from the field at 1:00 P.M., 9-7-1967 (light intensity = 10,800 ft-c). 4.0 ml of the extract was buzzed under air. The stability toward dilution at 30° of the phosphatase in the buzzed extract was 92%, while in the extract (not buzzed), it was 2%. Two preincubation mixtures, each containing 0.80 ml of the buzzed extract, 0.10 ml of the extract (not buzzed), and 0.10 ml of 0.10M glycolate, pH 7.0, were made up and held at 0° in 5 ml test tubes open to the air.[1]

■——■ and □——□ Control; the preincubation times were from the time of the addition of glycolate to the times at the start of the reactions.

○——○ Activity after 20-fold dilution at 30°; the preincubation times were from the time of the addition of glycolate to the times at the start of the dilutions for 1 hour at 30°.

[1]Assays for □ and ○ were run using the first preincubation mixture. Assays for ■ were run using the second preincubation mixture. The second preincubation was started 18 minutes after the first.


Phosphatase activity, μmoles Pi per min. per ml
4.0
3.0
2.0
1.0
Control
After dilution at 30°
1/4
1
4
16
8
10
20
22
Minutes
Hours
Preincubation time

buzzing under O_2, and the reversal of the stabilized phosphatase to the unstable enzyme, were performed during the lag period within which the post homogenization time had no effect on the stability of the phosphatase. Thus, no part of the changes in the stability which were observed was attributable to the post homogenization time required for these stabilization and reversal experiments.

Stabilization of the Phosphatase by O_2

The stabilization of the enzyme by "buzzing" was dependent on oxygen (Figure 22), which suggests that the stabilization of the phosphatase by Waring blendor extraction, post homogenization time, Sephadex G-25 column chromatography, and precipitation by acetone may have also been dependent on oxygen. The Waring blendor treatment, which was under aerobic conditions (Figure 21), mechanically resembled the "buzzing" treatment. Post homogenization time did not stabilize the phosphatase unless the extracts were held under aerobic conditions. Sephadex G-25 column chromatography was more effective in stabilizing the phosphatase (Figure 23) than was precipitation by acetone (Figures 24 and 42). Stabilization by the latter two methods could have been partly because of the removal of endogenous reductants and the resupplying of O_2, which was dissolved in the buffer which was used. Precipitation by acetone might be about as effective as one theoretical

plate in the Sephadex G-25 column.

The stabilization of a phosphatase by O_2 seems unique. It is therefore possible that the stabilized activity was a function only of P-glycolate phosphatase. In some extracts, the stabilized activity was almost equal to the total initial P-glycolate phosphatase activity.

Oxidized or Reduced P-glycolate Phosphatase

The following evidence suggests that the phosphatase stable toward dilution at 30° is oxidized while the phosphatase unstable toward dilution at 30° is reduced:

1. Oxygen stabilized the phosphatase.

2. Fresh ascorbate caused the stabilized phosphatase to be unstable.

3. Glycolate also caused the stabilized phosphatase to be unstable. OHPMS prevented the glycolate from being effective (Figure 28) which suggests that glycolate may have been acting as a reductant by donating electrons through a glycolate oxidase-type of enzyme.

4. Glycolate was more effective in converting the stabilized phosphatase to the unstable enzyme under anaerobic conditions than under aerobic conditions (Figure 28).

Glyceraldehyde-3-phosphate dehydrogenase is another enzyme which exists in both an oxidized and a reduced state, and which, under certain conditions at least, exhibits phosphatase activity. Highly purified

and crystalized glyceraldehyde-3-phosphate dehydrogenase hydrolyzes acetyl phosphate to acetate and P_i. While the dehydrogenase reaction proceeds readily under conditions which maintain free sulfhydryl groups, maximum hydrolytic activity occurs under conditions which oxidize or block sulfhydryl groups. It seems as if the development of hydrolytic activity were due to a change in the nature of the active site on the enzyme. The loss of at least one sulfhydryl group is involved, as shown by activation of the hydrolytic acitivity with iodoacetic acid or preparation of the enzyme in the absence of -SH activators. This change seems to involve a modification of the normal site rather than the creation of new sites (186).

Specific and Reversible Mechanism

The following evidence suggests that the reduction of P-glycolate phosphatase to an enzyme unstable toward dilution at 30°, and the oxidation of the phosphatase to an enzyme stable toward the same conditions, are by a specific reversible mechanism, mediated by a specific enzyme. The latter enzyme seems to be either one of the two presently known forms of glycolate oxidase, both of which are inhibited by OHPMS (12), or an enzyme with properties similar to glycolate oxidase.

1. In algae, OHPMS is not specific for inhibition of glycolate oxidase (235). In the higher plants such as

tobacco, however, the hydroxymethanesulfonates do have a high specificity for glycolate oxidase inhibition (117, 267, 269). The initially unstable tobacco phosphatase was stabilized by OHPMS while the phosphatase activity was not affected (Figure 25).

2. Glycolate inhibited the stabilization of the phosphatase by oxygen (Figures 25 and 26).

3. To the extent tested, glycolate seemed specific for converting the stable phosphatase to the unstable but just as active enzyme. Other compounds tested were ineffective except for ascorbate, and glyoxylate, which is also a substrate of glycolate oxidase (201), or which could have been converted in the extracts to glycolate. Ascorbate seemed to act nonspecifically because it greatly reduced the activity of the phosphatase, even though the concentration of ascorbate, which was 10^{-2}M during preincubation, was reduced to 10^{-4}M during the assays.

4. The phosphatase was rapidly converted to the unstable form by glycolate (Figure 29).

5. OHPMS completely inhibited the glycolate dependent conversion of the stable phosphatase to the unstable enzyme (Figure 28).

6. The phosphatase which had been converted to the unstable form by glycolate slowly restabilized with time under aerobic conditions (Figure 29).[1]

---

[1]The restabilization rate of the phosphatase shown in Figure 29 was not as great as the stabilization rate

Endogenous Tricarboxylic Acids

The evidence suggests that the stabilization of the phosphatase in the extracts by post homogenization time was not due to a time dependent binding of endogenous tricarboxylic acids. In particular, Sephadex G-25 column chromatography should have at least partially prevented the stabilization of the phosphatase by such a mechanism. Instead, Sephadex G-25 column chromatography stabilized the enzyme. In a previous section of this thesis, evidence for the stabilization of the phosphatase by endogenous tricarboxylic acids is presented. But that stability is with respect to the 45° test in which the enzyme is not diluted. The initial stability toward dilution at 30° of the phosphatase in fresh extracts was often at or near 0%. Thus, the dilution must have reduced the concentration of the endogenous tricarboxylic acids to levels that were ineffective in stabilizing the reduced enzyme toward dilution at 30°. It is concluded that the mechanism by which the phosphatase was stabilized toward dilution at 30° is independent of the mechanism by which the phosphatase was stabilized during the 45° test by the tricarboxylic acids.

---

shown in Figure 19. In a second experiment conducted as described for Figure 29, the restabilization rate was not even as great as the rate shown in Figure 29. The slower restabilization rate compared to the original stabilization rate may have been because of the presence of glycolate (Figure 26), rather than to a difference in the stabilization mechanism.

But the stability of the enzyme at 45° may have been partly a function of the stabilization by oxygen. Thus, the enzyme in fresh extracts was only 90% stable at 30°, without dilution, for one hour (Table 2), while the oxidized partially purified enzyme was 100% stable at 45°, without dilution, for one hour (Table 3).

Advantages of the Assay

The observations on the effect of dilution (p. 225) suggest that any stabilization (oxidation) reaction which was going on in fresh extracts was stopped at the instant of dilution. For the same reason, and also because glycolate was required for the reversal of stability, any reversal (reduction) reaction occuring in the preincubation mixtures was probably also stopped at the instant of dilution. Thus, it seems that the reactions which were studied took place in the fresh extracts or preincubation mixtures up to the instant of dilution.

The conditions for measuring the stability toward dilution at 30° were convenient because they utilized the standard phosphatase assay reaction mixture and temperature. They also were optimum because the stability varied from 0% to 100%.

In oxidized extracts, 100% stability was often attained, which suggests that the activity of the stabilized enzyme molecules was not decreased by the 1 hour of

dilution at 30°. Therefore, the activity after dilution at 30° may have provided an undiminished measure of the state of the phosphatase at the instant of dilution. In fresh extracts, 0% stability was often obtained, suggesting that reduced enzyme molecules were completely inactivated by the 1 hour of dilution at 30°.

Limitations of the Assay

Both the time required, and the precipitation procedures used during purification, stabilized the phosphatase. Therefore, it was advisable to study the stabilization of the enzyme toward dilution at 30° using fresh extracts, rather than purified preparations. However, the increased stabilization rate of the phosphatase after it had been sharply precipitated by a small increase in acetone concentration, from 38% to 41% (Figure 24), suggests that the stabilization system survived at least the first acetone purification step. Continued purification of the phosphatase would make the dilution at 30° test difficult to use without at least some modification, because with continued purification past the first acetone fractionation, the phosphatase became less stable toward dilution at 30° (Table 3), even though all purification procedures were conducted under aerobic conditions. It was previously pointed out that the stability of the phosphatase toward dilution at 30° is not a function of the endogenous

tricarboxylic acids. Something else, specific or nonspecific, seemed to be lost from the phosphatase preparations during the purification. This loss could have been because less enzyme was used during the 1 hour of dilution at 30° as the enzyme was purified (Table 3), or something could have been lost from the enzyme during purification which was necessary to help stabilize the oxidized phosphatase toward dilution at 30°.

The Site of the Stabilization

With respect to the stability toward dilution at 30°, the term "phosphatase" may include more than the phosphatase per se. It is possible that the site of the stabilization was another molecule or enzyme which was bound to the phosphatase, and that stability was the result of a change in this other molecule or enzyme. However, the pronounced change in the stability toward dilution at 30° from 0% to 100% which occured for the enzyme in some extracts suggests that the site responsible for the stabilization is on the phosphatase itself, and that it may well be at or near the active site of P-glycolate phosphatase.

It is also possible that the stabilization mechanism involved the binding of the phosphatase to another molecule(s) in such a way that dilution did not separate the phosphatase from the molecule(s). However, the evidence that the stabilization of the phosphatase by O_2 may

have required at least one other molecule (p. 225) does not rule out the possibility that the stabilizing change per se, which converted the phosphatase to an enzyme stable to dilution at 30°, was an intramolecular event.

Protection of the Reduced Phosphatase

The evidence suggests that stabilization by oxidation did not continue after the start of the one hour of dilution at 30°. Thus, it is necessary to distinguish between treatments which stabilized the phosphatase by oxidation of the enzyme in the extracts, and treatments which protected the reduced phosphatase during the 1 hour of dilution at 30°. The stabilization by 6×10^{-3}M cis-aconitate during the 1 hour of dilution at 30° (Figure 27) can best be explained by a protection of the reduced phosphatase. Cis-aconitate might best compensate for the unstable reduced configuration of the enzyme if the site of oxidation and reduction were at or near the cis-aconitate binding site. Because cis-aconitate is a competitive inhibitor of the phosphatase (Figure 18), and because the tricarboxylic acids are analogous in structure to P-glycolate (Figure 47), the stabilization of the reduced phosphatase by cis-aconitate suggests that the site of oxidation and reduction may be at or near the active site of the phosphatase.

Most of the inactivation of the phosphatase during

the one hour of dilution at 30° occured in the first 10 minutes (Figure 6). Yet, the control activities for the phosphatase, which took 10 minutes to measure, were greater before the phosphatase was stabilized by post homogenization time than after (Figure 19). This was true even though the dilution of the phosphatase during the control assays was 1.5 times greater than the dilution of the phosphatase during the 1 hour of dilution at 30°. These observations suggest that the substrate may also protect the fully reduced phosphatase against inactivation by dilution at 30°. This suggests that the site of oxidation and reduction may be at or near the active site of the phosphatase.

The partial protection of the reduced phosphatase by $MgSO_4$ during the 1 hour of dilution at 0° is consistent with the enhanced stabilization by citrate or isocitrate with $MgSO_4$ (Table 11). Citrate and isocitrate were present in greater quantities than cis-aconitate (Figures 13 and 14). A reaction of the type

$$\text{enzyme} + \text{tricarboxylic acid} + Mg^{++} \rightleftharpoons$$
$$\text{enzyme} - \text{tricarboxylic acid} - Mg$$

would be forced to the right by increasing the concentration of $MgSO_4$. Thus, $MgSO_4$ may have acted in the same way as cis-aconitate in partially protecting the reduced phosphatase.

A Model for the Reversible Stability

Most of the experimental observations are consistent, at least partially, with a simple tentative model (Figure 30). The justification for this tentative model is its usefulness in correlating the existing observations.

Oxidation by oxygen or endogenous oxidants is assumed to occur through site b, which might include a flavin (Figure 30). In this way, both glycolate and the phosphatase could be oxidized. The anaerobic enhancement of the glycolate dependent reversal of the stable phosphatase to the unstable form (Figure 28) could thus have been due to the elimination of O_2 as a competitor for electrons, while the inhibition by FMN of the glycolate dependent reversal could have been due to the increased competition for electrons. By isolating the phosphatase from the source of electrons while not disturbing the coupling to O_2, the inhibition of the oxidase at site a[1] could explain the observed stabilization of the phosphatase by OHPMS (Figure 25). This could also explain the inhibition of the glycolate dependent reversal of the oxidized stable phosphatase to the unstable form (Figure 28) by the same inhibitor. The stabilization of the

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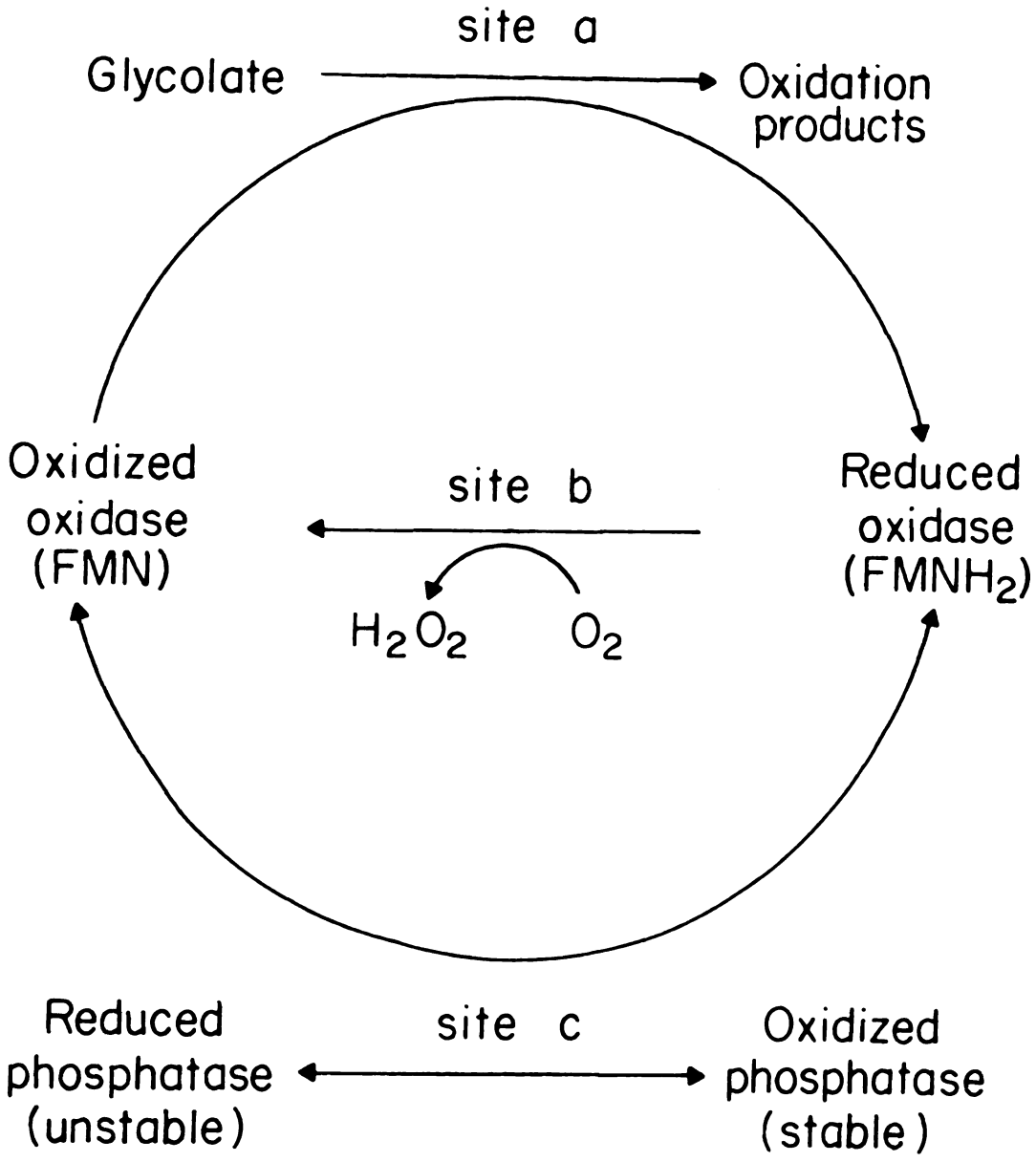
[1]Sodium hydroxymethanesulfonate is a competitive inhibitor of glycolate oxidase, and the competition is with respect to glycolate. The inhibition has been explained on the basis of the structural analogy between sodium hydroxymethanesulfonate and glycolate (267). OHPMS is about as effective as sodium hydroxymethanesulfonate in inhibiting glycolate oxidase (269). Therefore, site a (Figure 30) is considered to be the glycolate binding site and the OHPMS binding site of the system.

Figure 30

A Model for the Stabilization by Oxidation of P-glycolate Phosphatase, and for the Conversion by Glycolate of the Stable Phosphatase to the Unstable Enzyme


site a
Glycolate
Oxidation products
Oxidized oxidase (FMN)
site b
Reduced oxidase ($FMNH_2$)
H_2O_2
O_2
Reduced phosphatase (unstable)
site c
Oxidized phosphatase (stable)

phosphatase by OHPMS under anaerobic conditions (Figure 25) may have been caused by endogenous oxidants which were at too low a concentration to offer significant competition for electrons unless the source of electrons was essentially completely blocked. The inhibition by glycolate of the stabilization of the phosphatase by oxygen (Figures 25 and 26) also fits the model of Figure 30.

The Diurnal in the Stabilization of the Phosphatase

The diurnal in the stabilization of the phosphatase by post homogenization time under aerobic conditions partially matched the pH diurnal (Figure 20). The higher pH values in the extracts from leaves harvested during daylight occured along with greater stabilization rates of the phosphatase in these extracts, and visa versa for the extracts from leaves harvested at night. However, the phosphatase in an extract from leaves harvested during the day under dim light conditions did not stabilize with time even though the pH of the extract was 6.4 (Figure 24). This suggests that pH was not the decisive variable for the diurnal in the stabilization of the phosphatase by post homogenization time. Furthermore, the stabilization by post homogenization time was decreasing at 6 P.M., 7-19-1966, while the pH was increasing (Figure 20), which also suggests that pH was not the decisive variable.

The diurnal in the stabilization of the phosphatase

(Figures 20 and 26) could be explained by an inhibitor of oxidation which was more concentrated in tobacco leaves at night than during the day, or by the requirement for a compound which acted with oxygen to stabilize the phosphatase and which was only present during the day. The stabilization of the phosphatase by quick passage through Sephadex G-25, even though the extracts were from leaves harvested at night (Figure 23), suggests that the diurnal in the stabilization was caused by a diurnal in the concentration of an inhibitor of oxidation, and that it was not due to a diurnal in the concentration of a compound which acted with oxygen to stabilize the phosphatase. The increased stabilization rate of the phosphatase after acetone fractionation and the dependence of the stabilization rate after acetone fractionation on the rate before fractionation (Figures 24 and 42) also suggest that the stabilization diurnal was due to a diurnal in the concentration of an inhibitor of oxidation. On the basis of the Sephadex G-25 and acetone precipitation results, the inhibition of oxidation could have been at site b or site c (Figure 30). However, the less the extent of the stabilization by oxygen, the greater was the extent of the reversal by glycolate (Table 17). This observation is inconsistent with the diurnal inhibitor acting at site c, but it is consistent with it acting at site b. At the relatively high oxygen concentrations obtained by Waring

blendor treatment or buzzing under air[1] or O_2, this inhibition could be partially overcome. It is tentatively concluded that the stabilization by quick passage through Sephadex G-25 or by acetone fractionation was at least in part because of the removal of an inhibitor of oxidation which was more concentrated in extracts from tobacco leaves harvested at night than in extracts from tobacco leaves harvested during the day, and which acted at site b (Figure 30).

Variations in Initial Stabilities and Stabilization Rates

The great variation in the initial stability of the phosphatase, from 0% to 95% (p. 208), suggests that the phosphatase can exist in either the oxidized or the reduced form *in vivo*.

Since glycolate synthesis is favored by high light intensities (233), the greater initial instability of the phosphatase and the lengthier post homogenization time required to stabilize the phosphatase in extracts from leaves grown in the field in the summer compared to the phosphatase in extracts from leaves grown in the greenhouse in the winter (Figure 19) are consistent with the

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[1]The Waring blendor and buzzing treatments caused frothing. Thus, oxygen was not only dissolved in the solutions, probably to the saturation point, but the gas phase was thoroughly mixed with the solutions. At saturation at 0°, the oxygen concentration in water is 1/20 as great as in air (reference; footnote, p. 102).

possibility that the initial instability of the phosphatase in fresh extracts is due to the presence of endogenous glycolate. The stabilization of the phosphatase in fresh extracts by 10 minutes of preincubation with OHPMS (Figure 25) is consistent with the same possibility. On the other hand, much evidence is opposed to this possibility. It is doubtful that endogenous glycolate would have lasted long enough to explain the long lag in the stabilization of the enzyme, unless glycolate were being generated in the extracts from other metabolites. Second, glycolate added to fresh extracts partially stabilized the phosphatase (Figures 25 and 26), and possibly for the same reason, glycolate did not give complete reversal (Figure 28). (However, glycolate added to the extracts could have an effect different from glycolate generated in the extracts.) Third, the phosphatase in extracts from leaves harvested at night was almost as unstable initially as the enzyme in extracts from leaves harvested during the day, even though glycolate is not synthesized in the dark *in vivo* (268).

Under aerobic conditions, reductants have been used to protect enzymes which are sensitive to O_2 (259). By reducing the availability of oxygen, endogenous reductants could at least partially account for the long lag in the stabilization of the phosphatase by post homogenization time. The longer lag during the summer compared to

winter is consistent with this possibility. The buzzing or Waring blendor treatments under aerobic conditions could rapidly oxidize such reductants, and Sephadex G-25 column chromatography or acetone precipitation could separate them from the phosphatase.

The very low diffusion rate of O_2 in H_2O was probably an important factor in the long lag in the stabilization of the phosphatase by post homogenization time. The diffusion of O_2 is approximately 10,000 times slower in water than in air (244). Mixing the extracts by Waring blendor or vortex mixer would compensate for the slow diffusion rate by greatly reducing the distance that O_2 was required to travel to saturate the extracts.

An endogenous compound(s), other than glycolate, which could undergo oxidation and reduction, acting at site b, for example, might partially explain what has been observed about the initial stability of the phosphatase. Quinones and phenolic compounds have been postulated as possible electron acceptors for glycolate oxidase (133), and many of these compounds may be at higher concentrations in plants grown at high light intensities than in plants grown at low light intensities (134), which possibly could explain the seasonal variation in the initial stability of the phosphatase. It is also possible that the oxidation inhibitor, which was discussed earlier, could be this same endogenous compound(s).

The Endogenous Species Which Acts Synergistically with Glycolate

The enhancement by heated fresh extract of the glycolate dependent reduction of the phosphatase (Figure 28) could have been by inhibition of electron flow at site b, or by coupling at site c (Figure 30). The species which is this inhibitor or coupling factor was inactivated by the buzzing procedure, but not by a boiling water bath treatment.

Effect of Cysteine

The inhibition of the glycolate dependent reduction of the phosphatase by a combination of cysteine and Fe^{++} may be related to the strong inhibition of the phosphatase reaction by cysteine (202), and to the stabilization of the phosphatase by cysteine (Tolbert and Yu, unpublished data).

Secondary Effects

Although the changes of greatest magnitude in the state of the phosphatase can be explained by a simple oxidation-reduction scheme (Figure 30), changes of lesser magnitude cannot be so simply explained. The partial but inconsistent stabilization of the phosphatase by orthophenanthroline, and to a lesser extent by EDTA, and the partial but inconsistent enhancement of the glycolate

dependent reversal of the stable phosphatase to the less stable form by Fe^{++}, Cu^{++}, or to a lesser extent by Zn^{++}, suggest that one or more of these metals may play a secondary role in the stabilization. Also, stabilization by post homogenization time, by buzzing under air or O_2, or by mixing under air in a Waring blendor, caused some loss of the phosphatase activity. Stabilization by preincubation with OHPMS had no effect on the activity, while stabilization by Sephadex G-25 column chromatography consistently,and by acetone precipitation sometimes, increased the phosphatase activity. These relatively minor differences in activation seemed to be secondary phenomena superimposed on the major stabilization phenomenon.

The Effect of Arsenite and Cd^{+2}

Activation or inhibition by preincubation with low concentrations of Cd^{++} or arsenite has been considered diagnostic for the presence of vicinal sulfhydryls (84, 85, 217). Functional vicinal sulfhydryl groups, defined by high sensitivity to arsenite and reversal by added dithiol compounds, have been found only with enzymes which are concerned with oxidative reactions (84). Since Cd^{++} and arsenite had no stabilization effect on the initially unstable phosphatase and had no effect on the stabilization of the phosphatase by oxygen, vicinal

sulfhydryls are probably not part of the system involved in the reversible stabilization of the phosphatase toward dilution at 30°. However, the slight activation of the phosphatase by arsenite or Cd^{++} (Table 16) could mean that vicinal sulfhydryl groups are on the phosphatase, or on an enzyme which was bound to the phosphatase. The activation might have been the result of an uncoupling of the phosphatase reaction from interaction with these sulfhydryls. A similar explanation has been proposed for the activation of mitochondrial ATPase by 10^{-4}M arsenite (84).

Glycolate reduced the phosphatase without affecting the activity of the enzyme, while ascorbate reduced the phosphatase and also decreased its activity. This additional effect of ascorbate also suggests the existence of sulfhydryls on the phosphatase which are not involved in the stabilization of the enzyme.

Arsenite at low concentrations is a potent inhibitor of photosynthetic CO_2 fixation. Arsenite does not affect photosynthetic ATP formation or NADP reduction, nor does it appear to affect any individual enzyme of the photosynthetic carbon cycle. Gibbs et al (95) have reviewed evidence which led them to propose that the enzymes of the photosynthetic carbon cycle might form a structural complex <u>in vivo</u>, and that the complex itself may be the site of arsenite inhibition. The findings and

proposals reviewed by Gibbs et al may be relevant to the slight activation of P-glycolate phosphatase by arsenite.

In Vivo Function of the Phosphatase

Throughout this work, there has been the concern that P-glycolate phosphatase activity is not the true function of the enzyme. That the enzyme can exist in a stable form, in which it seems to be oxidized, or an unstable form, in which it seems to be reduced, leads to the speculation that *in vivo*, the enzyme may catalyze a more involved reaction. Such speculation is consistent with evidence that this phosphatase seems to be loosely associated with the chloroplast and may function in the excretion of glycolate by this particle (202, 260, 265). Furthermore, the oxidation-reduction behavior of the phosphatase may be related to the requirement for O_2 for the synthesis and excretion of glycolate (literature review, pp. 8-10). It may also be related to the fact that every possible glycolate precursor which is an intermediate of the photosynthetic carbon cycle (this cycle is the most probable source of glycolate (literature review, pp. 4-34)) would require at least one oxidation step in the course of the synthesis of glycolate (literature review, p. 23).

In view of the localization of P-glycolate phosphatase in or on the chloroplasts (literature review),

and evidence that at least some of the glycolate oxidase of leaf tissue is associated with the peroxisome, which is a cytoplasmic organelle (237), coupling between the phosphatase and the oxidation of glycolate (Figure 30) might seem to be unlikely *in vivo*. However, recent electron micrographs indicate that in some leaves, cytosomes, which are thought to be peroxisomes, may be appressed to the chloroplasts.[1] The contact between chloroplasts and the cytosomes is particularly striking in tobacco leaves. Contact between chloroplasts and peroxisomes would be consistent with the proposed excretory function of P-glycolate phosphatase, and with evidence that the peroxisomes are concerned with the metabolism of glycolate and related compounds (237). The scheme of Figure 30 is also of interest in relation to evidence that P-glycolate phosphatase and glycolate oxidase functionally seem to be sequential enzymes (literature review, pp. 25-26 and p. 34). A more detailed study will be required, however, to determine whether the reactions of the type depicted in Figure 30, either by direct or by indirect coupling, occur *in vivo*.

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[1]Frederick, Sue Ellen, and Eldon H. Newcomb. 1968. Microbody-like cytosomes in leaf cells. (personal communication).

Practical Considerations

The phosphatase, in those preparations in which it is unstable toward dilution at 30°, should be assayed by initiating the reaction with enzyme, since pre-equilibrating such an enzyme at 30° without substrate destroys much of the activity. Also, in view of the findings on the stability of the enzyme toward dilution, a minimum amount of water should be used for the extraction of the phosphatase from leaves.

The mortar and pestle or meat grinder method of extraction seems more advantageous than the Waring blendor method. Less inactivation of the phosphatase resulted (Figures 8 and 21), and the specific activity increase from the first acetone fractionation was about twice as great (p. 161). The stabilization by Waring blendor extraction was a temporary advantage only, because the enzyme extracted by meat grinder (Table 3) or by mortar and pestle (Figures 24 and 42) was stabilized by precipitation with acetone. Furthermore, the Waring blendor extraction method caused considerable frothing while the other two methods did not. While there is no evidence that surface denaturation of the phosphatase occured, it seems advantageous to choose an extraction method that does not cause frothing. Finally, there is no mechanical requirement for added water with the mortar and pestle or meat grinder extraction methods. Depending

on the turgidity of the leaves, usually more than one weight of water per fresh weight of leaf tissue must be added with the broken up leaf blades during Waring blendor extraction. Since the inclusion of only one weight of water per fresh weight of leaf tissue in the preparation of the extracts insures sharp acetone fractionation (pp. 161-164), the method of extraction can impose the lower limit on how much water must be used.

Aconitase, and Relationships Between Aconitase and P-glycolate Phosphatase

Unless noted otherwise, purification and experiments were started with 2 to 4 medium sized tobacco leaves (see Table 1), and the leaf tissue was homogenized in a mortar and pestle with sand and 2 weights of water. All acetone which was used for enzyme purification was reagent grade.

pH Optimum

The pH optimum for aconitase from the leaves of Swiss chard[1] was found to be broad, extending from about pH 5.7 to about 9.0. Activity fell off on the alkaline

[1]The reaction mixtures contained the following in a volume of 1.00 ml: 0.033M cacodylate, 0.033M glycyl-glycine, 0.01M $MgSO_4$, 2.0×10^{-4}M neutralized Na cis-aconitate, and 40 µl of enzyme. The reactions were initiated by the addition of cold enzyme. Ten buffer mixtures, each containing 0.10M cacodylate and 0.10M glycyl-glycine, had previously been made up and adjusted to the following pH values: 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5. The aconitase for this experiment was purified by one acetone precipitation essentially as described in Figure 31.

side of pH 9.0, and it fell off very sharply on the acid side of pH 6.0. The activity showed a peak at about pH 6.0 to pH 6.5, which was about 1.2 to 1.3 times the activities which were found from pH 7.5 to 9.0. In the latter alkaline region, the pH optimum curve was found to be flat.

Although a pH optimum experiment was not performed for aconitase from tobacco leaves, it was found that the initial activity of the enzyme from this source at pH 6.3 with cacodylate as the buffer, was 1.5 to 1.7 times greater than the activity at pH 7.5 with TES as the buffer.[1] However, it was found that the reaction rates were more linear at pH 7.5 than at pH 6.3. After about 15 minutes, the activity at pH 6.3 with cacodylate as the buffer was only about 1.1 times greater than the activity at pH 7.5 with TES as the buffer.

For several reasons, pH 7.5 and TES were selected as the standard pH conditions for the assays in subsequent experiments. Although there appear to be no significant competing reactions in crude pig heart tissue extracts which might lead to error in the assay of aconitase (5), the enzyme aconitic hydrase, which catalyzes the intercon-

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[1]The reaction mixtures contained either 0.033M cacodylate, pH 6.3, or 0.033M TES, pH 7.5. They also included 0.02M ammonium sulfate, 2.5×10^{-4}M neutralized Na cis-aconitate, and 20 µl of enzyme in a final volume of 1.20 ml. The tobacco aconitase was purified by one fractionation with acetone. The reactions were initiated by the addition of cis-aconitate.

version of citrate and cis-aconitate but is not active toward isocitrate, has been reported in cucumber (171), and the enzyme cis-aconitate decarboxylase has been reported in Aspergillus terreus (184). Both enzymes may be assayed by following the change in absorption at 240 mμ. However, both of these enzymes have a pH optimum on the acid side of neutrality. The activity of aconitic hydrase is minimal at pH 7.5 (171), while cis-aconitate decarboxylase from A. terreus shows zero activity above pH 7.0 (184). Second, the reaction rates of the tobacco aconitase were found to be more linear at pH 7.5 than at 6.3. Third, a common method for assaying aconitase is to link it to isocitrate dehydrogeanse (usually from pig heart) and follow the increase in absorption at 340 mμ. The isocitrate dehydrogenase from pig heart has a pH optimum of 7.4 (219). Thus, the linked assay could be performed with a minimal change in the reaction protocols. Fourth, with cis-aconitate as substrate and with $MgSO_4$ present, pH 7.5 is in the middle of the optimum range for the enzyme from the leaves of Swiss chard. Until the pH optima with the other two substrates and under other conditions, and for the enzyme from different plants, have been determined, 7.5 seems like a reasonable pH to use.

Activators

Passage of partially purified aconitase from Swiss chard leaves through a Sephadex G-10 column inactivated the enzyme. The enzyme could be reactivated by recombining it with fractions which emerged later (Figure 31). The Rf (elution volume/void volume) of the endogenous activator(s) on the G-10 column was 1.4. The exclusion limit for Sephadex G-10 is at a molecular weight of 700 (Sephadex G-10 brochure, Pharmacia Fine Chemicals, Inc.). Thus the endogenous activator(s) must be of low molecular weight. Similar results were obtained with the enzyme from tobacco leaves. Although no further attempt was made to identify the endogenous factor(s) which activated aconitase from the leaves of tobacco or Swiss chard, the inactivated enzyme from either plant could be reactivated with the anions which were tested (Figures 31, 32, and 33). Sulfates were more effective at lower concentrations than chlorides (Figure 32). The optimum concentration of ammonium sulfate was about 0.03M, with greater concentrations resulting in lower activity (Figure 32). $MgSO_4$ showed this same effect (Figures 31 and 32). The activation by sulfate was pronounced at both pH values tested, i.e. 6.3 (Figure 32) or 7.5 (Figures 31 and 33). Aconitase in fresh extracts from tobacco leaves was also activated at pH 7.5 by 0.02M ammonium sulfate, with about 2-fold activation being typical. The cations which were

Figure 31

Activation of Aconitase by $MgSO_4$ or by Factor(s) Removed by Sephadex G-10 Column Chromatography

The extract was prepared from Swiss chard leaves from the growth chamber. After centrifugation at 15,000 g for 8 minutes, the active supernatant was adjusted to pH 7.5. The aconitase in 34 ml of this supernatant was precipitated with 68 ml of cold acetone, taken up with 1.7 ml of cold water, and recentrifuged to give an active clarified supernatant. 1.0 ml of this enzyme was added to a 1.0 cm x 10.8 cm Sephadex G-10 column, which had been pre-equilibrated with cold H_2O, and was eluted with cold water. The void volume of H_2O was discarded, and then 9 fractions were collected. Fraction 1 contained 1.5 ml, while the other 8 fractions contained 1.0 ml each.

All reaction mixtures contained 1.5×10^{-4}M sodium cis-aconitate, pH 7.5. In addition, each reaction mixture contained:

Figure A: 20 µl of the indicated fraction.

Figure B: 20 µl of fraction 1 plus 50 µl of one of the other fractions, as indicated. In order to show the activation of the enzyme in fraction 1 by fraction 2 or 4, the values for 2 alone and 4 alone ($2\frac{1}{2}$ x values of Figure A) have been subtracted from the rates obtained with 1 + 2 and 1 + 4.

Figure C: 20 µl of fraction 1 plus $MgSO_4$ to give the reaction concentrations of 10^{-3}M, 5×10^{-3}M, 10^{-2}M, and 5×10^{-2}M.

Reactions were initiated by the addition of the indicated fraction (Figure A) or of fraction 1 (Figures B and C). The final volume of all reaction mixtures was 1.0 ml.

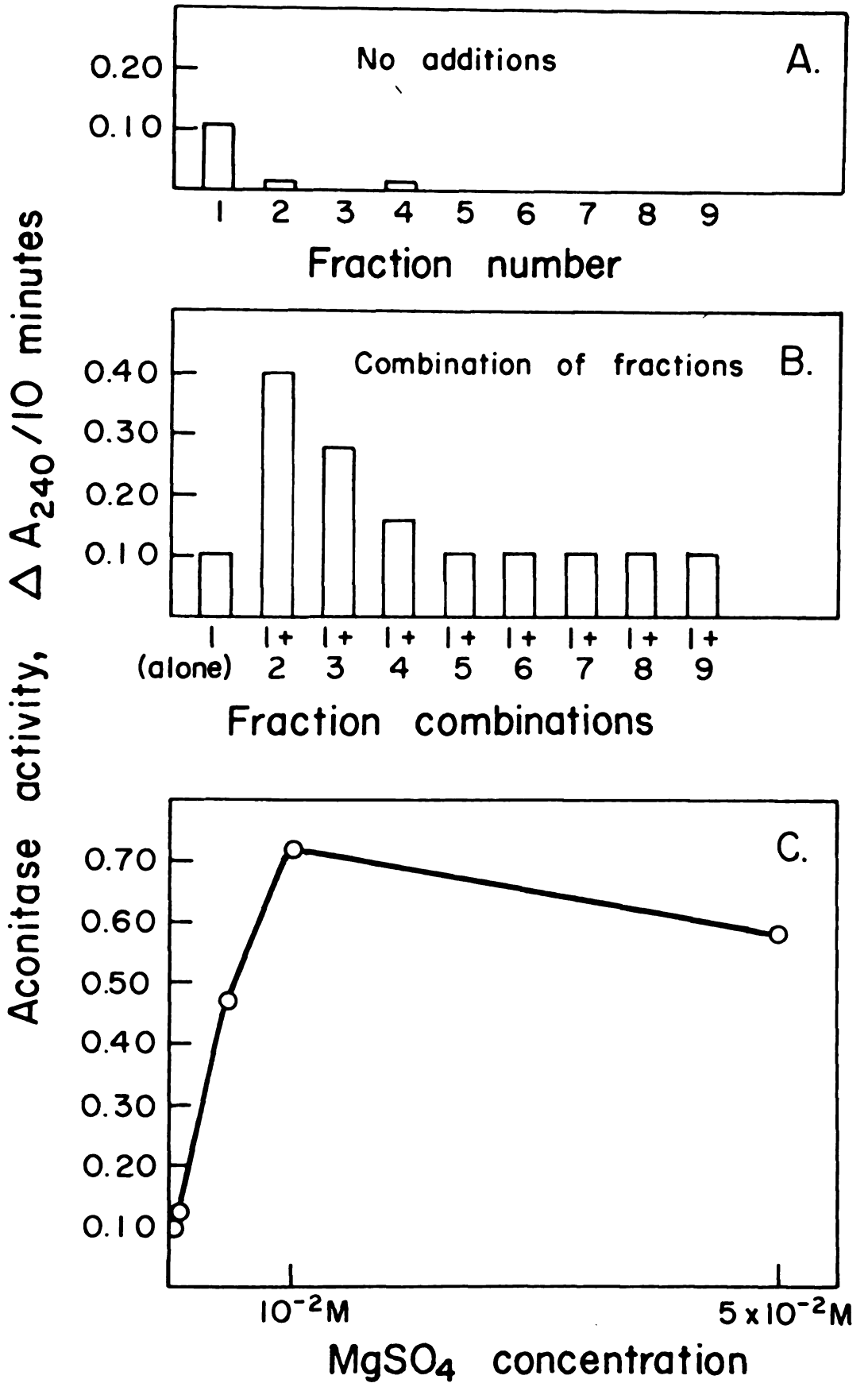
No additions
A.
0.20
0.10
1 2 3 4 5 6 7 8 9
Fraction number
Combination of fractions
B.
0.40
0.30
0.20
0.10
1 (alone) 1+2 1+3 1+4 1+5 1+6 1+7 1+8 1+9
Fraction combinations
Aconitase activity, Δ A240/10 minutes
C.
0.70
0.60
0.50
0.40
0.30
0.20
0.10
10-2M
5x10-2M
MgSO4 concentration

Figure 32

Activation of Aconitase by Sulfates or Chlorides

The aconitase for this experiment was prepared as described in Figure 31, except the Swiss chard was grown in the greenhouse, the extract was not adjusted with respect to pH, and 0.02M cacodylate, pH 6.3, rather than water, was used to resuspend the acetone precipitated enzyme.

All reaction mixtures contained 0.050 ml of 0.20M cacodylate, pH 6.3, 0.008 ml of 0.01M sodium cis-aconitate, pH 7.5, salt as indicated below, 0.005 ml of enzyme, and enough water to bring the reaction volume to 0.30 ml. The reactions were initiated by the addition of cis-aconitate.

O——O $MgSO_4$. ●——● $(NH_4)_2SO_4$. △——△ NH_4Cl.

□——□ NaCl. ■——■ KCl.

For NH_4Cl, NaCl, and KCl, the ionic strength is equal to the concentration in moles/liter. For $(NH_4)_2SO_4$, the ionic strength may be converted to the concentration in moles/liter by dividing the ionic strength by 3, and for $MgSO_4$, by dividing the ionic strength by 4.

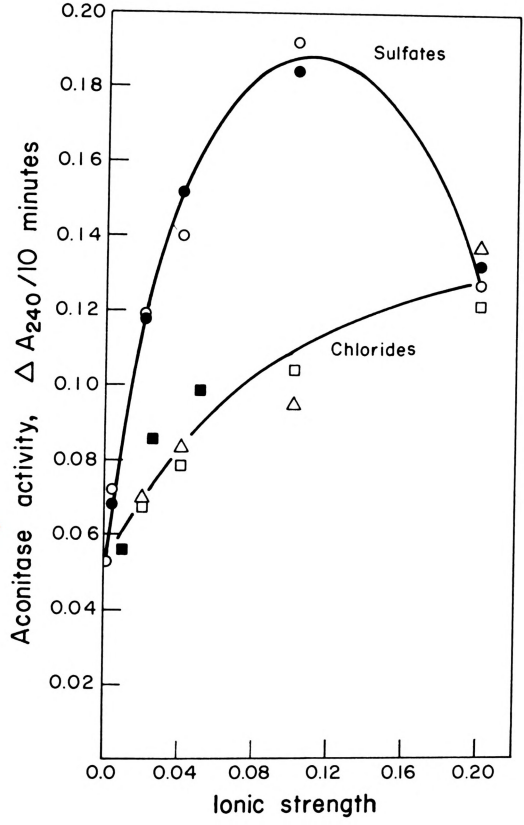
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
Aconitase activity, Δ A240/10 minutes
Sulfates
Chlorides
0.0
0.04
0.08
0.12
0.16
0.20
Ionic strength

Figure 33

Activation with Citrate, Isocitrate, or Cis-aconitate as Substrate

The acetone precipitated enzyme described in Figure 39 was used. All reaction mixtures contained 0.40 ml of 0.10M TES, pH 7.5, 0.020 ml of enzyme, substrate and $(NH_4)_2SO_4$ as described below, and enough water to bring the reaction volumes to 1.20 ml. Reactions were initiated with substrate.

Cis: reaction mixture contained 0.030 ml of 10^{-2}M sodium cis-aconitate, pH 7.5 (reaction concentration = 2.5×10^{-4}M).

Iso: reaction mixture contained 0.12 ml of 0.10M isocitrate, pH 7.5 (reaction concentration = 10^{-2}M).[1]

Citrate: reaction mixture contained 0.36 ml of 0.10M citrate, pH 7.5 (reaction concentration = 3×10^{-2}M).

Closed bars: reaction mixtures contained 0.24 ml of 0.10M $(NH_4)_2SO_4$, pH 7.5 (reaction concentration = 0.02M).

Open bars: reaction mixtures contained no $(NH_4)_2SO_4$.

Figure A: absolute rates.

Figure B: normalized rates.

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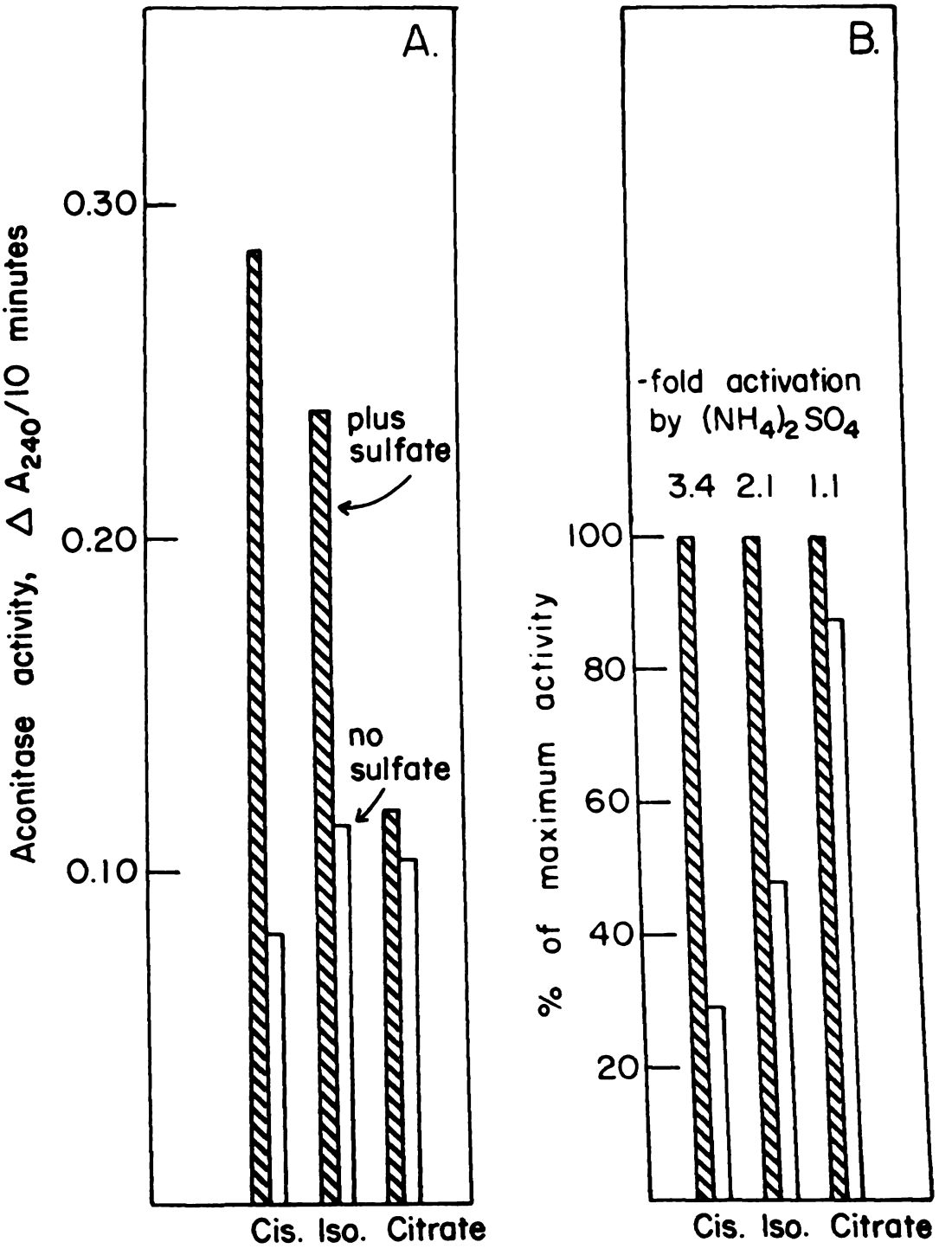
[1] 10^{-2}M was the total isocitrate concentration. The concentration of the naturally occuring isomer, threo-Ds-isocitrate, is thus assumed to have been 0.5×10^{-2}M.


A.
B.
Aconitase activity, Δ A_{240}/10 minutes
0.30
0.20
0.10
plus sulfate
no sulfate
Cis. Iso. Citrate
-fold activation by $(NH_4)_2SO_4$
3.4 2.1 1.1
% of maximum activity
100
80
60
40
20
Cis. Iso. Citrate

present with the chlorides or sulfates did not seem to exert as pronounced an effect on the plant aconitase as did the anions (Figure 32). Sulfate was found to activate aconitase from tobacco leaves with any of the three tricarboxylic acids as substrate. However, the amount of activation depended on which tricarboxylic acid was used as substrate and/or the concentration of the acid (Figure 33).

Classically, when an endogenous activator is present with an enzyme, the velocity vs. enzyme concentration plot shows an upward curvature. However, including the activator in the reaction mixtures straightens out the plot (68). Thus, the linear plot of velocity vs. aconitase concentration which was found with SO_4^{-2} present (Figure 5) is not unexpected.

Peters (191) found that increasing amounts of NaCl dramatically increased the activity of aconitase from pig heart, while KCl at low concentrations increased the activity and at high concentrations decreased the activity. To my knowledge, the activating effect of anions other than substrate on aconitase from plants has not been previously reported. However, it has been reported that increased isocitrate concentrations activate the aconitase from mustard (185).

From a control standpoint, it may be of significance that passage of acetone precipitated aconitase

through a Sephadex G-10 (Figure 31) or G-25 column reversibly inactivated the enzyme, while passage of fresh extracts through a Sephadex G-25 column activated the phosphatase (Figure 23).

Relative Reaction Rates with Citrate or Isocitrate

The rate of formation of cis-aconitate from isocitrate was considerably greater than the rate of formation of cis-aconitate from citrate, even though the concentration of citrate was 6 times greater than the concentration of threo-Ds-isocitrate (Figure 33). Although the data of Figure 33 do not give V_{max} values, they are consistent with the finding that for beef liver aconitase, the relative maximum velocity for the disappearance of isocitrate was 1.7 times greater than for the disappearance of citrate (114).

The Adequacy of Aconitase

In 5 experiments with tobacco leaves harvested from the field, the aconitase activity in the extracts varied from 0.40 to 1.2 μmoles of cis-aconitate converted/gram fresh weight of leaf blade tissue/minute. In these same experiments, the ratio of P-glycolate phosphatase to aconitase activity, each expressed as μmoles of substrate

converted/minute/ml of extract, varied from 13 to 23,[1] with the average ratio being 19. The adequacy of P-glycolate phosphatase has already been discussed. From the activities present in the extracts of leaves, it is estimated that enough phosphatase activity is present *in vivo* to allow all the newly fixed CO_2 to be acted upon by this enzyme. The respiratory evolution of CO_2 in plants in the dark has been estimated to be about 1/25 the rate of photosynthetic CO_2 fixation (120). For both P-glycolate phosphatase and aconitase, the enzyme must catalyze one reaction per 2 CO_2 molecules fixed or respired. Therefore, the amount of aconitase activity which was measured in the extracts from tobacco leaves seems adequate to account for *in vivo* dark respiration rates.

The Fractionation of Aconitase and P-glycolate Phosphatase

It was found that P-glycolate phosphatase

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[1]P-glycolate phosphatase assays were by the standard procedure. All aconitase reaction mixtures contained 0.033M TES, pH 7.5, 0.02M ammonium sulfate, and 2.5×10^{-4}M cis-aconitate in a volume of 1.2 ml. The estimates of the aconitase activities must be considered approximations of the *in vivo* activities at 30° for at least the following reasons. The activities were converted from those which were measured at 25° to the values given, which are the estimated activities at 30°, by multiplying by 1.41 (this was based on an estimated doubling of the rate for each 10° increase in the 25 to 30° range). The substrate for aconitase for dark respiration is citrate, and the rate with citrate is considerably less than with cis-aconitate (Figure 33) (114). Although the inactivation rate of aconitase in the extracts was significant (Figures 40 and 42), no allowance was made for the inactivation which occured before the aconitase assays were run.

and aconitase from tobacco leaves were fractionated in parallel by acetone. Significant recoveries were obtained for both enzymes (Figures 34 and 35). Although protein concentrations were not determined in the experiments shown in Figures 34 and 35, in every other similar fractionation of extract which had been prepared by mortar and pestle and in which protein concentrations were measured, the purification of the phosphatase was in excess of 20-fold. In a preliminary experiment similar to those described in Figures 34 and 35, aconitase and the phosphatase from Swiss chard leaves were also fractionated in parallel by acetone (Figure 36). It seems significant that although more than twice as much acetone was required to precipitate the two enzymes from Swiss chard leaves than from tobacco leaves, they fractionated together as they did from tobacco. In the experiment with Swiss chard, activities of glycolate oxidase and NADPH glyoxylate reductase were also measured. Unlike P-glycolate phosphatase and aconitase, most of the glycolate oxidase and of the reductase precipitated in the 7.50-8.75 fraction (data not shown).

Under conditions which included at least some exposure of tobacco leaves to light before homogenization, aconitase and the phosphatase, after a preliminary purification by an acetone fractionation, also fractionated in parallel during DEAE-cellulose chromatography (Figures 37

and 38).[1]

When any exposure of field grown tobacco leaves to light was completely avoided for 3 to 4 hours before harvest and homogenization, after a preliminary purification by an acetone fractionation, aconitase and the phosphatase did not fractionate in parallel during DEAE-cellulose chromatography (Figure 39). The slight shoulder on the aconitase peak of Figure 39 may reflect an affinity between the two enzymes, or it could be fortuitous, or because of possible inaccuracies in the assays. When aconitase and the phosphatase were separated, isocitrate was no longer found to be associated with the phosphatase. (Although isocitrate was with aconitase (Figure 39), the shapes of the curves do not strongly suggest binding between aconitase and isocitrate.) In contrast, the position of one of the isocitrate peaks was found to coincide with the phosphatase peak during chromatography on DEAE-

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[1]No significant amount of either enzyme from leaves which had been harvested in strong sunlight was retained by the column (Figure 37). (A second nearly identical experiment in which the leaves were harvested from the field when the light was at 11,000 ft-c gave the same results, i.e. after a preliminary purification by acetone fractionation, neither enzyme was retained by the DEAE-cellulose column. The column had been prepared as described for Figure 39.) In contrast, all of the aconitase and the phosphatase, from leaves which were exposed to no light for at least 3 to 4 hours before homogenization, was retained by an equivalent DEAE-cellulose column, even though a greater amount of protein was applied in the latter case (Figure 39). Also, the enzymes from leaves which were exposed to only a small amount of light before homogenization were retained by a DEAE-cellulose column (Figure 38).

Figure 34

Acetone Fractionation of P-glycolate Phosphatase and Aconitase
(Leaves Harvested from the Field During Daylight)

The tobacco leaves were harvested from the field at 2 P.M. on 7-20-1967 (light intensity = 2700 ft-c). Soon after centrifugation (15,000 g, 15 min), 20 ml of the extract was fractionated at 0°. The fractions were from 0-2, 2-3, 3-4, 4-5, 5-6, 6-7, 7-8, and 8-10 ml of acetone/10 ml of extract. After each aliquot of cold acetone had been slowly added in about 3 minutes with stirring, the mixture was allowed to sit at 0° for 5 more minutes. Then the mixture was centrifuged at about 6000 g for 8 minutes. Each supernatant was poured off for the subsequent addition of the next aliquot of cold acetone. Each precipitate was washed with H_2O and then taken up with 4.0 ml of cold 0.02M cacodylate, pH 6.3. Those resuspended preprarations which were turbid were recentrifuged at 6000 g for 8 minutes to give clear active preparations.

Open bars: P-glycolate phosphatase by the standard assay.

Closed bars: aconitase. The reaction mixtures contained the following in a volume of 1.20 ml: 0.033M TES, pH 7.5, 0.02M ammonium sulfate, 2.5 x 10^{-4}M Na cis-aconitate, pH 7.5, and either 80 or 160 µl of enzyme. The reactions were initiated by the addition of cis-aconitate.

Both enzyme activities, expressed as µmoles of substrate converted/min/ml of enzyme, were normalized, with the activity in the most active fraction designated as 100%. The most active fraction (5-6) had the following activities under the assay conditions which were used: 13.0 µmoles of P_i released/min/ml of enzyme and 0.20 µmoles of cis-aconitate converted/min/ml of enzyme.

The recovery of aconitase in all 8 fractions was 52% of the activity which was in the extract which was fractionated. The recovery of the phosphatase was not determined.

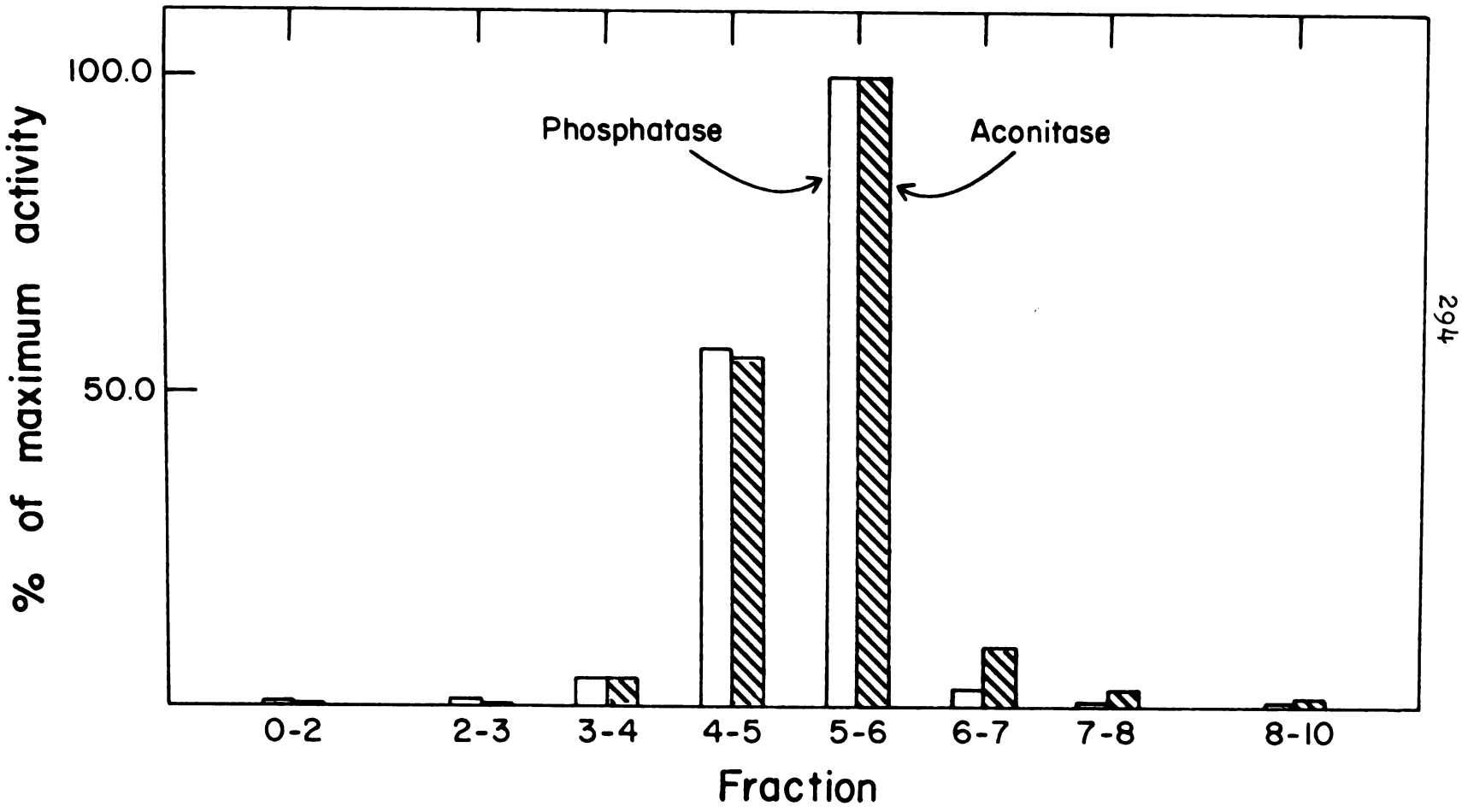

100.0
50.0
% of maximum activity
Phosphatase
Aconitase
0-2
2-3
3-4
4-5
5-6
6-7
7-8
8-10
Fraction

Figure 35

Acetone Fractionation of P-glycolate Phosphatase and Aconitase (Leaves Harvested from the Field During the Night)

The experiment was performed as described for Figure 34, with the following exceptions: The tobacco leaves were harvested at midnight, 7-25-1967, and were kept in complete darkness until after the homogenization. The fractions were from 0-2, 2-3, 3-5, 5-6, 6-7, 7-8, and 8-9 ml of acetone/10 ml of extract.

Open bars: P-glycolate phosphatase.

Closed bars: aconitase. The reactions were initiated by the addition of 30 to 80 µl of cold enzyme.

The most active fraction (5-6) had the following activities under the assay conditions which were used: 18.3 µmoles of P_i released/min/ml of enzyme and 0.33 µmoles of cis-aconitate converted/min/ml of enzyme.

The recovery of the phosphatase in all 7 fractions was 89% of the activity which was in the extract which was fractionated, while the recovery of aconitase was 54%.

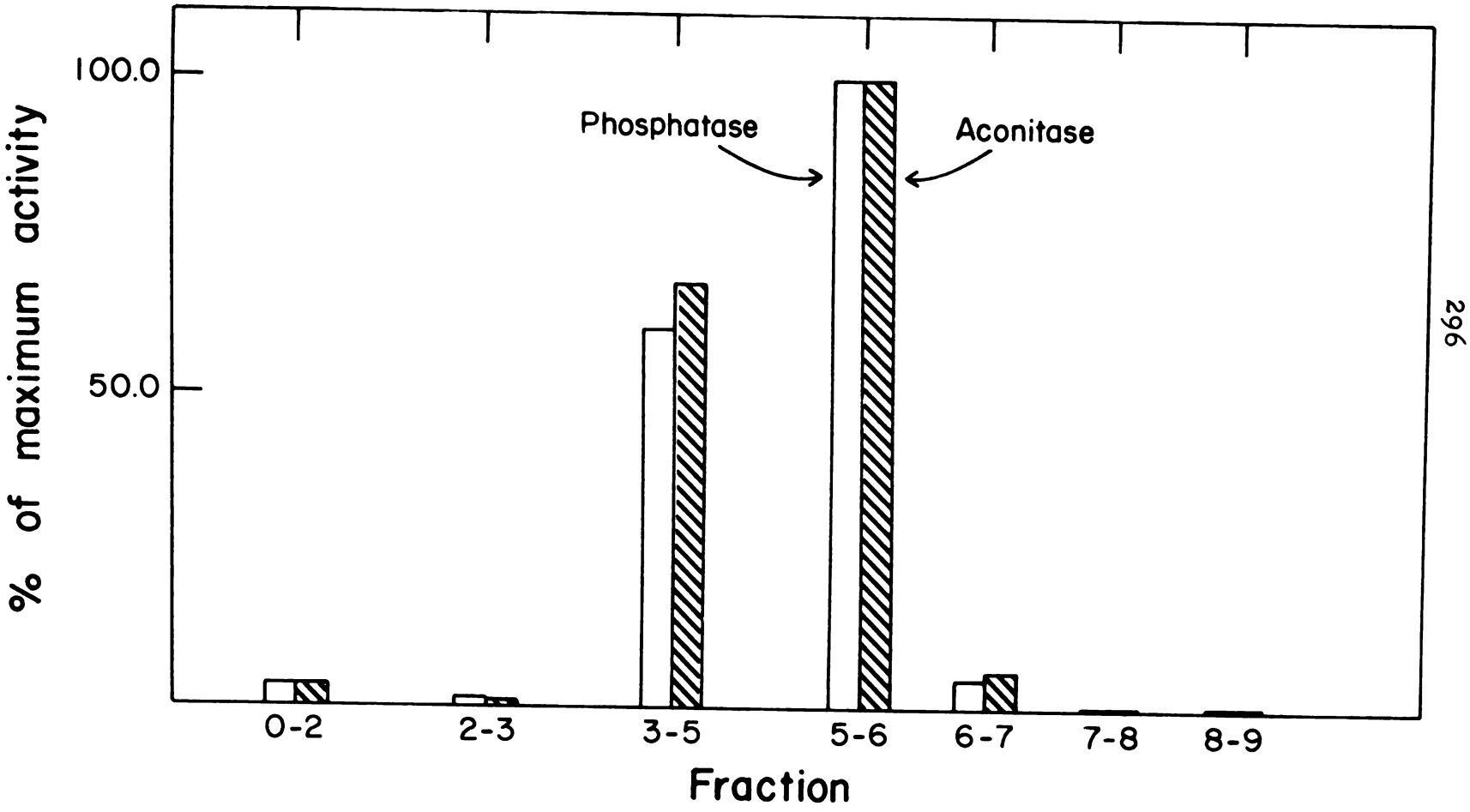

100.0
50.0
% of maximum activity
Phosphatase
Aconitase
0-2
2-3
3-5
5-6
6-7
7-8
8-9
Fraction

Figure 36

Acetone Fractionation of P-glycolate Phosphatase and Aconitase from Swiss Chard Leaves

Swiss chard leaf tissue from leaves harvested 2/9/1967 was homogenized in a Waring blendor for 20 seconds with 1.5 weights of a pH 7.5 solution containing 0.02M citrate, 0.02M glycyl glycine, 10^{-3}M EDTA, and 10^{-3}M $MgCl_2$. After the brei was squeezed through cheesecloth, the sap was adjusted to pH 7.5 and was then centrifuged. Twenty ml of the active supernatant was fractionated at 0°. The fractions were from 0-5, 5-6.25, 6.25-7.50, 7.50-8.75, 8.75-10.0, and 10.0-12.5 ml of acetone/10 ml of extract. The remainder of the procedure for the preparation of the 6 fractions was qualitatively similar to the procedure described for Figure 34. The first two fractions were taken up in 2 ml, and the last 4 fractions in 1 ml, of buffer.

Open bars: P-glycolate phosphatase.

Closed bars: aconitase. The reaction mixtures contained the following in a volume of 2.0 ml: 1.5×10^{-4}M Na cis-aconitate, pH 7.5, and either 50 or 200 µl of enzyme. The reactions were initiated by the addition of cold enzyme.

Both enzyme activities, expressed in µmoles of substrate converted/min/ml of enzyme, were normalized, with the activity in the most active fraction designated as 100%.

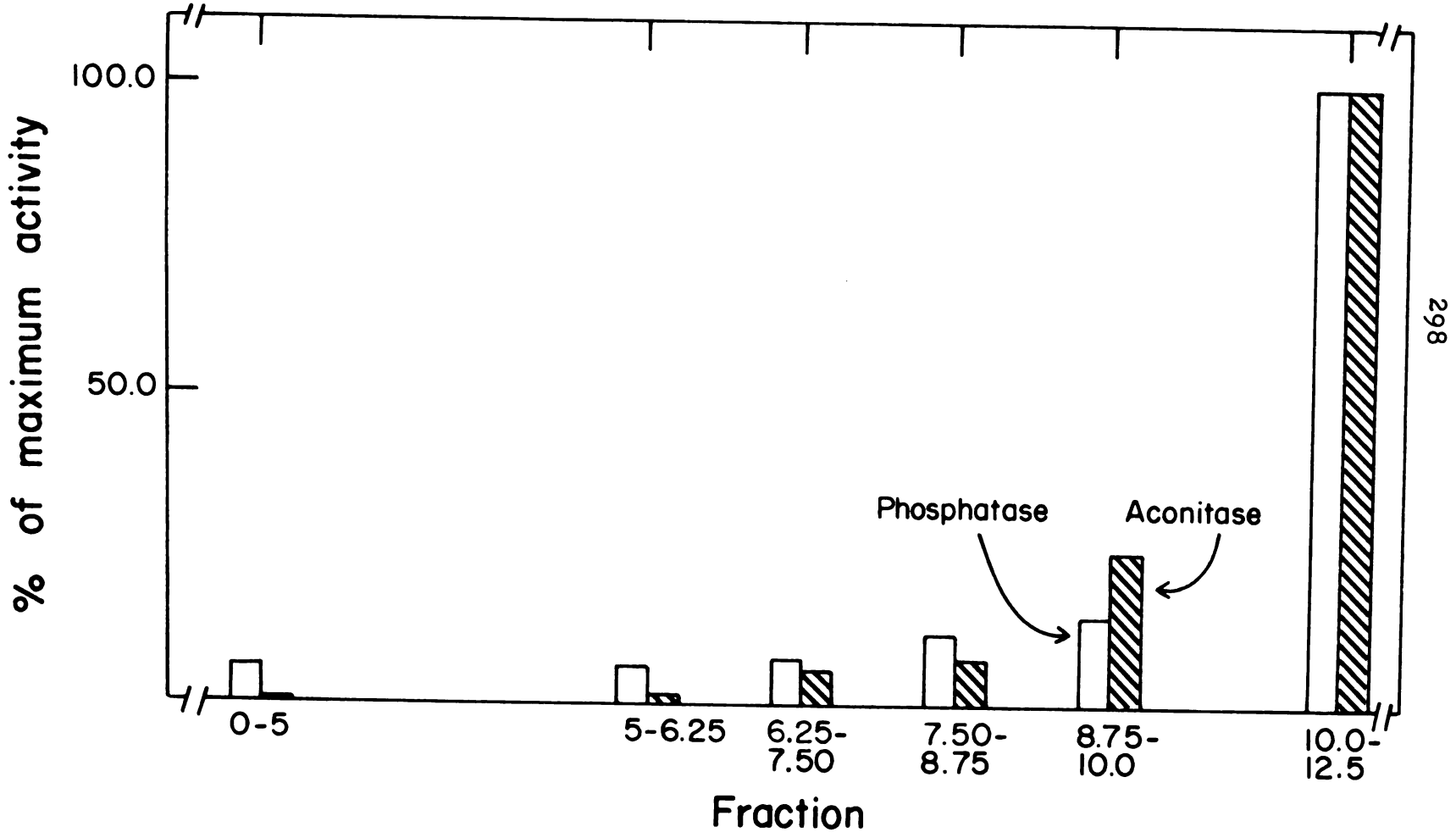

100.0
50.0
% of maximum activity
Phosphatase
Aconitase
0-5
5-6.25
6.25-
7.50
7.50-
8.75
8.75-
10.0
10.0-
12.5
Fraction

Figure 37

DEAE-Cellulose Chromatography of P-glycolate Phosphatase and Aconitase
(Leaves Harvested from the Field During Daylight)

The tobacco leaves were harvested from the field at 4 P.M. on 9/24/1967 (light intensity was 11,400 ft-c). One weight of water was used in the homogenization by mortar and pestle. Twenty ml of the extract was fractionated at 0°. The material precipitated by the slow addition of 6.0 ml of cold acetone was collected by centrifugation and discarded. The slow addition of another 10.0 ml of cold acetone to the supernatant precipitated aconitase and the phosphatase, which were collected by centrifugation. The precipitate was carefully washed with water and resuspended in 2 ml of cold 0.02M cacodylate, pH 6.3 . This suspension was recentrifuged to give a clear active supernatant.

DEAE which had been prepared as described by Peterson and Sober (192) was suspended in 0.02M cacodylate, pH 6.3, and the pH was adjusted to 6.3. This DEAE was washed with about 40 ml of cold 0.02M cacodylate, pH 6.3, in a 0.7 cm diameter column that was 3 cm high after washing. 0.25 ml of the enzyme from the acetone fractionation was layered onto the DEAE column and was eluted with 20.0 ml of 0.02M cacodylate, pH 6.3, containing NaCl in a linear gradient from 0.00M to 0.50M. Twenty-four fractions of about 0.8 ml each were collected.

o——o P-glycolate phosphatase.

△——△ Aconitase. The assays were as described for Figure 34, except the reactions were initiated by the addition of 80 µl of cold enzyme.

Both enzyme activities, expressed as μmoles of substrate converted/min/ml of enzyme, were normalized, with the activity in the most active fraction designated as 100%. The most active fraction (2) had the following activities under the assay conditions which were used: 6.7 μmoles of P_i released/min/ml of enzyme and 0.080 μmoles of cis-aconitate converted/min/ml of enzyme.

The yield of phosphatase for the acetone fractionation was 90%, and for the DEAE-cellulose column chromatography (fraction 2), 69%, giving an overall yield of 63%. The corresponding figures for aconitase were 378%[1] and 19%, giving an overall yield of 72%. The overall purifications for the phosphatase and aconitase in fraction 2 were 20 and 23-fold respectively.

[1] See pp. 334-337.

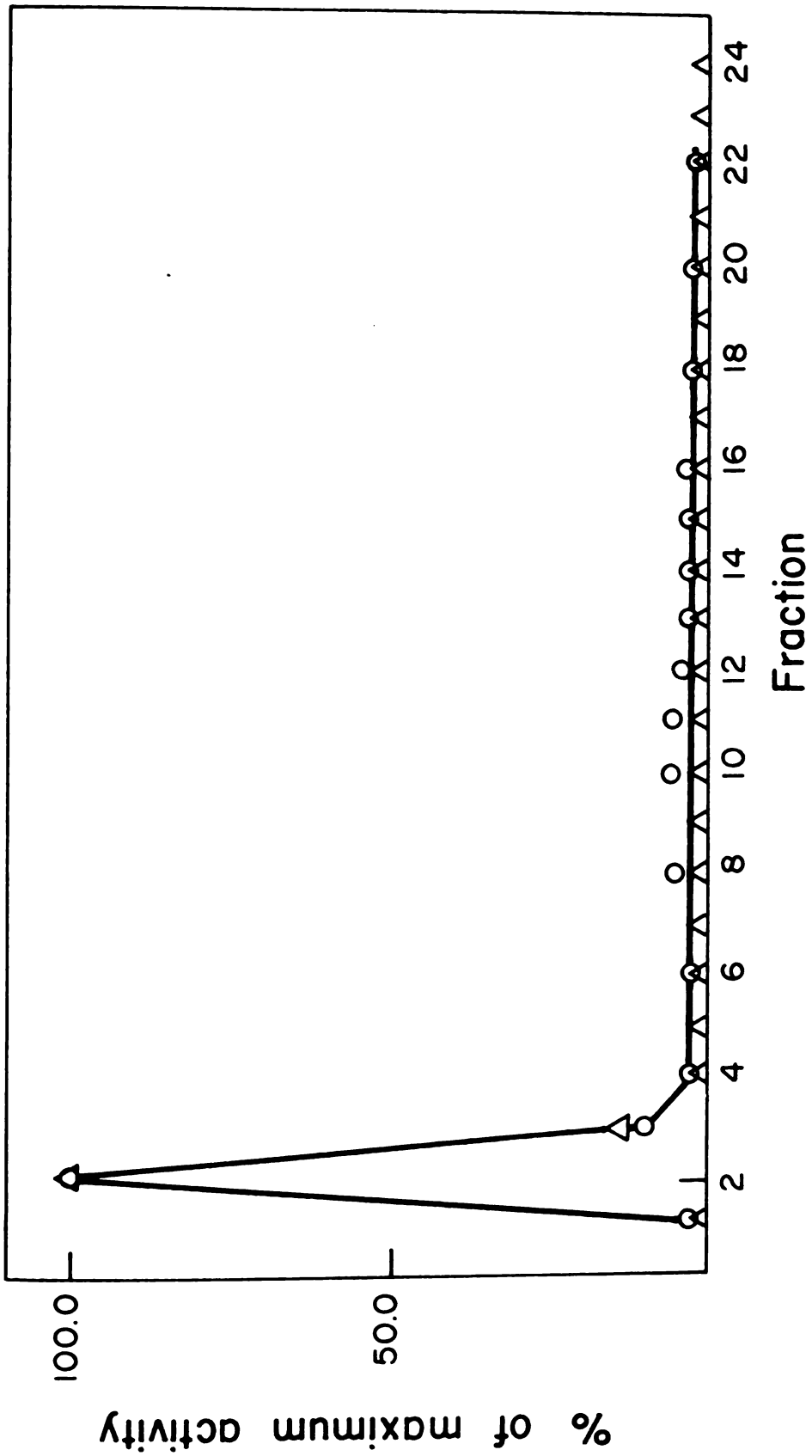

% of maximum activity
100.0
50.0
2
4
6
8
10
12
14
16
18
20
22
24
Fraction

Figure 38

DEAE-Cellulose Chromatography of P-glycolate Phosphatase and Aconitase (Leaves Harvested from the Growth Chamber During the Dark)

The tobacco leaves were harvested from the growth chamber at 3 A.M. on 2/6/1967 (the growth chamber had been dark for 8 hours prior to harvest). During the period of 6 to 7 minutes that the leaves were transported through the halls and were prepared for homogenization in the cold room, they were exposed to light of about 10 to 100 ft-c. After centrifugation (15,000 g, 8 min), the extract, which had a pH of 6.2, was adjusted to pH 7.6. Then 39 ml of cold acetone was slowly added with stirring to 39 ml of this extract. The precipitate was collected by centrifugation (6000 g, 8 min), washed with H_2O, and resuspended in 4.0 ml of H_2O. The suspension was recentrifuged (15,000 g, 6 min) to give an active clarified supernatant. A small DEAE-cellulose column had been prepared as described for Figure 37, except the DEAE had been suspended in H_2O, adjusted to pH 7.5, and washed with about 100 ml of H_2O.

4.0 ml of the enzyme preparation from the acetone precipitation was layered onto the DEAE column, and was eluted with 20.0 ml of water containing NaCl in a linear gradient from 0.00M to 0.50M. Thirteen fractions of about 1.5 ml each were collected.

○——○ P-glycolate phosphatase, by the standard assay.

△——△ Aconitase. The reaction mixtures contained the following in a volume of 2.0 ml: 1.5×10^{-4}M Na cis-aconitate, pH 7.5, and either 200 or 700 µl of enzyme. The reactions were initiated by the addition of enzyme.

Much of the aconitase and phosphatase activity did not stick to the DEAE and emerged with the first protein peak in fractions 1 and 2. Only the activities which stuck to the DEAE and were eluted with NaCl were normalized. The activity in the most active eluted fraction was designated as 100%. The aconitase activity in fraction 1 was 700% of the maximum normalized aconitase value (in fraction 5), while the P-glycolate phosphatase activity in fraction 1 was 123% of the maximum normalized phosphatase value (in fraction 6). Before they were normalized, all activities were expressed as μmoles of substrate converted/min/ml of enzyme.

The yield of phosphatase for the acetone precipitation was 73%, and the recovery from the DEAE-cellulose column (in all 13 fractions) was 65%. The corresponding figures for aconitase were 135% and 20%.

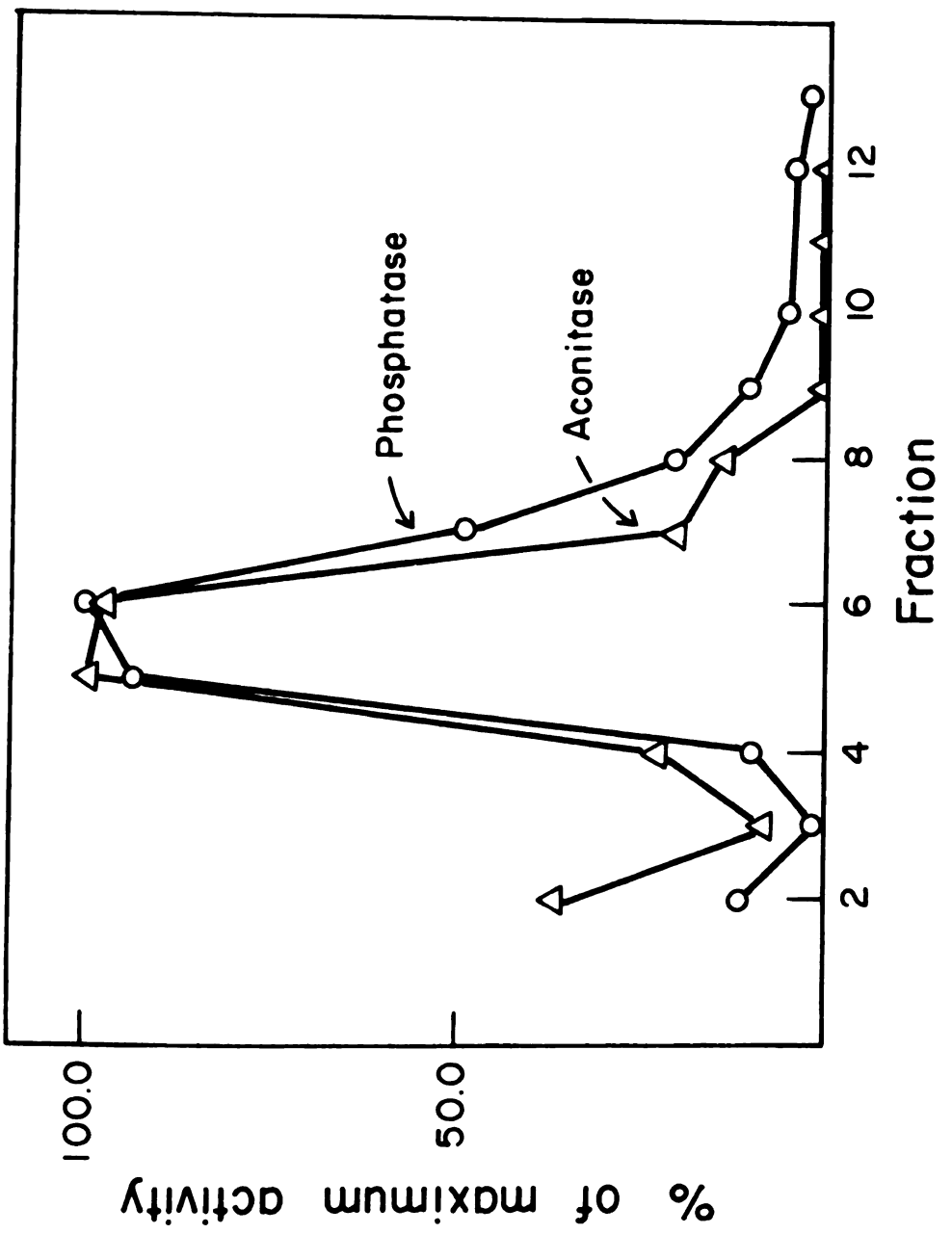

% of maximum activity
100.0
50.0
Phosphatase
Aconitase
2
4
6
8
10
12
Fraction

Figure 39

DEAE-Cellulose Chromatography of P-glycolate Phosphatase, Aconitase, and Isocitrate (Leaves Harvested From the Field During the Night)

Up until the time of assaying the fractions from the DEAE-cellulose, the experiment was performed as described for Figure 37, with the following exceptions. The tobacco leaves were harvested at midnight, 7/12/1967, and were kept in darkness until after the homogenization. The homogenization was with two weights of water. The centrifugation of the sap was at 15,000 g for 15 minutes. Again the fractionation was from 0-3 ml and 3-8 ml of acetone/10 ml of extract, with the latter precipitate containing the two enzymes. EDTA was used in the first steps when the DEAE was prepared as described by Peterson and Sober (192). But as in Figure 37, EDTA was not used when the DEAE column itself was washed. A 0.75 ml aliquot of the enzyme preparation from the acetone fractionation was applied to the DEAE column 21 hours after the enzyme was precipitated by acetone. Twenty-nine fractions of about 0.7 ml each were collected.

O——O P-glycolate phosphatase by the standard assay.

△——△ Aconitase. Reactions were initiated by the addition of cis-aconitate, pH 7.5, (final assay concentration of 2.5×10^{-4}M) to the reaction mixtures, which included 80 µl of enzyme.

■——■ Aconitase. Reactions were initiated by the addition of DL-isocitrate, pH 7.5, (final assay concentration of 10^{-2}M. The concentration of the natural threo-Ds-isomer was therefore 5×10^{-3}M) to the reaction mixtures containing 80 µl of enzyme.

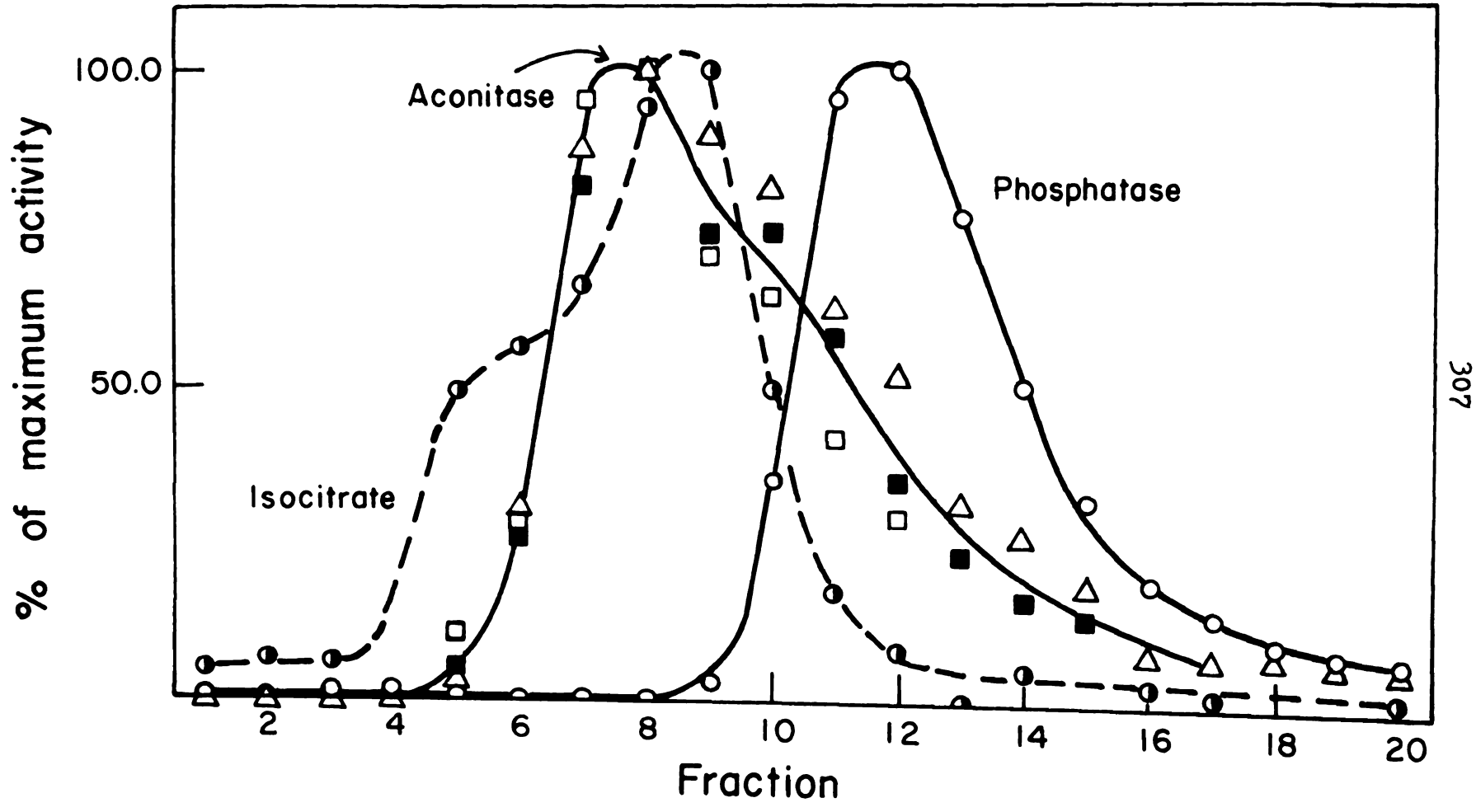
□——□ Aconitase. Reactions were initiated by the addition of citrate, pH 7.5, (final assay concentration of 3×10^{-2}M) to the reaction mixtures containing 160 µl of enzyme.

All reaction mixtures for the assay of aconitase also contained 0.033M TES, pH 7.5, and 0.02M ammonium sulfate in the reaction volume of 1.20 ml.

◑---◑ Isocitrate. Aliquots of 150 µl of the designated fractions (250 µl of fraction 8) were added to 6 x 50 mm test tubes, followed by 6 µl of 1.0N HCl. After 11½ hours at 0-4°, 6 µl of 1.0N NaOH was added to each 156 µl mixture. The aliquot from fraction 8 was checked for aconitase activity before and after the acidification and neutralization. Although aconitase was very active before the acid-base treatment, there was no detectable aconitase activity after the treatment. Enzymatic determinations of isocitrate, as described in the methods section, were made on the aliquots. Except for the aliquots from fractions 3 and 4, the blank absorbances did not increase with time, which indicates that there was no endogenous isocitrate dehydrogenase activity in these aliquots. For an unknown reason, the blank absorbances increased with time for the aliquots from fractions 3 and 4. For fraction 4, the time dependent increase for the blank was so great that the value for the concentration of isocitrate was discarded.

Isocitrate recovery was tested, from one fraction, essentially as described in the legend of Figure 16, except that the stock isocitrate was added before the addition of HCl. This test aliquot, which was taken from fraction 20, was then treated as the other aliquots had been treated. The amount of isocitrate which was recovered was the same as the amount added.

Fractions 21 through 29 contained no significant amounts of P-glycolate phosphatase, aconitase, or isocitrate. The enzymes were eluted at the following NaCl concentrations: aconitase, 0.07M; phosphatase, 0.14M. Before they were normalized, the activities were expressed as µmoles of P-glycolate hydrolyzed, cis-aconitate converted, or cis-aconitate formed/min/ml of enzyme. Fraction 8 had the following activity: 0.07 µmole of cis-aconitate converted/min/ml of enzyme. The concentration of isocitrate in fraction 9 was 3.2×10^{-5}M. Fraction 12 had the following activity: 8.0 µmoles of P_i released/min/ml of enzyme. The yield of phosphatase for the acetone fractionation was 94% and from the DEAE-cellulose column (fractions 10 through 15) 88%, giving an overall yield of 83%. The corresponding figures for aconitase were 45% (24% of this was lost during the 21 hours of storage at 0°) and 91% (fractions 6 through 13), giving an overall yield of 31%. The purification of the phosphatase was 20-fold from the acetone fractionation and 2.9-fold in the 6 fractions from the DEAE-cellulose column giving an overall purification of 58-fold. The corresponding figures for aconitase were 9.5-fold (7.3-fold after the 21 hours of storage) and 2.5-fold (in the 8 fractions) giving an overall purification of 18-fold.


100.0
50.0
% of maximum activity
Aconitase
Isocitrate
Phosphatase
2
4
6
8
10
12
14
16
18
20
Fraction

cellulose of purified enzyme which had come from field grown tobacco leaves harvested in the light (Figures 16 and 17).

Although the data suggest that exposure of leaves to light or dark before harvest and homogenization could be a factor in the determination of whether aconitase and the phosphatase or isocitrate and the phosphatase fractionate together, and in the determination of the salt concentration required to elute these enzymes during DEAE-cellulose chromatography, such effects of light are at present only hypotheses. Since effects of light were not anticipated, the experiments discussed here were not adequately controlled with respect to the possible effects of light. But no matter what factors determined whether isocitrate was to fractionate with the phosphatase as in Figures 16 and 17, or separately from the enzyme as in Figure 39, and whether the two enzymes were to fractionate together in a significantly parallel fashion as in Figures 34 through 38, or separately from each other as in Figure 39, all of the data suggest that the parallel fractionation of isocitrate and P-glycolate phosphatase is closely related to the parallel fractionation of aconitase and P-glycolate phosphatase.

Evidence for Aconitase Activity

Cis-aconitate was used as substrate in most of the experiments. A cis-aconitate dependent decrease in absorption at 240 mμ is not definitive evidence for conversion

to citrate or isocitrate. However, the activities with citrate, isocitrate, and cis-aconitate essentially paralleled each other (Figure 39), which suggests that the enzyme was aconitase. Furthermore, the enzyme fractionated with the phosphatase, and relatively large amounts of endogenous citrate, isocitrate, and cis-aconitate also fractionated with the phosphatase.

If the aconitase function required two enzymes in tobacco leaves, for the conversion of citrate to isocitrate through cis-aconitate, or if aconitic hydrase (169, 170, 171) were also present, then with cis-aconitate as substrate, two activity peaks might be expected from fractionations. But, in all the fractionations with cis-aconitate as substrate (see for example Figures 34 through 39), except in the case of a DEAE-cellulose column being overloaded, the activity fractionated as a single peak, suggesting that in tobacco leaves, this activity is from a single protein species. From the previous paragraph, this single protein was aconitase. Thus, the fractionations provided no evidence for the presence in tobacco leaves of aconitic hydrase, or for the aconitase function requiring two enzymes. Palmer (185) has presented evidence that aconitase from mustard is a single protein species, and a great amount of evidence has been presented that the aconitase of animal tissues is a single protein species, with the interconversion between all three substrates

being catalyzed by a single active site (literature review).

The Stability of Aconitase

Under aerobic conditions, aconitase in some fresh extracts from field grown tobacco leaves became inactive at a rapid rate (Figures 40 and 42).[1] Extrapolation of the plots to the time of homogenization permitted the estimation of the post homogenization time required for aconitase to lose half the activity it had at the time of homogenization ("$t_{\frac{1}{2}}$"). In the experiments of Figures 40 and 42, this time was approximately 2 hours. In three other similar experiments, the $t_{\frac{1}{2}}$ values from an experiment in August and from one in September were also approximately 2 hours, but the $t_{\frac{1}{2}}$ from a July experiment was about 24 hours. In all 5 experiments, although the tobacco leaves were harvested during daylight hours, the time of day for the harvest did not seem to be a determining factor in the rate of inactivation of aconitase. In three experiments in February, March, and April, conducted with leaves from Swiss chard grown in the growth chamber or greenhouse, the inactivation rates of aconitase were greatly reduced. Furthermore, when an aliquot of the extract from one of these latter experiments was mixed for

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[1] Breidenbach and Beevers found that aconitase in crude preparations from the endosperm of germinated castor beans also lost activity rapidly at 0° (36).

2 minutes under aerobic conditions in a Waring blendor, the activity of the aconitase was not affected. Thus, under aerobic conditions, the inactivation rate of aconitase in fresh extracts from leaves may in part be a function either of the kind of plant or the conditions used for the culture of the plants.

When the extracts from tobacco leaves were made 10^{-2}M with glycolate, the inactivation rate increased significantly, while making the extracts 10^{-2}M with OHPMS greatly decreased the rate of inactivation (Figure 40). Gassing fresh extracts with N_2 also resulted in a decreased rate of inactivation of aconitase compared to a control left under aerobic conditions (data not shown). The data suggest the possibility that glycolate oxidase and its endogenous substrate(s) are involved in the rapid inactivation of aconitase in extracts from tobacco leaves.

In those experiments in which both the activity of aconitase and the stability toward dilution at 30° of P-glycolate phosphatase were assayed in fresh extracts from field grown tobacco leaves, the phosphatase began to stabilize at about the same time that aconitase became inactive (Figure 42). This suggests the possibility that active aconitase may have been necessary to maintain the phosphatase in the reduced state in these extracts, either as a redox buffer, or by furnishing a metabolite which kept the phosphatase reduced.

Figure 40

Inactivation Rate of Aconitase

Tobacco leaves were harvested from the field at 11 A.M. on 9-23-1967 (light intensity was 10,600 ft-c). Thirty minutes after the homogenization, preincubation mixtures were made up as follows:

Description	ml H_2O	ml Glycolate	ml OHPMS	ml Extract
Control	0.400	-	-	3.600
Glycolate	-	0.400	-	3.600
OHPMS	-	-	0.400	3.600

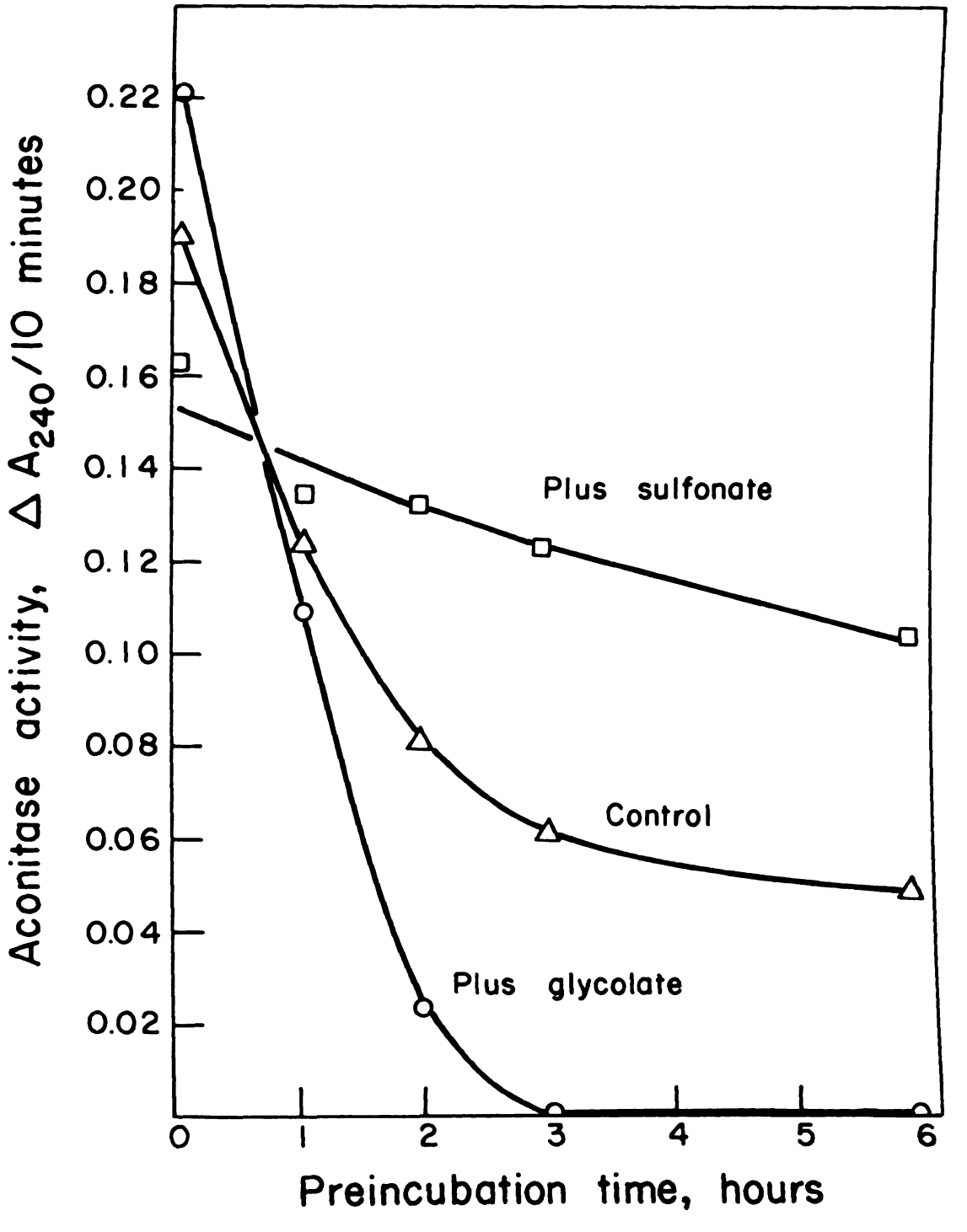
The stock glycolate and OHPMS solutions were both 0.10M, pH 7.0, so that the concentration of these compounds was 10^{-2}M during the preincubations. The preincubation mixtures were exposed to the air in medium sized test tubes, at 0^{o}.

△——△ Control.

○——○ Preincubation mixture containing glycolate.

□——□ Preincubation mixture containing OHPMS.

All reaction mixtures contained the following in a reaction volume of 1.20 ml: 0.033M TES, pH 7.5, 0.02M ammonium sulfate, and 2.5 x 10^{-4}M cis-aconitate. All reactions were initiated by the addition of 30 µl of the appropriate preincubation mixture.


Aconitase activity, Δ A240/10 minutes
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
Plus sulfonate
Control
Plus glycolate
0
1
2
3
4
5
6
Preincubation time, hours

Although exposure to the atmosphere of aliquots of fresh extracts from field grown tobacco leaves resulted in inactivation of aconitase compared to a control aliquot held under nitrogen, this exposure caused the enzyme to become more stable toward dilution at 30° (Figure 41).[1] Buzzing of such aliquots under air further decreased the activity and further increased the stability toward dilution at 30°. Not only did the activity after dilution at 30° increase as a percent of the activity before dilution, but also the absolute value of the activity after dilution at 30° increased. Only when aliquots were buzzed under O_2 did the activity before dilution decrease to such an extent that the absolute value of the activity after dilution at 30° decreased (Figure 41).

When aliquots of the extracts were gassed with CO_2, the aconitase was essentially completely inhibited (Figure 41).[2] Attempts to reverse this inhibition by CO_2 by regass-

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[1]The initial stability toward dilution at 30° of aconitase in fresh extracts from leaves harvested in the summer from field grown tobacco was 10% to 20%. However, in other experiments in March and April with the leaves of Swiss chard grown in the growth chamber or greenhouse, the stability of aconitase toward dilution at 30°, in 5 separate determinations, varied from 101% to 113%. Thus, the stability toward dilution at 30° of aconitase in fresh extracts from leaf tissue may be a function, at least partially, either of the kind of plant, or the conditions used for the culture of the plants.

[2]Carbon dioxide as the bidentate carbonate ion participates in inorganic complex formation of transition metal ions, and carbon dioxide facilitates the binding of iron to transferrin and conalbumin (1). The importance of iron in aconitase from animal tissues has been well documented, but the role of iron in the enzyme from plants remains obscure (literature review and pp. 321-333). Dickman and Cloutier found that although bicarbonate did not inhibit animal aconitase, it did prevent the reactivation

Figure 41

Effect of Gasses of the Atmosphere on Aconitase

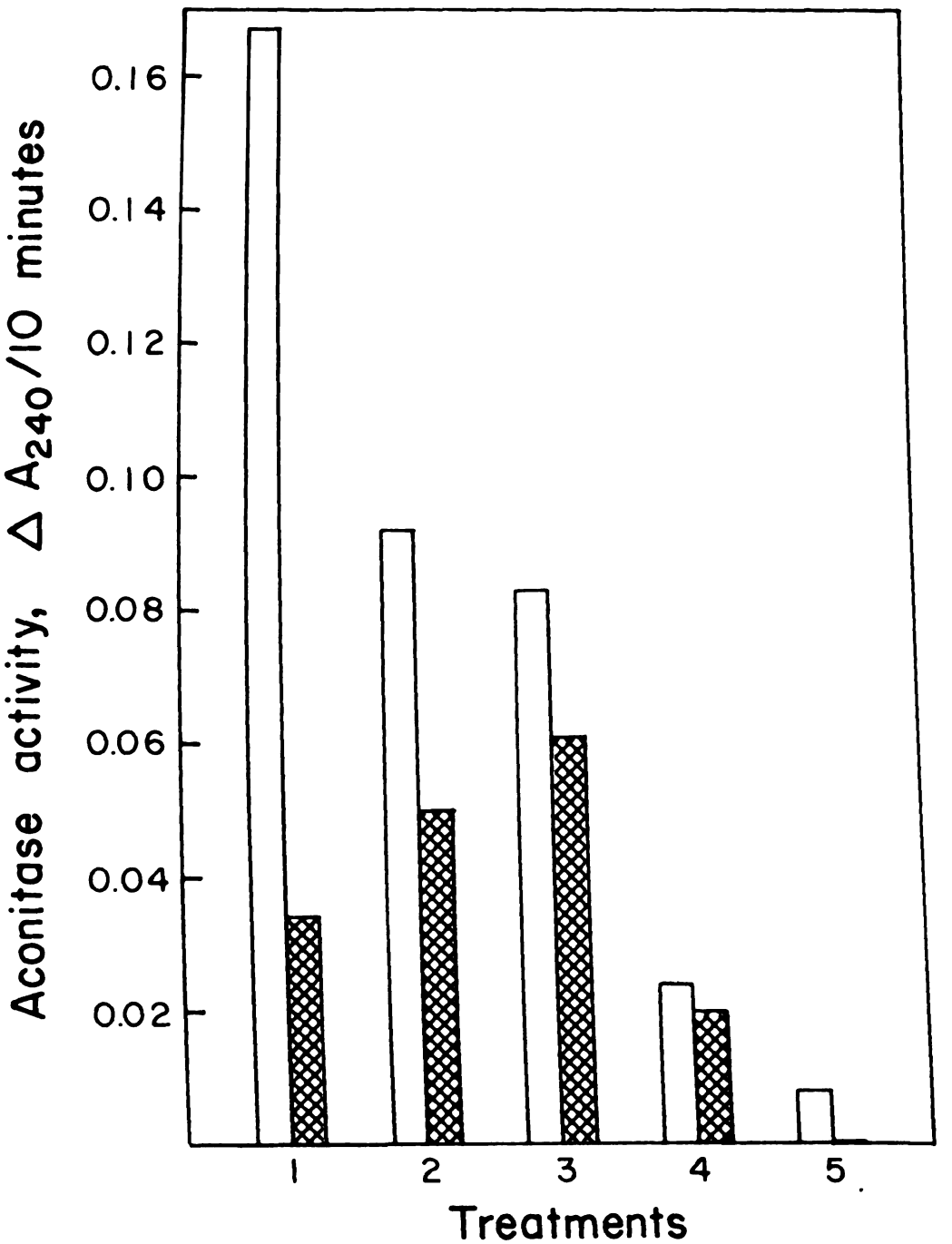
The five aliquots were those prepared for the experiment described in Figure 22 and were as follows:

1. Gassed with N_2. Buzzed.
2. Left open to the atmosphere. Not buzzed.
3. Left open to the atmosphere. Buzzed.
4. Gassed with O_2. Buzzed.
5. Gassed with CO_2. Buzzed.

All aconitase reaction mixtures contained 0.033M TES, pH 7.5, 0.02M ammonium sulfate, 2.5×10^{-4}M cis-aconitate, pH 7.5, and 30 µl of the appropriate extract, in a final reaction volume of 1.20 ml.

Open bars: The reactions were initiated by the addition of cold enzyme.

Closed bars: The reaction mixtures (in the reaction cuvettes), less substrate but including enzyme, were held in a 30° bath for one hour, then in the 25° chamber of the Beckman D.U. spectrophotometer for 10 more minutes. The reactions were then initiated by the addition of 30 µl of 10^{-2}M cis-aconitate.


Aconitase activity, Δ A240/10 minutes
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
1
2
3
4
5
Treatments

ing with N_2 were unsuccessful (data not shown). The inhibition by CO_2 is of interest in view of the photorespiratory evolution of CO_2 in tobacco and the possibility of the inhibition of aconitase in some plants in the light (literature review, and pp. 334-337).

The effects of the various treatments on aconitase (Figure 41) were qualitatively the same as the effects of the same treatments on P-glycolate phosphatase in the same aliquots (compare Figure 22 with Figure 41). But quantitatively, a large effect on one enzyme was accompanied by a small effect on the other. Thus, in these aliquots, oxygen caused a great decrease in the activity of aconitase and a small decrease in the activity of the phosphatase, it caused a great increase in the activity after dilution at 30° of the phosphatase and a smaller increase for aconitase, and carbon dioxide caused a great decrease in the activity of aconitase, and a smaller decrease in the activity of the phosphatase.

Precipitation of aconitase from extracts by acetone greatly decreased the rate of inactivation of the enzyme (Figure 42). However, acetone precipitated aconitase stored under aerobic conditions was still not nearly as stable as similarly prepared P-glycolate phosphatase stored under the same conditions. For example, when stored at 0° under aerobic conditions, aconitase which had been purified by a single acetone fractionation and resuspended in 0.02M cacodylate, pH 6.3, lost about ¼ of its

Figure 42

The Effect of the Precipitation by Acetone on Aconitase and P-glycolate Phosphatase

Tobacco leaves were harvested from the field at 1:30 P.M. on 8-9-1967 (light intensity was 4400 ft-c). An aliquot of the extract was fractionated with acetone essentially as described in Figure 37. Eight ml of the acetone purified preparation was placed in a 20 ml beaker, and 8 ml of the remaining extract was placed in a second 20 ml beaker. Both aliquots were held near 0°, under air, and were stirred about every 3/4 hour during the course of the experiment.

Figure A, extract.

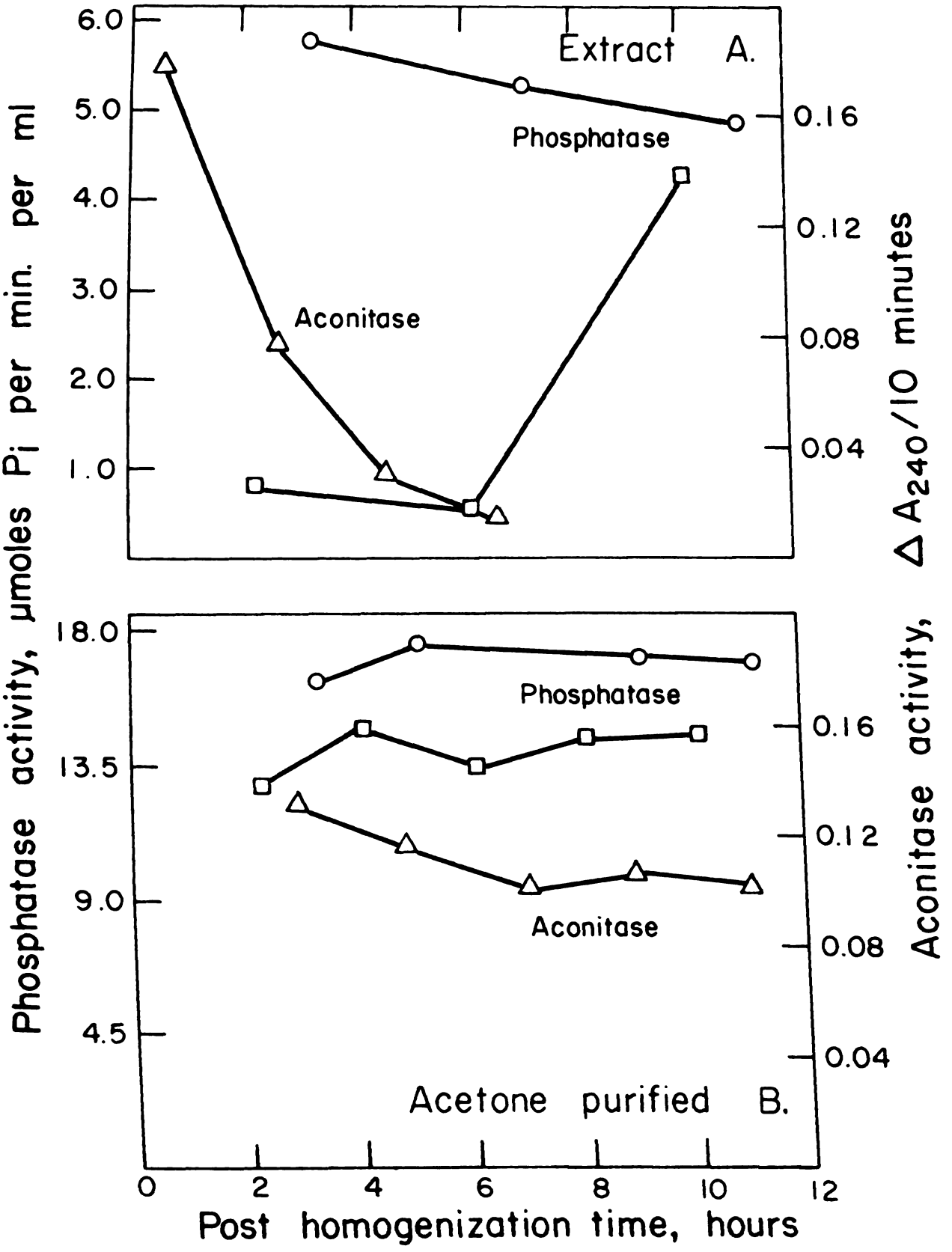
Figure B, acetone purified enzyme.

○——○ Phosphatase, controls.

□——□ Phosphatase, activity after 33 1/3-fold (Figure A) or 125-fold (Figure B), dilution at 30°.

△——△ Aconitase. All reaction mixtures contained 0.033M TES, pH 7.5, 0.02M ammonium sulfate, and 2.5×10^{-4}M cis-aconitate, pH 7.5, in a reaction volume of 1.20 ml. Reactions were initiated with 30 µl of extract of 10 µl of acetone precipitated enzyme.

The fractionation by acetone was performed from 45 minutes to 2 hours and 15 minutes after the homogenization, i.e. the fractionation was half over at 1½ hours after the homogenization. Based on the aconitase activity in the extract and in the acetone purified fraction (the latter obtained by extrapolation) at 1½ hours after homogenization, the yield of aconitase in the acetone purification step was 75% while the purification of the enzyme was 13.6-fold. The corresponding figures for the phosphatase are 84% and 15.4-fold.


Extract A.
Phosphatase
Aconitase
Phosphatase activity, μmoles Pi per min. per ml
Δ A240/10 minutes
6.0
5.0
4.0
3.0
2.0
1.0
0.16
0.12
0.08
0.04
Acetone purified B.
Phosphatase
Aconitase
Aconitase activity,
18.0
13.5
9.0
4.5
0.16
0.12
0.08
0.04
0
2
4
6
8
10
12
Post homogenization time, hours

activity in about 1 day (legend of Figure 39). This result was fairly typical for the enzyme from tobacco, and also for aconitase from Swiss chard at the same degree of purification and stored under essentially the same conditions. By contrast, when the phosphatase from tobacco was purified and stored in essentially the same way, it lost little or no activity in 2 weeks (p. 177).

When aconitase was fractionated by acetone, the enzyme in the various fractions did not lose activity at the same rate. For example, in the fractionation described in Figure 35, the activity ratio of fractions 3-5:5-6 was 0.68 in the first set of assays made soon after the fractionation, but gradually increased to 0.85 several hours later. The values plotted are those which were obtained immediately after fractionation. Also, using the aconitase activities which prevailed at the time of precipitation by acetone gave better matching between aconitase and phosphatase yields and purifications than would otherwise have been possible (legend of Figure 42).

In the experiment depicted in Figure 37, the rate of inactivation of aconitase in the most active fraction from the DEAE-cellulose column (fraction 2) was compared with the rate for the enzyme which had been fractionated by acetone, and which was applied to the column. Aconitase in fraction 2 was found to have a considerably greater inactivation rate than the enzyme which was applied to the column. Instability of aconitase after

DEAE fractionation under the conditions which were used may account for the pattern of activities exhibited by the trailing half of the aconitase peak shown in Figure 39. In this experiment, all the assays with cis-aconitate were performed first, followed by the assays with isocitrate, followed finally by the assays with citrate. All the aconitase assays took a total of 6 hours. Thus, it appears that the greatest relative activities with cis-aconitate, the intermediate activities with isocitrate, and the lowest relative activities with citrate which were observed were a consequence of the inactivation of aconitase during the 6 hour period. In particular, the pattern cannot be explained by two enzymes, one catalyzing the interconversion of citrate and cis-aconitate, and the other catalyzing the interconversion of cis-aconitate and isocitrate. Such an explanation would require that the activities with cis-aconitate as substrate would be between those obtained with the other two acids as substrate.

Partial Reactivation of Aconitase

Partially purified aconitase from field grown tobacco leaves was reactivated 8-fold by preincubation with fresh cysteine and ferrous ions (Figure 46). In solutions extracted from the acetone powder described in Figure 46, the ratio of P-glycolate phosphatase to aconitase activity, after reactivation, was 409. (Each activity

was expressed as μmoles of substrate converted/minute/ml of solution.) Although aconitase activities were not measured during the purification of this preparation, the average phosphatase to aconitase activity ratio of 19 (p. 289) in fresh extracts permits the estimation that the most active of the reactivated aconitase (Figure 46) had approximately 19/409 or 5% of the activity relative to the phosphatase that it had in the fresh extracts. In an experiment with Swiss chard, a preparation which had been precipitated by acetone and passed through a Sephadex G-10 column (fraction 1 of Figure 31) lost 99½% of its activity while stored at 0° in air for 3½ days.[1] (An aliquot which was not passed through the G-10 column retained a substantial amount of activity after the 3½ days period. Thus, the G-10 gel filtration chromatography not only removed aconitase activators, but rendered the protein less stable to storage at 0° under aerobic conditions.) The preparation containing ½% of its fresh activity was activated 20 fold by preincubation at 0° for 1½ hours with 10^{-2}M cysteine and 5 x 10^{-3}M ferrous ammonium sulfate. However, the reactivated activity was only 11% of the fresh activity.

Under the preincubation conditions described in Figure 46, the system was essentially saturated with Fe^{++}

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[1]All assays included $SO_4^=$ in the reaction mixtures. Thus, the loss of activity was not due to loss of anions.

at 10^{-3}M. In the same experiment, preincubation with either cysteine (10^{-2}M) or Fe^{++} (4×10^{-3}M) alone resulted in no activation of the aconitase, and inclusion of Zn^{++} (10^{-3}M) in the preincubation mixture with Fe^{++} and cysteine almost completely inhibited the reactivation (data not shown). Cysteine alone in the preincubation mixture also failed to reactivate the aconitase from Swiss chard, while Mg^{++} plus cysteine or Mn^{++} plus cysteine did not give significant reactivation compared to the results with Fe^{++} plus cysteine.

The reactivation with cysteine and iron required an alkaline pH (Figure 43). Of the concentrations of fresh cysteine tested, 10^{-2}M during preincubation was found to be optimal. Higher concentrations of cysteine resulted in less reactivation (Figure 44). One hour at 0° was found to be an adequate preincubation time (Figure 45).

In a preliminary experiment, glutathione, thioglycolate, Cleland's reagent, mercaptoethanol, and sulfide were compared with cysteine (all in combination with iron) in the reactivation of aconitase. Of these 6 compounds, cysteine was found to be the most effective. Ascorbate was also found to be considerably less effective than cysteine.

Cysteine alone in the reaction mixtures resulted in partial inhibition of aconitase. For example, cysteine at 5×10^{-4}M gave about 10% inhibition. Inhibition by

Figure 43

Reactivation of Aconitase as a Function of the pH During Preincubation

Samples of enzyme as an acetone powder (described on p. 140 under "second acetone fractionation"), weighing 14.2 mg each, were separately suspended in six 0.53 ml aliquots of cold buffer, each 0.02M in glycyl-glycine and 0.02M in cacodylate. The pH values of the buffers are indicated on the horizontal axis. After 3 hours, the suspensions were centrifuged at 6000 g for 10 minutes, and the clear supernatants were saved as the non preincubated enzyme. Six preincubation mixtures of 200 µl contained 120 µl of non preincubated enzyme, a final concentration of 10^{-1}M ammonium sulfate, 10^{-2}M cysteine, and 5×10^{-3}M $FeSO_4$. Six ammonium sulfate and six 0.1M cysteine solutions had previously been made up and adjusted to the designated pH values. The preincubations were for 1½ hours at 0°.

O—O Reaction mixtures contained 50 µl of the designated preincubation mixture.

△—△ Reaction mixtures contained 30 µl of the designated non preincubated enzyme.

Other than enzyme, all reaction mixtures contained 0.033M cacodylate, pH 6.3, 0.02M ammonium sulfate, and 1.67×10^{-4}M neutralized cis-aconitate in a final volume of 1.20 ml. Reactions were initiated by the addition of cis-aconitate.

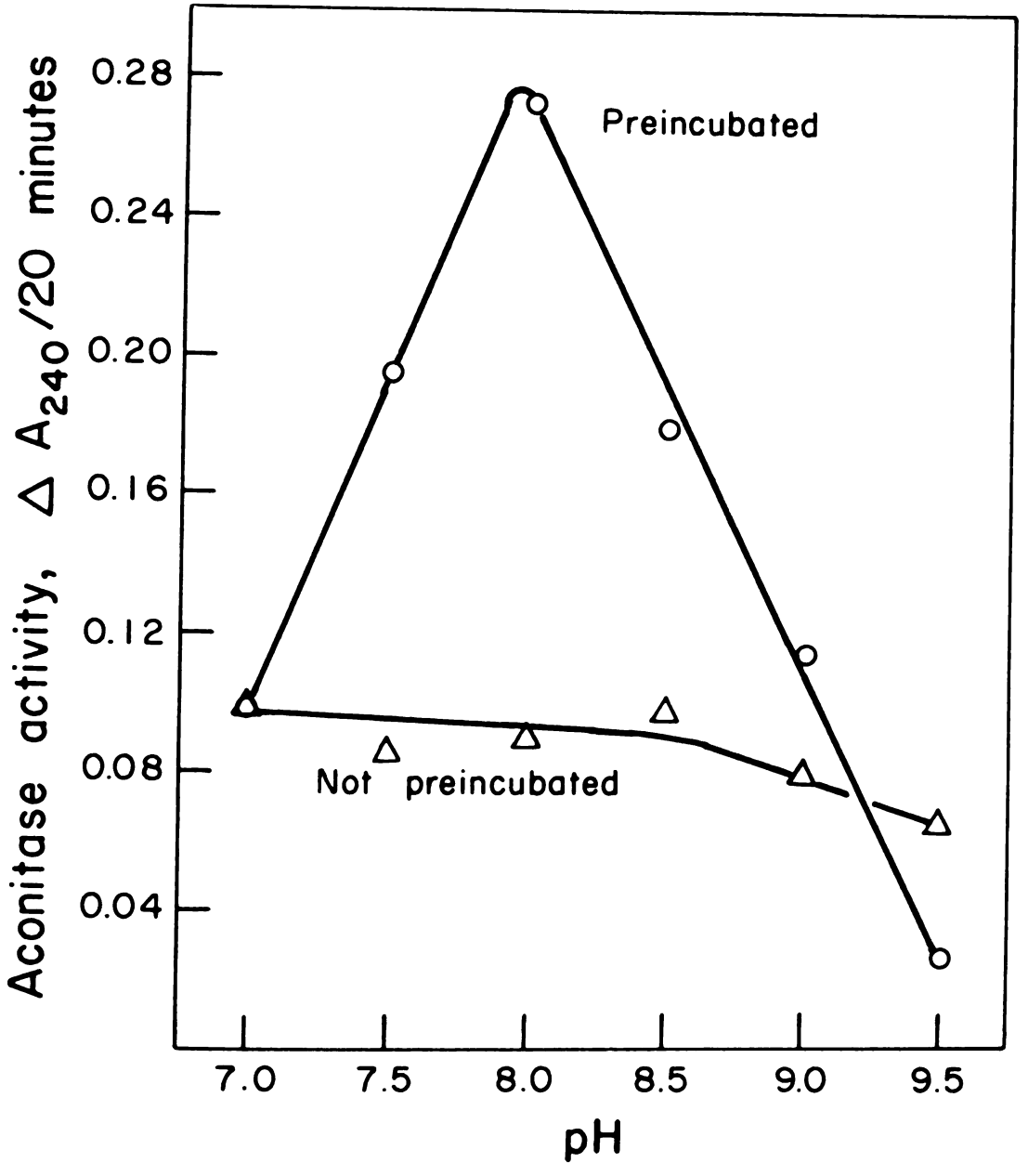

Aconitase activity, Δ A240/20 minutes
0.28
0.24
0.20
0.16
0.12
0.08
0.04
Preincubated
Not preincubated
7.0
7.5
8.0
8.5
9.0
9.5
pH

Figure 44

Reactivation of Aconitase as a Function of Cysteine During Preincubation

Enzyme as 26.8 mg of acetone powder (described on p. 140 under "second acetone fractionation") was suspended in 1.0 ml of cold pH 8 buffer, 0.02M in glycyl-glycine and 0.02M in cacodylate. After $5\frac{1}{2}$ hours at 0°, the suspension was centrifuged at 6000 g and the supernatant was saved as the non-preincubated enzyme. Preincubation mixtures of 250 µl contained 137.5 µl of non-preincubated enzyme, a final concentration of 10^{-1}M ammonium sulfate, 10^{-3}M $FeSO_4$, and cysteine at the designated molarities. The cysteine and iron solutions had been freshly prepared and the cysteine and ammonium sulfate solutions had been adjusted to pH 8. Preincubations were for 1 hour at 0°.

Aconitase reaction mixtures contained 0.033M cacodylate, pH 6.3, 0.02M ammonium sulfate, 1.67×10^{-4}M neutralized cis-aconitate, and either 55 µl of the appropriate preincubation mixture, or 30 µl of the non-preincubated enzyme, in a final volume of 1.20 ml. Reactions were initiated by the addition of cis-aconitate.

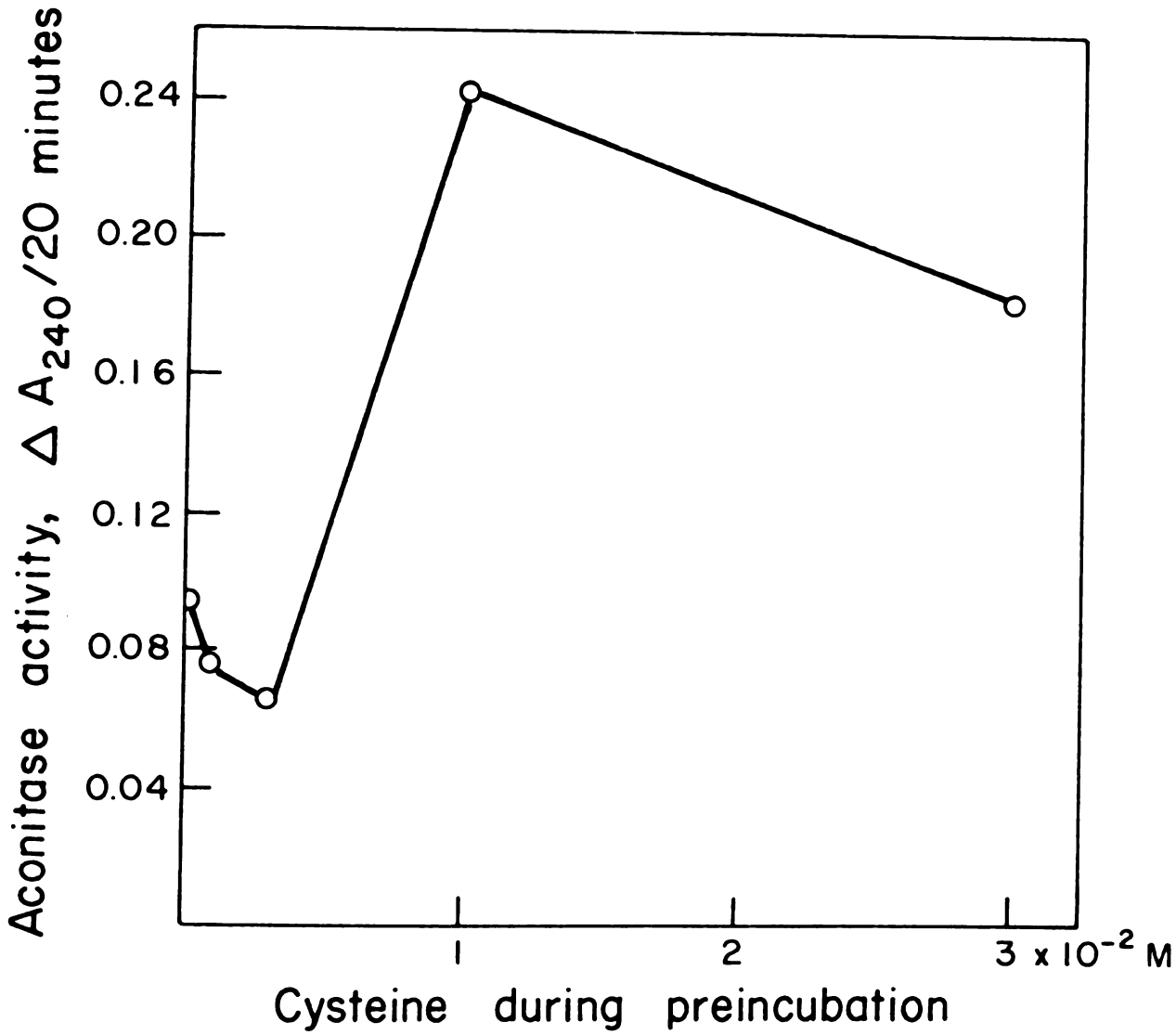

Aconitase activity, Δ A240/20 minutes
0.24
0.20
0.16
0.12
0.08
0.04
1
2
3 x 10-2 M
Cysteine during preincubation

Figure 45

Reactivation of Aconitase as a Function of Preincubation Time

Non-preincubated enzyme was prepared essentially as described for Figure 44. The preincubation mixture of 1.00 ml contained 600 µl of non-preincubated enzyme, a final concentration of 10^{-1}M ammonium sulfate, 10^{-2}M cysteine, and 4×10^{-3}M $FeSO_4 \cdot 7H_2O$. The cysteine and iron solutions had been freshly prepared, and the cysteine and ammonium sulfate solutions were adjusted to pH 8. The preincubations were for the times indicated, at 0°.

Aconitase reaction mixtures contained 0.033M cacodylate, pH 6.3, 0.02M ammonium sulfate, 1.67×10^{-4} neutralized cis-aconitate, and either 50 µl of the preincubation mixture or 30 µl of the non-preincubated enzyme in a final volume of 1.20 ml. Reactions were initiated by the addition of enzyme.

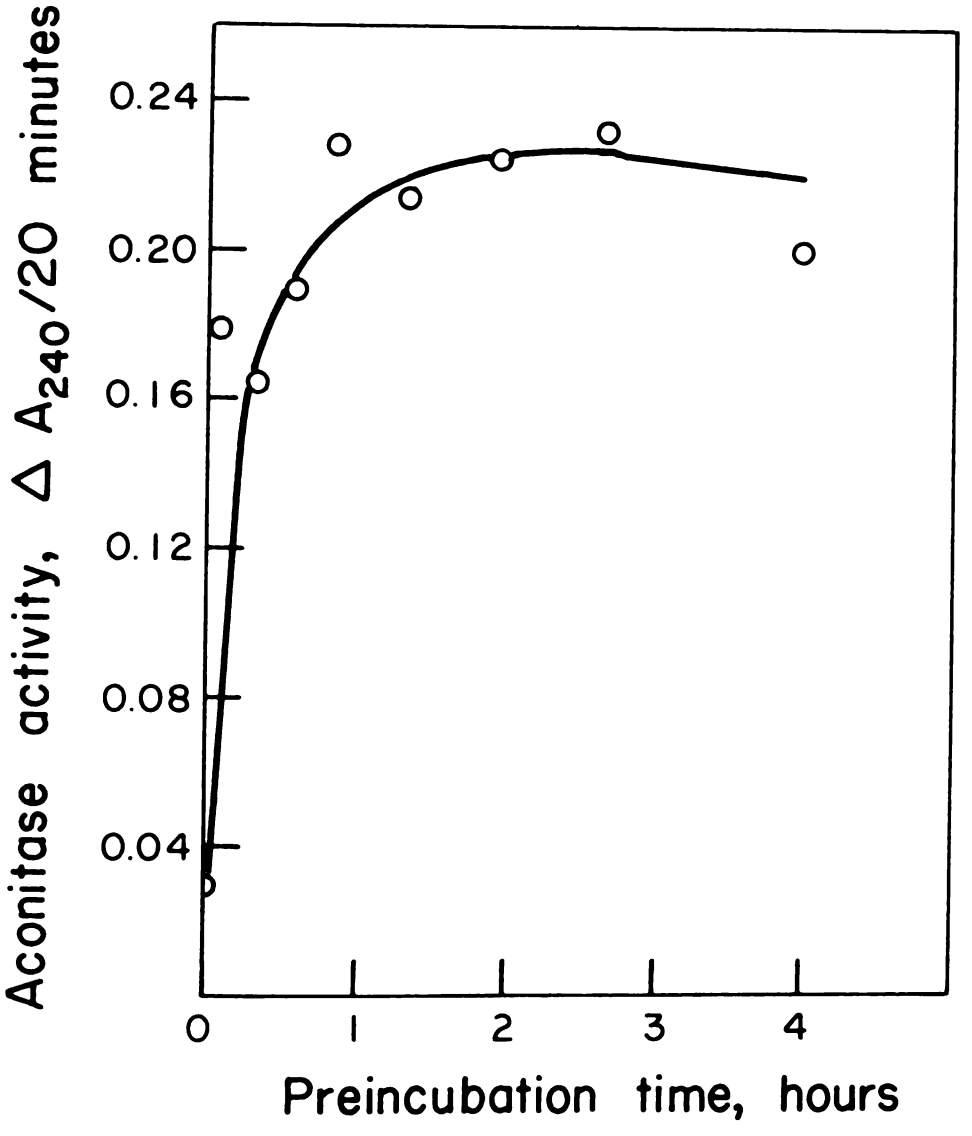
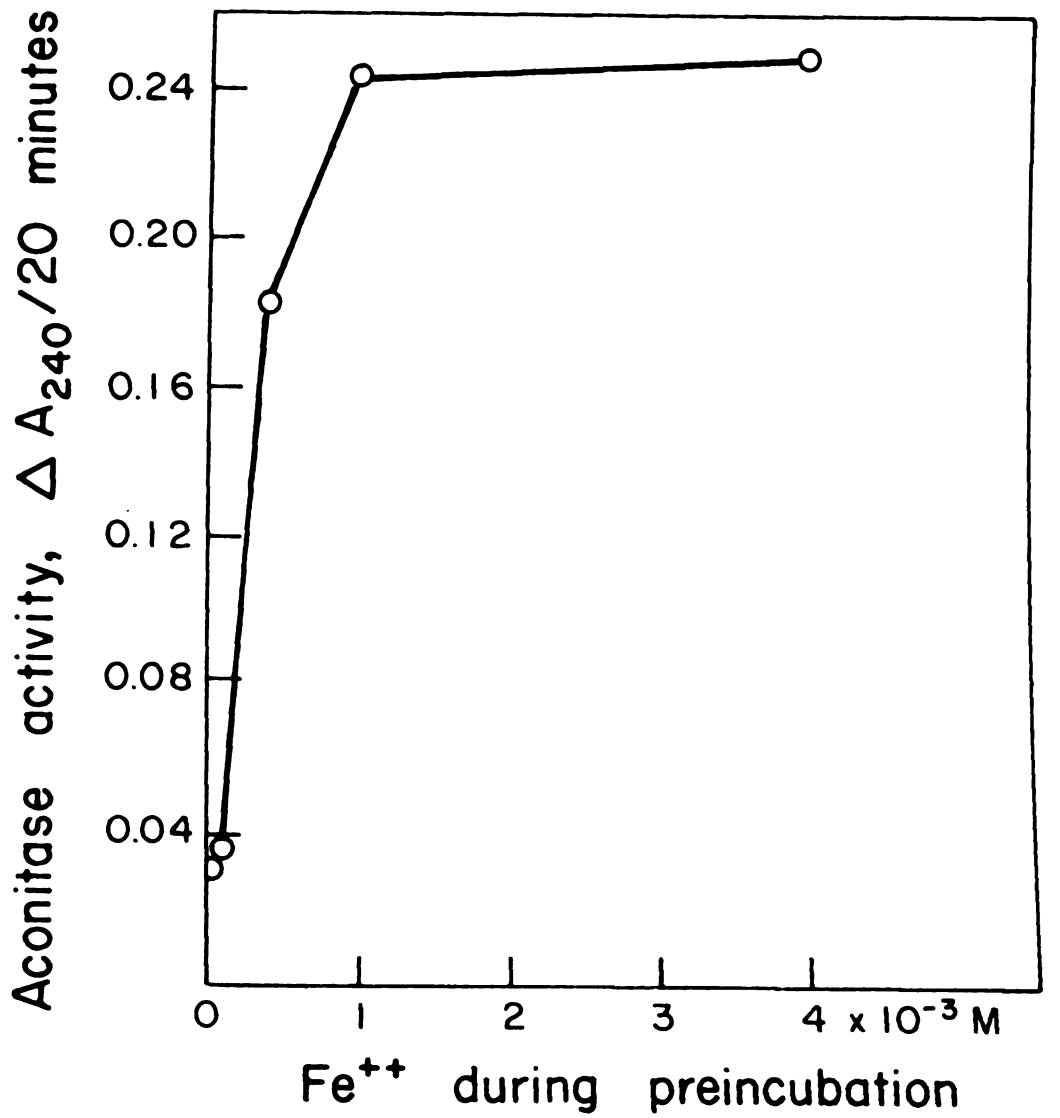

Aconitase activity, Δ A_{240}/20 minutes
0.24
0.20
0.16
0.12
0.08
0.04
0
1
2
3
4
Preincubation time, hours

Figure 46

Reactivation of Aconitase as a Function of the Iron Concentration During Preincubation

The enzyme as 54 mg of acetone powder (described on p. 140 under "second acetone fractionation") was suspended in 2.0 ml of cold pH 8 buffer, 0.02M in glycyl-glycine and 0.02M in cacodylate. After 75 minutes, the suspension was centrifuged at 6000 g for 10 minutes. The supernatant was the non-preincubated enzyme. Preincubation mixtures of 0.333 ml contained 200 µl of non-preincubated enzyme, a final concentration of 10^{-1}M ammonium sulfate (preadjusted to pH 8), 10^{-2}M freshly prepared cysteine (preadjusted to pH 8), and $FeSO_4 \cdot 7H_2O$ at the designated final concentrations. The preincubations were for 1 hour at 0^{o}.

Aconitase reaction mixtures contained 0.033M cacodylate, pH 6.3, 0.02M ammonium sulfate, 1.67×10^{-4}M neutralized cis-aconitate, and either 50 µl of the appropriate preincubation mixture or 30 µl of non-preincubated enzyme, in a final volume of 1.20 ml. Reactions were initiated by the addition of cis-aconitate.


Aconitase activity, Δ A240/20 minutes
0.24
0.20
0.16
0.12
0.08
0.04
0
1
2
3
4 x 10-3 M
Fe++ during preincubation

cysteine might explain the reduced activation from high cysteine concentrations during preincubation (Figure 44).

Although ammonium sulfate was included as a component in many of the preincubation mixtures, it was found to have little effect on the outcome of the preincubations.

When enzyme which had been reactivated by preincubation with iron and cysteine was added to otherwise complete reaction mixtures, an increase in absorption (240 mμ) occured for about 2 minutes. This increase did not occur if cis-aconitate were absent. Similar observations have been made by Herr et al (115) and by Morrison (163). As observed by the latter author, after about 2 to 3 minutes, this non enzymatic change in absorbance ceased and it was possible to distinguish the aconitase dependent change. These later values are the ones which have been plotted.

Although the role of iron in aconitase from animal tissues is well documented, its role in the aconitase from plant tissues is still not well defined (literature review). The partial reactivation of the partially purified enzyme from tobacco and Swiss chard by preincubation with iron plus cysteine is not inconsistent with the findings of Bacon et al (10), Palmer (185), and Rahatekar and Rao (198). The findings with the enzyme from tobacco and Swiss chard would be much more significant if the enzyme which lost its activity could be reactivated to its original activity. Since a ferrothiol system obtains rapid equilib-

rium with other reversible oxidation reduction systems (259), the iron and cysteine may not be acting as cofactors, but rather as a reduction system to reduce oxidized and inactive aconitase to a reduced and active form. Another possibility which is consistent with the very limited reactivation of the partially purified aconitase is that the enzyme requires an endogenous cofactor, which can be reduced by the cysteine-Fe^{+2} system, and which is gradually lost during purification of the enzyme.

The Effect of Divalent Metal Ions on the Activity of Aconitase

The effects described here were due to metals included in the reaction mixtures at the indicated concentrations. Thus, the effects were immediate, and were not because of any preincubation.

In a preliminary experiment with partially purified aconitase from Swiss chard leaves, $CaCl_2$ at 10^{-3}M gave 5% inhibition, while $MnCl_2$ at 10^{-3}M gave 20% inhibition, and at 4×10^{-3}M gave 50% inhibition. $SO_4^=$ was not included in the reaction mixtures with Ca^{++} or Mn^{++}. Between 10^{-2}M and 4×10^{-2}M, the inhibition by $MnCl_2$ was only between 30 and 50%, possibly because of the stimulatory effect of chloride at the higher concentrations. In a preliminary experiment with partially purified aconitase from tobacco leaves, the reactivated enzyme was inhibited 7% by 2×10^{-3}M $MgCl_2$. This latter reaction mixture did contain $SO_4^=$.

In several experiments with reactivated aconitase from tobacco leaves, 10^{-3}M $ZnSO_4$ gave activation, about 20% being typical. These latter reaction mixtures also contained 0.02M ammonium sulfate, so that the stimulation was seemingly due to the Zn^{++}. At concentrations as high as 10^{-2}M, $ZnSO_4$ was inhibitory in these experiments.

Experiments with Fe^{++} were difficult to interpret because iron added directly to the reaction mixtures caused more pronounced changes in absorbance at 240 mμ than did the addition of enzyme which had been preincubated with iron. Nevertheless, in several experiments, Fe^{++} at 10^{-4}M appeared to be inhibitory.

Speculation about an Endogenous Aconitase Inhibitor(s)

In reviewing many experiments, some evidence for an endogenous aconitase inhibitor was noted from those experiments in which tobacco leaves were harvested from the growth chamber[1] and in which the reaction mixtures contained no buffer or sulfate.[2] In two out of three such experiments, and in which the leaves had been harvested in the dark, when the aconitase reactions were initiated with

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[1]The extracts, after centrifugation at 15,000 g for 8 to 10 minutes, were adjusted to pH 7.5.

[2]The concentration of cis-aconitate was 1.5×10^{-4}M in these reaction mixtures.

extract, an approximately 8 minute lag, during which the reaction rate was zero, occured before the rate gradually started to increase to a maximum. In the third such experiment, no lag was noted. In all three experiments, after the aconitase had been precipitated by acetone and resuspended in water, no lag occured when the reactions were initiated with the enzyme. In the only such experiment in which tobacco leaves were harvested from the growth chamber in the light, when the aconitase reaction was initiated with extract, the reaction rate remained zero during the entire observation period of 1 hour. But after the aconitase had been precipitated by acetone and resuspended in water, no lag occured when the reaction was initiated with the enzyme, and the rate was about the same per gram of leaf as the rate obtained with the acetone precipitated enzyme from the leaves harvested in the dark. When the assays of the latter experiment were repeated after 3 hours, the same results were obtained. In all the above experiments, cis-aconitate was the substrate. These results could be explained by assuming that an endogenous inhibitor was reversibly bound to aconitase, and that due to the dilution which occured in the reaction mixtures, the inhibitor dissociated from the enzyme. Precipitation by acetone apparently also removed the hypothetical inhibitor. The data suggest that the concentration of this inhibitor may be greater in the light than in the

dark. Evidence has been presented (literature review) for a lag in the aconitase reaction, but such a lag has only been reported for the interconversion of the hydroxy-acids, presumably because of a buildup of cis-aconitate as a partially obligate intermediate. Such a lag was reported absent when cis-aconitate itself was the substrate (164). The observations reported here are of interest in view of evidence (literature review) which could be interpreted to suggest that aconitase may be inhibited in the light in some photosynthetic cells.

Contrary to the findings with tobacco leaves, in three experiments with Swiss chard leaves from the growth chamber,[1] in which the reaction mixtures contained no buffer or sulfate,[2] and in which the leaves were harvested in the light, there was no evidence for an endogenous inhibitor of aconitase.

Experiments with tobacco leaves from the field[3] in which the reaction mixtures did contain buffer and sulfate[4]

---

[1]The extracts, after centrifugation at 15,000 g for 8 to 10 minutes, were adjusted to pH 7.5.

[2]The concentration of cis-aconitate was 1.5×10^{-4}M in these reaction mixtures.

[3]The extracts had been centrifuged at 15,000 g for 15 to 20 minutes, and the pH of the extracts was not adjusted.

[4]Reaction mixtures included 0.033M TES, pH 7.5, 0.02M ammonium sulfate, and 2.5×10^{-4}M cis-aconitate.

provided little evidence for an endogenous inhibitor. In most of these experiments, no lag followed the initiation of the reaction with extract.

None of the experiments had been designed for the observation of an endogenous inhibitor. Therefore, care was not taken to control the light impinging on the leaves between harvest and homogenization, or to establish other controls designed for observations on the hypothetical inhibitor.

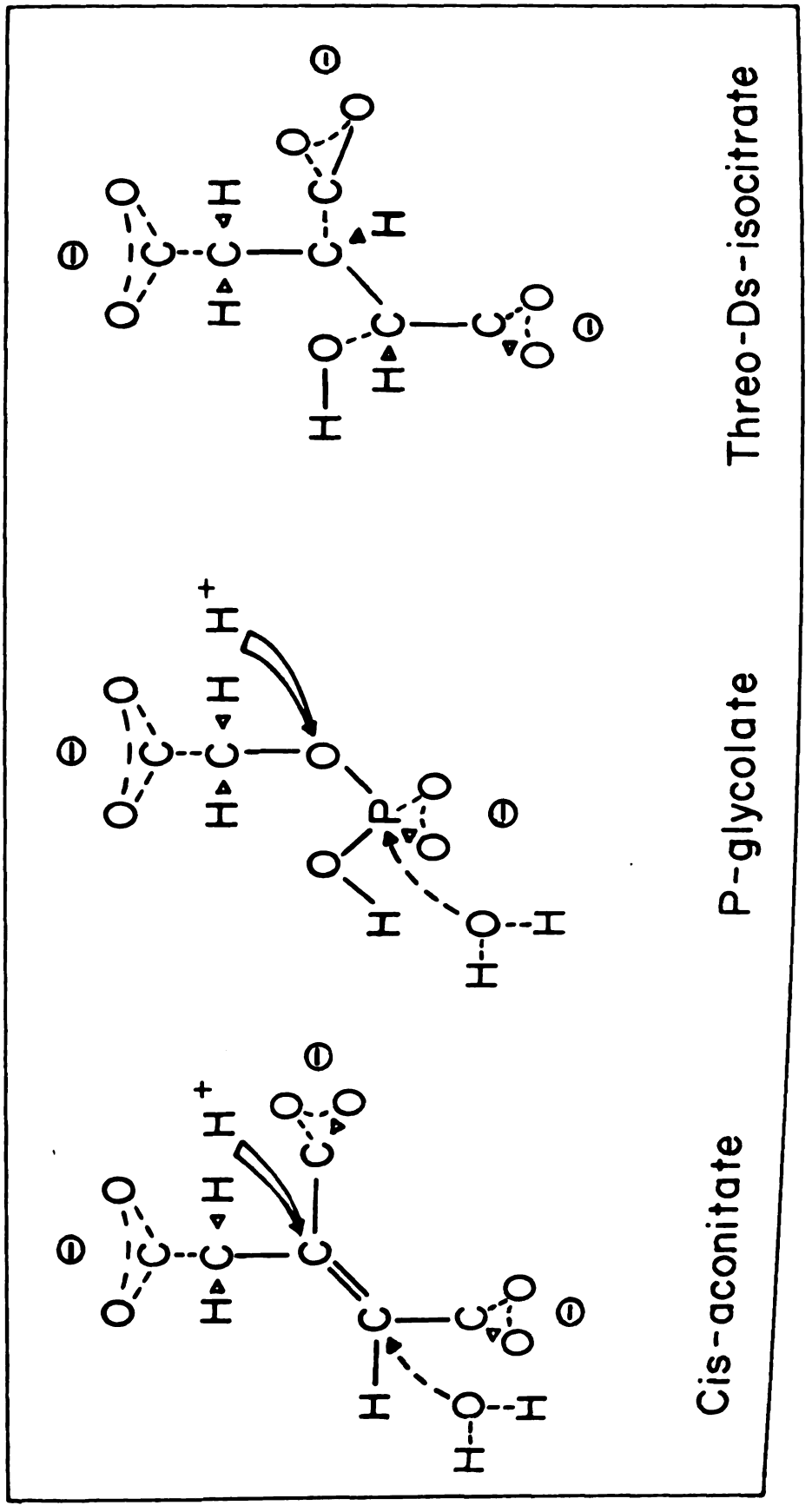
Similarities Between the Substrates and Reaction Mechanisms of P-glycolate Phosphatase and Aconitase

P-glycolate shows structural analogies to cis-aconitate and isocitrate. Furthermore, assuming that the phosphatase cleaves P-glycolate between the phosphorous and oxygen atoms, as has been found for all phosphate monoesterases which so far have been studied (70 , 213, 260), the reaction mechanism for the conversion of cis-aconitate to isocitrate shows analogies to the reaction mechanism for the hydrolysis of P-glycolate (Figure 47). The structures of the tricarboxylic acids and the mechanism for the interconversion of cis-aconitate and isocitrate, which are shown in Figure 47, fit the stringent stereospecificity requirements of aconitase (literature review). The conformations depicted in Figure 47 have been arbitrarily assigned for the purpose of illustrating

Figure 47

Similarities Between the Substrates and Reaction Mechanisms of P-glycolate Phosphatase and Aconitase

The molecules are shown with accepted configurations but arbitrary conformations. For all three molecules, the plane through the methylene carbon and the two atoms alpha and beta to it is in the plane of the paper.


Threo-Ds-isocitrate
P-glycolate
Cis-aconitate

the similarity between the substrates and the possible similarity between the reaction mechanisms of aconitase and P-glycolate phosphatase. Other conformations could no doubt be found which would serve this same illustrative purpose. In any case, the conformations which are represented are imagined as those which might occur while the molecules are bound to the enzyme. For each of the molecules, there are of course an infinite number of conformations which could result from rotation about single bonds.

At pH 6.3 (pH optimum for the phosphatase), most of the P-glycolate molecules should bind one proton at one of the phosphate oxygens, while the other two ionizable groups, one on the phosphate and the other being the carboxyl group, would be fully ionized. (The pK_a values for phosphate are 2.1, 7.2, and 12.7, and for glycolate, 3.8.)[1] At pH 6.3 or higher (pH range for aconitase), most of any one of the tricarboxylic acids should be fully dissociated (the pK_a values for citrate are 3.1, 4.7, and 5.4).[1] The phosphate moiety of P-glycolate would contain two non-bridging oxygen atoms, which would share a single negative charge between them. Both isocitrate and cis-aconitate would contain a carboxylate group at this

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[1]Reference; footnote, p. 102.

analogous position, in which a single negative charge would also be distributed between two oxygen atoms. If attention is focused on the longest chain of atoms which includes this negative charge, the main difference between P-glycolate and cis-aconitate or isocitrate, apart from a phosphorous and an oxygen being substituted for two carbon atoms, is that one more atom (a carboxylate carbon) is in the tricarboxylic acid chain (Figure 47). Furthermore, "the chemistry of carbon and that of phosphorous are very closely connected, as would be expected from the diagonal relationship of these elements in the Periodic Table" (247).

In both the proposed phosphatase reaction mechanism and the reaction mechanism for the conversion of cis-aconitate to isocitrate by aconitase, a proton is added to the atom alpha to the carboxymethyl moiety and water is added to the atom which is beta to this moiety. The oxygen of P-glycolate which accepts the proton acts as a base by donating a pair of electrons. The two pi electrons of the double bond of cis-aconitate act in a similarly basic fashion. Using aconitase from pig heart, Rose and O'Connell have provided evidence that a proton from solution is passed by means of a basic group of the enzyme to cis-aconitate during its hydration to isocitrate or citrate (204).

The structure of citrate would likewise be expected to be analogous to that of P-glycolate. The -OH group of

isocitrate is in a position analogous to the -OH (on the phosphate) of P-glycolate, i.e. both are on atoms β to the carboxymethyl moiety (Figure 47). The position of the -OH of citrate is not as analogous. This difference may partly explain the observation that isocitrate was more effective in stabilizing the phosphatase than was citrate (Figure 15). The increased stabilization by $MgSO_4$ in conjunction with citrate or isocitrate, but not in conjunction with cis-aconitate (Table 11), suggests that Mg^{++} may bridge between the enzyme and the -OH group of citrate or isocitrate. An analogous link with the -OH of P-glycolate would be consistent with one of the known principles by which Mg^{++} catalyzes phosphatase reactions, i.e. by polarizing the phosphate group making it more subject to nucleophilic attack by water.

Discussion of Similarities, Differences, and Relationships Between Aconitase and P-glycolate Phosphatase

Stabilization by Tricarboxylic Acids

The substrates of aconitase have been found to be the most effective stabilizers of that enzyme (literature review). The substrates of aconitase were also found to be effective, as well as specific (to the extent tested), stabilizers of the phosphatase (Figure 15).[1] In the

[1]The order of effectiveness of the tricarboxylic acids in stabilizing the phosphatase (cis-aconitate somewhat more effective than isocitrate which was somewhat

literature review, 4 enzymes are described which are activated by at least one of the tricarboxylic acids, while 11 are described which are inhibited by one or more of the tricarboxylic acids. For none of these enzymes was it reported that any of the tricarboxylic acids stabilized the enzyme, nor to my knowledge is there a report that any enzyme other than the phosphatase and aconitase is stabilized by the tricarboxylic acids. Furthermore, in some cases where all 3 tricarboxylic acids were tested for inhibition or activation, not all of the tricarboxylic acids were found to be effective. For example, although citrate was found to be the most effective activator of acetyl CoA carboxylase, cis-aconitate was found to be without effect. While citrate was found to be an effective inhibitor of mitochondrial malate dehydrogenase, isocitrate and cis-aconitate were found to be ineffective. On the other hand, deoxyribose phosphate aldolase was found to be activated by citrate, isocitrate, cis-aconitate, and trans-aconitate (literature review). Thus, even among those enzymes which are affected by the tricarboxylic acids, the phosphatase and aconitase are somewhat unique in that

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more effective than citrate (Figure 15)) shows an apparent correlation with the order of the K_m values of aconitase for these acids (K_m for cis-aconitate < K_m for isocitrate < K_m for citrate (literature review)). The effect of trans-aconitate seems to be similarly correlated, since it was about as effective as citrate in stabilizing the phosphatase (Figure 15), while for aconitase the K_I for trans-aconitate was found to be about equal to the K_m for citrate (literature review).

they are significantly affected by citrate, isocitrate, cis-aconitate, and trans-aconitate.

Competitive Inhibition by Cis-aconitate

The competitive inhibition of the P-glycolate phosphatase reaction by cis-aconitate was not caused by chelation of the Mg^{++} which is required for the reaction (Figure 18). By contrast, the competitive inhibition of phosphoglucomutase and of intestinal alkaline phosphatase by citrate could be reversed by the addition of relatively high concentrations of Mg^{++}. It was therefore hypothesized that citrate exerted its effect by complexing the Mg^{++} required by the latter two enzymes as an activator (79, 275). In the case of P-glycolate phosphatase, the alternative is that cis-aconitate competes with P-glycolate by binding with the phosphatase itself. Product inhibition is usually competitive inhibition (149). For the conversion of any one tricarboxylic acid, the other two tricarboxylic acids should be competitive inhibitors of aconitase. Tomizawa (241) demonstrated that DL-isocitrate-2-^{14}C competitively inhibited the formation of unlabeled citrate from unlabeled cis-aconitate. Thus, aconitase and P-glycolate phosphatase are similar in that reactions of both enzymes can be competitively inhibited by cis-aconitate. The available evidence suggests that in both cases the site of inhibition is on the enzyme.

Substrates and Reaction Mechanisms

The substrates of P-glycolate phosphatase and aconitase seem to be structurally analogous, and the phosphatase reaction mechanism seems to be analogous to the mechanism for the conversion of cis-aconitate to isocitrate (Figure 47). These similarities are consistent with the observations on stability and competitive inhibition. They are also consistent with another property that the two enzymes have in common, i.e. each one is highly specific for its substrate(s) (literature review).

Effect of O_2

Oxygen had a qualitatively similar effect on the activity and stability toward dilution at 30° of P-glycolate phosphatase and aconitase in fresh extracts from tobacco leaves (p. 317). An important practical consideration concerning the stability toward dilution at 30° of both enzymes is that, except when it is desired to study this stability itself, the reactions should be initiated by the addition of cold enzyme to the otherwise complete reaction mixtures (including substrate).

Stability Toward Acetone Fractionation

Both enzymes were very stable to fractionation by acetone. High phosphatase recovery was obtained, and similarly high recovery of aconitase was obtained if

allowance was made for the rapid inactivation of the latter enzyme in the fresh extracts, by using the activity which prevailed at the time of the precipitation by acetone. Furthermore, when this same allowance was made, the phosphatase and aconitase were both purified many fold and about the same amount by acetone fractionation (p. 320). Stability toward precipitation from plant extracts by acetone is somewhat unique, since most of the protein present seems to be irreversibly denatured by the treatment (p. 166).

The phosphatase from tobacco leaves was unstable toward further acetone fractionation after an ammonium sulfate fractionation (p. 178). It may also be that aconitase from pig heart is stable to organic solvents before ammonium sulfate treatment, but not after (literature review, p. 62).

Effect of pH

Although the pH optimum of the phosphatase from Swiss chard has not been determined, the optimum of the enzyme from the leaves of the plants tested (wheat, spinach, or tobacco), with $MgSO_4$ present at 10^{-3}M, was found to be 6.3 (265). The pH optimum of partially purified aconitase from Swiss chard, with $MgSO_4$ present at 10^{-2}M and with cis-aconitate as substrate, was found to be between 6.0 and 6.5 (p. 278). Thus, under the assay conditions which were used, aconitase from the leaves of

Swiss chard is similar to the phosphatase from the leaves of the other higher plants tested, with respect to the pH optimum.

Marked loss of pig heart aconitase activity occured when the pH of the clarified crude extract was adjusted below pH 5.0 (162). The same observation was made for P-glycolate phosphatase in an extract from tobacco leaves (Figure 9).

Effect of Freezing and Thawing

During the early stages of purification, the phosphatase from tobacco leaves was found to be unstable to freezing and thawing, while the enzyme after the fifth purification step was found to be stable (p. 175). Like the phosphatase from tobacco leaves, aconitase from pig heart was unstable toward freezing at the earliest stage of purification, and the purified enzyme was unaffected by repeated freezing and thawing (162). However, unlike the phosphatase from tobacco leaves, highly purified aconitase from mustard leaves was totally inactivated by freezing (185). Association of the phosphatase and aconitase from tobacco leaves during the early stages, but not the last stage of purification, might explain the behavior of the phosphatase toward freezing, but this possibility has not been tested.

Effect of Sulfhydryl Reagents

P-glycolate phosphatase from tobacco was inhibited 90% by 10^{-3}M p-chloromercuribenzoate in the reaction mixture, but was not inhibited at all by 3 x 10^{-3}M iodoacetate in the reaction mixture (202). p-Mercuribenzoate at 0.53 x 10^{-6}M inhibited the animal aconitase 50% while iodoacetate of 10^{-2}M or arsenite at 10^{-2} gave no inhibition. The animal enzyme had been activated by Fe^{++} and ascorbate, and the inhibitors were at the concentrations indicated during a 5 minute preincubation at 30° (63). Arsenite at 10^{-2}M during a one hour preincubation at 0° resulted in a slight stimulation of P-glycolate phosphatase in fresh extracts from tobacco leaves (Table 16). Buchanan and Anfinsen (39) reported that cysteine alone, in the initial stages of the purification, showed a stabilizing effect on pig heart aconitase, but as the purification of the enzyme continued, cysteine alone strongly inhibited the enzyme. P-glycolate phosphatase from tobacco was inhibited 90% by 8 x 10^{-3}M cysteine in the reaction mixture, and was inhibited 84% by glutathione at 8 x 10^{-3}M in the reaction mixture (202). Cysteine partially stabilized the phosphatase from wheat, though not as effectively as did the tricarboxylic acids (Tolbert and Yu, unpublished data). Thus, the aconitase from animal tissues and P-glycolate phosphatase from higher plants are affected qualitatively in essentially the same way by the four sulfhydryl reagents

for which comparisons are available. Little such work has been done with aconitase from plants. However, Palmer (185) did find that preincubation of the aconitase from mustard with iodoacetate (final concentration 10^{-3}M) for 10 minutes had no effect on the activity, but he also found that cysteine had no stabilizing effect on the purified aconitase from mustard. In the present work, it was found that cysteine (without iron) in the reaction mixture inhibited the aconitase from tobacco leaves (p. 323).

Molecular Weight

The molecular weight of neither aconitase nor P-glycolate phosphatase has been determined. However, the s_{20} value for purified aconitase from mustard leaves was found to be 4.7 by Palmer (185). Using the $s_{20,w}$ value of 7.7 and M.W. of 150,000 for aldolase (58), and the $s = (\text{constant}) M^{2/3}$ relationship which is based on the assumption of spherical shape, a rough approximation of the molecular weight of the mustard aconitase is 70,000. The Rf of 1.6 for the phosphatase from tobacco leaves during Sephadex G-100 gel filtration chromatography (p. 173) allows a very rough approximation of its molecular weight of about 20,000 to 30,000.

Although aconitase from plants and animals seems to be a single protein species with a single active site

for both of the aconitase half reactions, there is some evidence from Saccharomyces that aconitase may comprise two polypeptide chains (literature review). Also, the phosphatase reaction seems to be analogous only to the cis-aconitate to isocitrate half reaction (pp. 337-342). These observations lead to the hypothesis that the phosphatase might have a considerably smaller molecular weight than aconitase.

Effect of Cations and Anions

P-glycolate phosphatase from the leaves of a variety of plants seems to be totally dependent on divalent cations in the reaction mixture for activity (202, 265), while aconitase from tobacco or Swiss chard leaves may be totally dependent on anions in the reaction mixture for activity (pp. 281-289).

Parallel Fractionation of the Enzymes

Concerning the parallel fractionation of aconitase and P-glycolate phosphatase (Figures 34 through 38), at least three hypotheses are worth consideration:

A: The two enzymes are not bound together, either directly or indirectly, i.e. they fractionated together by coincidence. The similarities in the properties of the two enzymes are consistent with this hypothesis. If this hypothesis were true, the parallel fractionation of the two

enzymes during acetone fractionation and during DEAE-cellulose chromatography would have to be considered as two additional similarities between the two enzymes. However, the separation of the two enzymes during DEAE-cellulose chromatography under certain conditions (Figure 39) would then have to be considered an anomoly.

B: The two enzymes are bound together indirectly, by being bound to a third structure. Since both enzymes have an affinity for the tricarboxylic acids, one or more of these could serve as the third structure.[1] Furthermore, the possibility of an unidentified third structure has not been ruled out.

C: The two enzymes are bound together directly. The similarities in the properties of the two enzymes are also consistent with this hypothesis.[2]

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[1] Isocitrate (Figures 16, 17 and 39) and the tricarboxylic acids in general (Figure 10) consistently showed either a double peak or a pronounced shoulder, while both enzymes consistently showed single peaks during fractionation (Figures 10, 11, 34, 35, 36, 37, 38 and 39). These data suggest that the tricarboxylic acids were not tightly bound to either enzyme, but may have been in equilibrium with one or both of them. Nevertheless, with enough matching binding sites on both enzymes, the tricarboxylic acids could have kept the enzymes bound together while the acid at any one pair of sites was in equilibrium with the medium.

[2] Studies with E. coli alkaline phosphatase (212), fumarase (121), and muscle or heart lactate dehydrogenase (130) have demonstrated that they are composed of identical or nearly identical subunits. From interallelic complementation studies with Neurospora (46), it is thought that a great number of enzymes are likewise composed of identical or nearly identical subunits. (See also comments by Dr. Lacks, Brookhaven Symp. Biol. 17, p. 174). Reversible dissociation into subunits of rabbit muscle aldolase

On the basis of present evidence, it is not possible to eliminate any of these three hypotheses. Furthermore, should hypothesis B or C be true, it would still be necessary to determine whether the binding is artifactual or of physiological significance.

Some of the apparent similarities between aconitase and P-glycolate phosphatase, especially in the early stages of purification, could have been because the enzymes were bound together. Even for the enzymes in the later stages of purification, this possibility cannot be excluded. Thus, the stabilization of the phosphatase by the tricarboxylic acids (Figures 10 and 15) could have been because of stabilization of aconitase which may have been bound to the phosphatase.[1] Also, competitive inhibition does not

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(58), E. coli alkaline phosphatase (212), and fumarase (121) has been demonstrated, and definitive evidence has been presented in the latter two cases that the subunits are held together by non covalent interactions (121, 212). The very rapid in vitro association of E. coli alkaline phosphatase subunits, even in crude cell extracts, points to the very high degree of recognition between the monomers (212). It seems likely that for multichain enzymes, the amino acid sequence in the polypeptide chains uniquely determines the chain conformation as well as the spatial relationships of the subunits in the native enzyme molecules (58, 211, 212). Thus, there exists ample precedence for peptide chains with identical or nearly identical structure having a highly specific affinity. These identical or nearly identical peptide chains should have identical or nearly identical properties.

[1]The phosphatase used for the experiment of Figure 10 had a specific activity of 161, or half of 333, which is the highest specific activity attained for the phosphatase. The additional protein could have included aconitase. However, in the experiment of Figure 15, the Rf of the phosphatase on the G-100 column which was used was

require that the substrate and the inhibitor bind at the same site. Conceivably, if aconitase were bound to the phosphatase, the binding of cis-aconitate at the aconitase substrate site could competitively inhibit the phosphatase reaction (Figure 18).[1] However, I know of no precedent in which the binding site of the competitive inhibitor is on an enzyme different from the one with the substrate binding site. Furthermore, the structural similarity between P-glycolate and cis-aconitate suggests that the site of inhibition by cis-aconitate is the P-glycolate binding site. Also, before the competitive inhibition experiment of Figure 18, the phosphatase preparation was tested for aconitase activity and was found to be essentially inactive, and aconitase reactivation attempts yielded negative results. Still, the enzyme might have been present in a conformation in which it could bind cis-aconitate but not catalyze the reaction.

Parallel Fractionation of the Phosphatase and the Tricarboxylic Acids

Because citrate, cis-aconitate, and isocitrate are the substrates of aconitase, the fractionation of large

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1.6. This Rf value on G-100 suggests that aconitase and the phosphatase were separated before or during the experiment. Still, the phosphatase was stabilized by the tricarboxylic acids (Figure 15).

[1]The enzyme used for the experiment of Figure 18 had a specific activity of 161, or half of 333, which is the highest specific activity attained for the phosphatase. The additional protein could have included aconitase.

quantities of these acids with the phosphatase under certain conditions of harvest and purification (pp. 137-147, 181-191, and 195-201) would seem to be closely related to the parallel fractionation of aconitase and the phosphatase under similar conditions of harvest and purification (Figures 34 through 38). The concept that the fractionation of the tricarboxylic acids with the phosphatase is related to the fractionation of aconitase with the phosphatase is strengthened by the evidence that isocitrate no longer fractionated with the phosphatase when aconitase was separated from the phosphatase (Figure 39).

There is some evidence that aconitase possesses multiple binding sites for the tricarboxylic acids. For most enzymes, the effect of anions at ordinary concentrations is negligible (71). However, enzymes such as fumarase (158), soluble malate dehydrogenase from pea seeds (253), and glyoxylate reductase from tobacco leaves (266), which are activated by anions, have each been found to be activated by many different anions. Thus, a role for tricarboxylic acid anions as activators of the plant aconitase would be consistent with the finding that chloride or sulfate activates aconitase from tobacco or Swiss chard leaves (Figures 31, 32, and 33). Isocitrate has in fact been found to activate the aconitase from mustard leaves, and an argument is presented that the activating effect by isocitrate is by combination with groups other than at the

active center (literature review). The activation of the tobacco aconitase by $SO_4^=$ supports the concept that the enzyme may possess groups, other than at the active center, capable of binding the tricarboxylic acids. Furthermore, Henson and Cleland (114) reported that citrate, which was added during purification for stability purposes, remained bound to beef liver aconitase even after passage through a Sephadex G-25 column. The amount of citrate that was bound to the enzyme was sufficient to require removal before kinetic experiments could be performed.

Since the phosphatase is stabilized by the tricarboxylic acids and is competitively inhibited by cis-aconitate, and since there is some evidence that the phosphatase might be activated by the tricarboxylic acids under certain conditions (footnote, p. 202), the phosphatase itself must also possess binding sites for the acids. Therefore, the fractionation of endogenous tricarboxylic acids with the phosphatase may also be partly due to properties of the phosphatase itself.

The two enzymes fractionating in parallel would offer more binding sites for the tricarboxylic acids than would the phosphatase alone. The ratio of 1 isocitrate per 10 to 15 amino acid residues of the phosphatase (pp. 195-201) is misleading if some of the isocitrate were bound to aconitase.[1]

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[1]Although the specific activity of the phosphatase depicted in Figures 16 and 17 was not measured, the specific

From the available data, it is not possible to know the nature of the relationship between aconitase, the tricarboxylic acids, and the phosphatase when they fractionate together. The finding that isocitrate was with aconitase and was not with the phosphatase when the two enzymes were separated (Figure 39) suggests as one possibility that the tricarboxylic acids might have fractionated with the phosphatase because they were bound to aconitase which fractionated with the phosphatase. However, although isocitrate was with aconitase, the shapes of the curves do not strongly suggest binding between aconitase and isocitrate (Figure 39). (The curves of Figures 16 and 17 are more suggestive of binding.) Therefore, as a second possibility, it might be that under some conditions isocitrate was bound to both enzymes, and that under other conditions it was bound to neither. Other possibilities also exist.

Effect of Light

Although the effect of light before harvest on the time dependent stabilization of P-glycolate phosphatase in tobacco extracts (Figure 20) could be related to the possible role of light as a factor in the determination of

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activity in a nearly identical preparation (both preparations had come from the same preparation of 3 times acetone purified phosphatase, and were further fractionated on nearly identical DEAE-cellulose columns) was 84.7 (Table 3), or $\frac{1}{4}$ of the highest specific activity of 333 which was obtained. Thus, the phosphatase fractions of Figures 16 and 17 contained enough other protein to include aconitase.

whether aconitase and P-glycolate phosphatase fractionate together (pp. 291-308), no direct evidence for such a relationship has been sought or obtained. However, these effects of light are of further interest in view of some evidence that aconitase may be inhibited in some plants in the light (pp. 334-337) and in view of the requirement for light for the synthesis of glycolate (literature review).

The Possibility of an Evolutionary Relationship

Rutter (207) has pointed out that the study of enzymes with related mechanisms may suggest meaningful evolutionary relationships. Thus, the similarity between the mechanism for the hydration of cis-aconitate to isocitrate and the proposed mechanism for the enzymatic hydrolysis of P-glycolate (Figure 47) leads to the speculation that an evolutionary relationship may exist between the two enzymes. Furthermore, the phosphatase and aconitase should contain homologous regions if they evolved from a single prototype gene (207). Many of the observations reported in this thesis concerning these two enzymes would be more or less expected with such an evolutionary relationship. Also, a possible evolutionary relationship between aconitase and the phosphatase might be of significance in view of the requirement for Mn^{++} for the development of aconitase activity in Aspergillus niger and in Chlorella vulgaris, and in view of the Mn^{++} requirement

for glycolate synthesis (literature review). It would be of interest to see if P-glycolate phosphatase has a similar Mn^{++} requirement for the development of its activity.

In Vivo Correlation Between the Phosphatase and Aconitase

The localization of P-glycolate phosphatase in or on the chloroplasts (literature review), together with the classical concept that aconitase is a mitochondrial enzyme, would seem to rule out the possibility of a physiologically significant binding between P-glycolate phosphatase and aconitase. However, the localization of aconitase in animal as well as in plant cells actually is controversial (literature review). Thus, it would be premature to consider an in vivo association of P-glycolate phosphatase and aconitase as being ruled out.

Further Observations on the Stability of P-glycolate Phosphatase

As discussed previously, both aconitase and isocitrate (and presumably the other tricarboxylic acids) were separated from the phosphatase under the conditions described for Figure 39. Yet, when the stability of the phosphatase in the 6 most active fractions (10 through 15) was tested, it was found that only 4 to 18% of the activity was lost by storage at 0-4° for 6 days. The average loss of activity was 8%. Thus, at this stage of purification,

the phosphatase was stable to storage at 0-4° even without the tricarboxylic acids. In contrast, when the tricarboxylic acids were removed from the phosphatase, which had been purified by 3 successive acetone fractionations, by passage through a Sephadex G-100 gel filtration column (p. 193), 66% of the activity was lost by storage at 0-4° for 4 days. These findings suggest that the loss of stability during the Sephadex G-100 gel filtration chromatography, after 3 acetone purification steps, may not have been only because of the separation of the tricarboxylic acids from the phosphatase.

Many of the observations on the stability of the phosphatase (pp. 175-178) might be explained by hypothesizing an unknown factor(s) which interacts with the enzyme independently from the tricarboxylic acids and from O_2. It could have been an unknown factor which made the enzyme stable even without the tricarboxylic acids, stable to acetone fractionation, and unstable to freezing. The factor was apparently not removed by acetone or DEAE fractionation, or by dialysis, but it apparently was removed by $(NH_4)_2SO_4$ fractionation, or when the history of the enzyme was that of the Bio-Gel P-60 preparation of Table 3. Removal of the unknown factor may have been the reason why the $(NH_4)_2SO_4$ fractionated enzyme was stable to freezing, unstable to acetone fractionation, and unstable to dialysis, and why the Bio-Gel P-60 enzyme of Table 3 was stable to

freezing with or without citrate, but was unstable without added tricarboxylic acids. Likewise, the presence of the unknown factor may have been the reason why the acetone purified enzyme was stable to repeated acetone fractionation, unstable to freezing, stable to and after dialysis, and stable without the tricarboxylic acids even after further purification by DEAE-cellulose chromatography. Furthermore, gradual loss of the unknown factor during purification could have been the reason for the decreasing stability toward dilution at 30° of the phosphatase in the later stages of the purification (p. 261).

It is worth noting that every preparation that behaved as though the hypothetical factor were present was colored (orange-brown), and every one that did not was not colored (or, in the case of the $(NH_4)_2SO_4$ purified enzyme, was relatively pale in color). As pointed out previously (p. 174), the colored compound(s) absorbed strongly at 254 mμ. Thus, the coincidence of a 254 mμ peak with the phosphatase peak (Figure 16) could possibly mean that a colored compound was bound to the phosphatase after 3 acetone fractionations and DEAE-cellulose chromatography.

SUMMARY

A purification procedure for P-glycolate phosphatase from tobacco leaves, which included three successive acetone fractionations, DEAE-cellulose chromatography, and gel filtration chromatography, was developed. The enzyme was purified 1000-fold to a specific activity of 333 μmoles of substrate hydrolyzed per minute per mg of protein.

During the last purification step, the tobacco phosphatase became unstable, as determined by heating the enzyme to 45° for 1 hour, but could be restabilized with fractions which emerged after the phosphatase from the gel filtration column. The endogenous stabilizing factors in these fractions were identified as citrate, isocitrate, and cis-aconitate. Commercial citrate, isocitrate, and cis-aconitate, as well as trans-aconitate, also stabilized the enzyme, but mono and dicarboxylic acids which were tested did not.

Cis-aconitate was found to be an inhibitor, competitive with respect to P-glycolate, of the phosphatase. The apparent K_I for cis-aconitate was 5.0×10^{-3}M when the Mg^{+2} was 2×10^{-3}M. Increasing the Mg^{+2} to 2×10^{-2}M, with cis-aconitate at 10^{-2}M, did not significantly affect the apparent K_I. The data suggest that cis-aconitate did not cause inhibition by complexing the Mg^{+2} required as an activator of the phosphatase. When the Mg^{+2} was 2×10^{-3}M,

the apparent K_m for P-glycolate was 6.7×10^{-5}M. Increasing the Mg^{+2} to 2×10^{-2}M did not significantly affect the apparent K_m. The apparent K_A for Mg^{+2} was 3.5×10^{-4}M.

When isocitrate and the phosphatase were assayed in fractions from the DEAE-cellulose chromatographic step, one of the two isocitrate peaks approximately coincided with the phosphatase peak. At this DEAE stage of purification, approximately 1 mole of isocitrate per 10 to 15 moles of amino acid of the phosphatase fractionated with the enzyme. Furthermore, comparison of the sizes of the spots on paper chromatograms suggests that more citrate than isocitrate fractionated with the phosphatase.

In most of the experiments, aconitase and P-glycolate phosphatase from tobacco leaves fractionated in parallel during acetone fractionation and during subsequent DEAE-cellulose chromatography. However, when field grown tobacco leaves were kept in darkness for 3 to 4 hours before harvest and homogenization, the phosphatase and aconitase, after a preliminary purification by acetone fractionation, were separated during DEAE-cellulose chromatography. In the latter case, isocitrate no longer fractionated with the phosphatase. Although in all the experiments in which the phosphatase and aconitase fractionated together and in which isocitrate fractionated with the phosphatase, the tobacco leaves had been exposed to at least some light in the period before harvest and/or homog-

enization, experiments controlled so as to determine the effect of light on the parallel fractionation of the two enzymes, or of the phosphatase and the tricarboxylic acids, were not performed. Nevertheless, the evidence suggests that the fractionation of the endogenous tricarboxylic acids with the phosphatase was closely related to the fractionation of aconitase with the phosphatase.

Although it is not known whether the parallel fractionation of the two enzymes was artifactual or of physiological significance, it is of interest that the tricarboxylic acids are also effective stabilizers of aconitase, and that any one tricarboxylic acid would be expected to competitively inhibit the interconversion of the other two. Thus, aconitase and P-glycolate phosphatase seem to possess at least two similarities, i.e. stabilization by the tricarboxylic acids and competitive inhibition by cis-aconitate. Other apparent similarities between the two enzymes are discussed.

Partially purified aconitase from tobacco or Swiss chard leaves could be deactivated by Sephadex G-10 or G-25 gel filtration chromatography and could be reactivated by fractions which emerged after aconitase from the column, or by sulfates or chlorides. The activation of aconitase by sulfates or chlorides is consistent with the concept that the enzyme may possess groups, other than at the active center, capable of binding citrate, isocitrate, or

cis-aconitate.

In fresh extracts from tobacco leaves, P-glycolate phosphatase was unstable toward dilution of 20 to 30-fold at 30° for 1 hour, but under aerobic conditions, the enzyme in the extracts slowly became stable toward this dilution at 30°. A variety of treatments of the extracts including mixing under air or O_2 (but not N_2 or CO_2), a 10 minute preincubation with 10^{-2}M OHPMS, or rapid passage through a Sephadex G-25 column, quickly and completely stabilized the phosphatase toward dilution at 30°. The stabilized enzyme could be partially and rapidly reconverted to the unstable but just as active enzyme by preincubation of the oxygenated extracts with glycolate. OHPMS completely inhibited, while anaerobic conditions enhanced, this glycolate dependent conversion. Glycolate also inhibited the stabilization toward dilution at 30° of the phosphatase by O_2. Other aspects of the stability toward dilution at 30° of the phosphatase are discussed. The data suggest that the phosphatase can exist in an oxidized or in a reduced state and that in the extracts, glycolate oxidase could be involved in the interconversion between the two states. A simple tentative model which is consistent with the experimental observations is presented.

Other findings concerning P-glycolate phosphatase and aconitase are discussed.

BIBLIOGRAPHY

1. Aisen, Philip, Roland Aasa, Bo G. Malmström, and Tore Vanngard. 1967. Bicarbonate and the binding of iron to transferrin. J. Biol. Chem. 242: 2484-2490.

2. Anderson, Louise and R. C. Fuller. 1967. Photosynthesis in Rhodospirillum rubrum. II. Photoheterotrophic carbon dioxide fixation. Plant Physiol. 42: 491-496.

3. Anderson, Donald E. and N. E. Tolbert. 1966. Phosphoglycolate phosphatase. In: Methods in Enzymology, Vol. IX. Willis A. Wood, ed. Academic Press, New York and London, pp. 646-650.

4. Anet, F. A. L. 1960. The configuration of deuterio-L-malic acid produced enzymatically. Synthesis of threo-3-deuterio-DL-malic acid. J. Am. Chem. Soc. 82: 994-995.

5. Anfinsen, Christian B. 1955. Aconitase from pig heart muscle. In: Methods in Enzymology, Vol. I. Sidney P. Colowick and Nathan O. Kaplan, eds. Academic Press Inc., New York, pp. 695-698.

6. Aronoff, S. and John Z. Hearon. 1960. Kinetic models of aconitase action. Arch. Biochem. Biophys. 88: 302-307.

7. Asada, Kozi and Zenzaburo Kasai. 1962. Inhibition of the photosynthetic carbon dioxide fixation of green plants by α-hydroxy sulfonates, and its effects on the assimilation products. Plant and Cell Physiol. 3: 125-136.

8. Atkinson, Daniel E. 1966. Regulation of enzyme activity. Ann. Rev. Biochem. 35: 85-124.

9. Atkinson, Daniel E. and Gordon M. Walton. 1967. Adenosine triphosphate conservation in metabolic regulation. Rat liver citrate cleavage enzyme. J. Biol. Chem. 242: 3239-3241.

10. Bacon, J. S. D., P. C. DeKock, and M. J. Palmer. 1961. Aconitase levels in the leaves of iron-deficient mustard plants (Sinapis alba). Biochem. J. 80: 64-70.

11. Bacon, J. S. D., M. J. Palmer, and P. C. DeKock. 1961. The measurement of aconitase activity in the leaves of various normal and variegated plants. Biochem. J. 78: 198-204.

12. Baker, A. L. and N. E. Tolbert. 1967. Purification and some properties of an alternate form of glycolate oxidase. Biochim. Biophys. Acta 131: 179-187.

13. Bandurski, Robert S. and Bernard Axelrod. 1951. The chromatographic identification of some biologically important phosphate esters. J. Biol. Chem. 193: 405-410.

14. Bassham, J. A. 1963. Recent kinetic studies on the carbon reduction cycle. In: Photosynthetic Mechanisms of Green Plants. NAS-NRC Publication 1145, pp. 635-647.

15. Bassham, J. A. 1964. Kinetic studies of the photosynthetic carbon reduction cycle. Ann. Rev. Plant Physiol. 15: 101-120.

16. Bassham, J. A. 1965. Photosynthesis: The path of carbon. In: Plant Biochemistry. James Bonner and J. E. Varner, eds. Academic Press, New York and London, pp. 875-902.

17. Bassham, J. A., A. A. Benson, Lorel D. Kay, Anne Z. Harris, A. T. Wilson, and M. Calvin. 1954. The path of carbon in photosynthesis. XXI. The cyclic regeneration of carbon dioxide acceptor. J. Am. Chem. Soc. 76: 1760-1770.

18. Bassham, J. A. and M. Calvin. 1957. The Path of Carbon in Photosynthesis. Prentice-Hall, Inc.

19. Bassham, J. A., Horst Egeter, Frances Edmonston, and Martha Kirk. 1963. The effects of 8-methyl lipoic acid on the evolution of oxygen and reduction of carbon dioxide during photosynthesis. Biochem. Biophys. Res. Comm. 13: 144-149.

20. Bassham, James A. and Richard G. Jensen. 1967. Photosynthesis of carbon compounds. In: Harvesting the Sun. Anthony San Pietro, Frances A. Greer, and Thomas J. Army, eds. Academic Press, New York and London, pp. 79-110.

21. Bassham, J. A. and Martha Kirk. 1962. The effect of oxygen on the reduction of CO_2 to glycolic acid and other products during photosynthesis by Chlorella. Biochem. Biophys. Res. Comm. 9: 376-380.

22. Bassham, J. A. and Martha Kirk. 1964. Photosynthesis of amino acids. Biochim. Biophys. Acta 90: 553-562.

23. Beevers, Harry. 1961. Respiratory Metabolism in Plants. Row, Peterson and Company, Evanston, Illinois, p. 213.

24. Benson, A. A. 1957. Sugar phosphates, paper and column chromatography. In: Methods in Enzymology, Vol. III. Sidney P. Colowick and Nathan O. Kaplan, eds. Academic Press, New York, pp. 110-129.

25. Benson, A. A., J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas, and W. Stepka. 1950. The path of carbon in photosynthesis. V. Paper chromatography and radioautography of the products. J. Am. Chem. Soc. 72: 1710-1718.

26. Benson, A. A. and M. Calvin. 1950. The path of carbon in photosynthesis. VII. Respiration and photosynthesis. J. Exptl. Bot. 1: 63-68.

27. Berenblum, Isaac and Ernst Chain. 1938. An improved method for the colorimetric determination of phosphate. Biochem. J. 32: 295-298.

28. Bertrand, Didier and Andre de Wolf. 1966. Influence des oligoelements fer et manganese dans le metabolisme de l-acide citrique et la synthese de l'aconitase chez Chlorella. Compt. Rend. D263: 1081-1083.

29. Björkman, Olle. 1966. The effect of oxygen concentration on photosynthesis in higher plants. Physiol. Plantarum 19: 618-633.

30. Björkman, Olle. 1966. Further studies of the effect of oxygen concentration on photosynthetic CO_2 uptake in higher plants. Carnegie Inst. Wash. Year Book 66: 220-228.

31. Block, Richard J., Emmett L. Durrum, and Gunter Zweig. 1958. Paper chromatography, quantitative methods. In: Paper Chromatography and Paper Electrophoresis. Second Edition. Academic Press, New York, p. 90.

32. Blostein, Rhoda. 1968. Relationships between erythrocyte membrane phosphorylation and adenosine triphosphate hydrolysis. J. Biol. Chem. 243: 1957-1965.

33. Bonner, James and J. E. Varner. 1965. The path of carbon in respiratory metabolism. In: Plant Biochemistry. James Bonner and J. E. Varner, eds. Academic Press, New York and London, pp. 213-230.

34. Bowling, J. D., J. E. McMurtrey, Jr., and D. E. Brown. 1952. Variety, description and performance tests with Maryland tobacco, measured by composition, yield and value. Bulletin A 73, University of Maryland.

35. Bradbeer, J. W. and C. M. A. Anderson. 1967. Glycolate formation in chloroplast preparations. In: Biochemistry of Chloroplasts, Vol. II. T. W. Goodwin, ed. Academic Press, London and New York, pp. 175-179.

36. Breidenbach, R. W. and Harry Beevers. 1967. Association of the glyoxylate cycle enzymes in a novel subcellular particle from castor bean endosperm. Biochem. Biophys. Res. Comm. 27: 462-469.

37. Breidenbach, R. W., Albert Kahn, and Harry Beevers. 1968. Characterization of glyoxysomes from castor bean endosperm. Plant Physiol. 43: 705-713.

38. Brummond, D. O. and R. H. Burris. 1954. Reactions of the tricarboxylic acid cycle in green leaves. J. Biol. Chem. 209: 755-765.

39. Buchanan, John M. and Christian B. Anfinsen. 1949. Partial purification of aconitase. J. Biol. Chem. 180: 47-54.

40. Buchanan, Bob B., Peter P. Kalberer, and Daniel I. Arnon. 1967. Ferredoxin-activated fructose diphosphatase in isolated chloroplasts. Biochem. Biophys. Res. Comm. 29: 74-79.

41. Buchanan, B. B., P. P. Kalberer, and D. I. Arnon. 1968. Ferredoxin-activated fructose diphosphatase of isolated chloroplasts. Fed. Proc. 27 (No. 2): 344.

42. Burns, Linda C., Robert M. O'Neal, and Roger E. Koeppe. 1967. Labeling patterns in glutamic acid in Nicotiana rustica L. from carbon-14 dioxide. J. Am. Chem. Soc. 89: 3938-3939.

43. Burton, David and P. K. Stumpf. 1966. Fat metabolism in higher plants. XXXII. Control of plant acetyl-CoA carboxylase activity. Arch. Biochem. Biophys. 117: 604-614.

44. Callery, A. G. and R. C. Fuller. 1967. Carboxylic acid cycle enzymes in Chloropseudomonas ethylicum. Biochem. J. 103: 74 P.

45. Calvin, Melvin and J. A. Bassham. 1962. The Photosynthesis of Carbon Compounds. W. A. Benjamin, Inc., New York.

46. Catcheside, D. G. 1964. Interallelic complementation. Brookhaven Symp. Biol. 17: 1-13.

47. Cennamo, C., G. Montecuccoli, and G. König. 1967. Inhibition of mitochondrial malate dehydrogenase by citrate. Biochim. Biophys. Acta 139: 514-516.

48. Chang, Wei-Hsien and N. E. Tolbert. 1965. Distribution of C^{14} in serine and glycine after $C^{14}O_2$ photosynthesis by isolated chloroplasts. Modification of serine-C^{14} degradation. Plant Physiol. 40: 1048-1052.

49. Cherbuliez, E., H. Probst, et J. Rabinowitz. 1962. Mecanismes de la scission alcaline des monoesters phosphoriques: hydrolyse ou β-elimination. Pharm. Acta Helv. 37: 396-400.

50. Clark, John M., Jr. 1964. Glucose-1-phosphate: enzymatic formation from starch. In: Experimental Biochemistry. John M. Clark, Jr., ed. W. H. Freeman and Company, San Francisco and London, pp. 30-35.

51. Clark, John M., Jr. 1964. Glucose-1-phosphate: chemical characterization. In: Experimental Biochemistry. John M. Clark, Jr., ed. W. H. Freeman and Company, San Francisco and London, pp. 36-39.

52. Cook, J. R. and Mary Carver. 1966. Partial photorepression of the glyoxylate by pass in Euglena. Plant and Cell Physiol. (Tokyo) 7: 377-383.

53. Coombs, J. and C. P. Whittingham. 1966. The mechanism of inhibition of photosynthesis by high partial pressures of oxygen in Chlorella. Proc. Roy. Soc. B. 164: 511-520.

54. Cossins, E. A. and S. K. Sinha. 1966. The interconversion of glycine and serine by plant tissue extracts. Biochem. J. 101: 542-549.

55. Cossins, E. A. and S. K. Sinha. 1967. Studies of glycollate utilization and some associated enzymes of C_1 metabolism in the endosperm of Ricinus communis L. J. Exp. Bot. 18: 215-228.

56. Davies, D. D., J. Hanford, and A. P. Wilkinson. 1959. The metabolism of aspartic acid, serine, and glycine in higher plants. Symp. Soc. Exp. Biol. XIII: 353-364.

57. Day, Clyde M. and Joel Selbin. 1962. Theoretical Inorganic Chemistry. Reinhold Publishing Corporation, New York, pp. 271-272.

58. Deal, W. C., W. J. Rutter, and K. E. Van Holde. 1963. Reversible dissociation of aldolase into unfolded subunits. Biochemistry 2: 246.

59. DeLuca, M., K. E. Ebner, D. E. Hultquist, G. Kreil, J. B. Peter, R. W. Moyer, and P. D. Boyer. 1963. The isolation and identification of phosphohistidine from mitochondrial protein. Biochem. Z. 338: 512-525.

60. Denison, F. W., Jr. and E. F. Phares. 1952. Rapid method for paper chromatography of organic acids. Anal. Chem. 24: 1628-1629.

61. Dennis, D. T. and T. P. Coultate. 1966. Phosphofructokinase, a regulatory enzyme in plants. Biochem. Biophys. Res. Comm. 25: 187-191.

62. Dennis, D. T. and T. P. Coultate. 1967. The regulatory properties of a plant phosphofructokinase during leaf development. Biochim. Biophys. Acta 146: 129-137.

63. Dickman, Sherman R. 1961. Aconitase. In: The Enzymes, Vol. 5. Second Edition. Paul D. Boyer, Henry Lardy, and Karl Myrbäck, eds. Academic Press, New York and London, pp. 495-510.

64. Dickman, Sherman R. and A. A. Cloutier. 1951. Factors affecting the activity of aconitase. J. Biol. Chem. 188: 379-388.

65. Dickman, Sherman R. and Joseph F. Speyer. 1954. Factors affecting the activity of mitochondrial and soluble aconitase. J. Biol. Chem. 206: 67-75.

66. Dilley, R. A. and L. P. Vernon. 1965. Ion and water transport processes related to the light-dependent shrinkage of spinach chloroplasts. Arch. Biochem. Biophys. 111: 365-375.

67. Dixon, Malcolm and Edwin C. Webb. 1964. Enzyme isolation. In: Enzymes. Second Edition. Academic Press, New York, pp. 27-53.

68. Dixon, Malcolm and Edwin C. Webb. 1964. Enzyme kinetics. In: Enzymes. Second Edition. Academic Press, New York, pp. 54-166.

69. Dixon, Malcolm and Edwin C. Webb. 1964. Enzyme classification. In: Enzymes. Second Edition. Academic Press, New York, pp. 167-198.

70. Dixon, Malcolm and Edwin C. Webb. 1964. Enzyme mechanisms. In: Enzymes. Second Edition. Academic Press, New York, pp. 259-314.

71. Dixon, Malcolm and Edwin C. Webb. 1964. Enzyme cofactors. In: Enzymes. Second Edition. Academic Press, New York, pp. 360-451.

72. Downes, R. W. and J. D. Hesketh. 1968. Enhanced photosynthesis at low oxygen concentrations: differential response of temperate and tropical grasses. Planta (Berl.) 78 (1): 79-84.

73. Ducet, Gaston and Albert Jean Rosenberg. 1962. Leaf respiration. Ann. Rev. Plant Physiol. 13: 27-44.

74. Egle, K. and H. Fock. 1967. Light respiration-correlations between CO_2 fixation, O_2 pressure and glycolate concentration. In: Biochemistry of Chloroplasts, Vol. II. T. W. Goodwin, ed. Academic Press, London and New York, pp. 79-87.

75. Eichhorn, Gunther L. 1960. The role of metal ions in enzyme systems. In: Metal-Binding in Medicine. Marvin J. Seven, ed. J. B. Lippincott Co., Philadelphia, Montreal, pp. 19-26.

76. Englard, Sasha. 1960. Configurational considerations in relation to the mechanisms of the stereospecific enzymatic hydrations of fumarate and cis-aconitate. J. Biol. Chem. 235: 1510-1516.

77. Englard, Sasha and Sidney P. Colowick. 1957. On the mechanism of the aconitase and isocitric dehydrogenase reactions. J. Biol. Chem. 226: 1047-1058.

78. Evans, M. C. W., Bob B. Buchanan, and Daniel I. Arnon. 1966. A new ferredoxin-dependent carbon reduction cycle in a photosynthetic bacterium. Proc. Natl. Acad. Sci. 55: 928-934.

79. Evered, D. F. and T. I. Steenson. 1964. Citrate inhibition of alkaline phosphatase. Nature 202: 491-492.

80. Fanshier, D. W., L. K. Gottwald, and E. Kun. 1964. Studies on specific enzyme inhibitors. VI. Characterization and mechanism of action of the enzyme-inhibitory isomer of monofluorocitrate. J. Biol. Chem. 239: 425-434.

81. Fewson, Charles A., Clanton C. Black, and Martin Gibbs. 1963. Further studies on the photochemical production of reduced triphosphopyridine nucleotide and adenosine triphosphate by fragmented spinach chloroplasts. Plant Physiol. 38: 680-685.

82. Fieser, Louis F. 1941. Experiments in Organic Chemistry. Part II. Second Edition. D. C. Heath and Company, pp. 395-396.

83. Fiske, C. H. and Y. Subbarow. 1925. The colorimetric determination of phosphorous. J. Biol. Chem. 66: 375-400.

84. Fluharty, Arvan and D. R. Sanadi. 1960. Evidence for a vicinal dithiol in oxidative phosphorylation. Proc. Natl. Acad. Sci. 46: 608-615.

85. Fluharty, Arvan L. and D. R. Sanadi. 1961. On the mechanism of oxidative phosphorylation. J. Biol. Chem. 236: 2772-2777.

86. da Fonseca-Wollheim, F., K. W. Bock, and H. Holzer. 1962. Preparation of "active glycolic aldehyde" (2-(1,2-dihydroxyethyl)-thiamine pyrophosphate) from hydroxypyruvate and thiamine pyrophosphate with a preparation of pyruvate oxidase from pig heart muscle. Biochem. Biophys. Res. Comm. 9: 466-471.

87. da Fonseca-Wollheim, F., H. Heesen, und H. Holzer. 1964. Oxidative Decarboxylierung von Hydroxypyruvat zu Glykolyl-Coenzym A. Biochem. Zeitschrift. 340: 383-389.

88. Forrester, Marlene L., G. Krotkov, and C. D. Nelson. 1966. Effect of oxygen on photosynthesis, photorespiration, and respiration in detached leaves. I. Soybean. Plant Physiol. 41: 422-427.

89. Forrester, Marlene L., G. Krotkov, and C. D. Nelson. 1966. Effect of oxygen on photosynthesis, photorespiration, and respiration in detached leaves. II. Corn and other monocotyledons. Plant Physiol. 41: 428-431.

90. Gawron, Oscar, Andrew J. Glaid, III, and Thomas P. Fondy. 1961. Stereochemistry of Krebs' cycle hydrations and related reactions. J. Am. Chem. Soc. 83: 3634-3640.

91. Gawron, Oscar and Kishan P. Mahajan. 1966. α-Methyl-cis-aconitic acid. Aconitase substrate. II. Substrate properties and aconitase mechanism. Biochemistry 5: 2343-2350.

92. Gest, Howard. 1966. Comparative biochemistry of photosynthetic processes. Nature 209: 879-882.

93. Gest, Howard, John G. Ormerod, and Kari S. Ormerod. 1962. Photometabolism of Rhodospirillum rubrum: light-dependent dissimilation of organic compounds to carbon dioxide and molecular hydrogen by an anaerobic citric acid cycle. Arch. Biochem. Biophys. 97: 21-33.

94. Gibbs, Martin. 1967. Photosynthesis. Ann. Rev. Biochem. 36: 658-784.

95. Gibbs, Martin, Elchanan S. Bamberger, Peter W. Ellyard, and R. Garth Everson. 1967. Assimilation of carbon dioxide by chloroplast preparations. In: Biochemistry of Chloroplasts, Vol. II. T. W. Goodwin, ed. Academic Press, London and New York, pp. 3-38.

96. Gibbs, Martin, Erwin Latzko, R. Garth Everson, and William Cockburn. 1967. Carbon mobilization by the green plant. In: Harvesting the Sun. Anthony San Pietro, Frances A. Greer, and Thomas J. Army, eds. Academic Press, New York and London, pp. 111-130.

97. Gomori, G. 1955. Preparation of buffers for use in enzyme studies. In: Methods in Enzymology, Vol. I. Sidney P. Colowick and Nathan O. Kaplan, eds. Academic Press, New York, p. 142.

98. Good, Norman E., G. Douglas Winget, Wilhelmina Winter, Thomas N. Connolly, Seikichi Izawa, and Raizada M. M. Singh. 1966. Hydrogen ion buffers for biological research. Biochemistry 5: 467-477.

99. Goore, Moshe Y. and John F. Thompson. 1967. γ-Glutamyl transpeptidase of kidney bean fruit. II. Studies of the activating effect of sodium citrate. Biochim. Biophys. Acta 132: 27-32.

100. Goulding, K. H. and M. J. Merrett. 1967. The role of glycollic acid in the photoassimilation of acetate by Chlorella pyrenoidosa. J. Exptl. Bot. 18: 620-630.

101. Graham, D. and Judith E. Cooper. 1967. Changes in levels of nicotinamide adenine nucleotides and Krebs cycle intermediates in mung bean leaves after illumination. Aust. J. Biol. Sci. 20: 319-327.

102. Graham, D. and D. A. Walker. 1962. Some effects of light on the interconversion of metabolites in green leaves. Biochem. J. 82: 554-560.

103. Gregolin, Carlo, Elena Ryder, Albrecht K. Kleinschmidt, Robert C. Warner, and M. Daniel Lane. 1966. Molecular characteristics of liver acetyl CoA carboxylase. Proc. Natl. Acad. Sci. 56: 148-155.

104. Groth, D. P. 1966. Deoxyribose-5-phosphate aldolase. II. Purification and properties of the rat liver enzyme. J. Biol. Chem. 242: 155-159.

105. Hanes, C. S. and F. A. Isherwood. 1949. Separation of the phosphoric esters on the filter paper chromatogram. Nature 164: 1107-1112.

106. Hanson, Kenneth R. and Irwin A. Rose. 1963. The absolute stereochemical course of citric acid biosynthesis. Proc. Natl. Acad. Sci. 50: 981-988.

107. Harrop, Linda C. and H. L. Kornberg, F. R. S. 1967. The role of isocitrate lyase in the metabolism of algae. Proc. R. Soc. B. 166: 11-29.

108. Hartley, R. D. and G. J. Lawson. 1960. Improved methods for the paper chromatography of organic acids. J. Chromatog. 4: 410-413.

109. Hatch, M. D. 1968. Distribution of the C_4-dicarboxylic acid pathway of photosynthesis and its occurance in dicotyledonous plants. Phytochemistry 7: 375-380.

110. Hatch, M. D. and C. R. Slack. 1966. Photosynthesis by sugar-cane leaves. A new carboxylation reaction and the pathway of sugar formation. Biochem. J. 101: 103-111.

111. Hatch, M. D. and C. R. Slack. 1967. Further studies on a new pathway of photosynthetic carbon dioxide fixation in sugar-cane and its occurrence in other plant species. Biochem. J. 102: 417-422.

112. Hatch, M. D. and C. R. Slack. 1967. The participation of phosphoenolpyruvate synthetase in photosynthetic CO_2 fixation of tropical grasses. Arch. Biochem. Biophys. 120: 224-225.

113. Havel, Richard J., Howard A. Eder, and Joseph H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Invest. 34: 1345.

114. Henson, Carl P. and Cleland, W. W. 1967. Purification and kinetic studies of beef liver cytoplasmic aconitase. J. Biol. Chem. 242: 3833-3838.

115. Herr, Earl B. Jr., James B. Sumner, and David W. Yesair. 1956. A study of the kinetics of aconitase inhibition and activation. Arch. Biochem. Biophys. 62: 29-39.

116. Hess, John L. 1966. A study and comparison of glycolic acid metabolism in tobacco and green algae. Ph.D. Thesis, Michigan State University.

117. Hess, J. L. and N. E. Tolbert. 1966. Glycolate, glycine, serine, and glycerate formation during photosynthesis by tobacco leaves. J. Biol. Chem. 241: 5705-5711.

118. Hess, J. L. and N. E. Tolbert. 1967. Glycolate pathway in algae. Plant Physiol. 42: 371-379.

119. Hess, J. L. and N. E. Tolbert. 1967. Changes in chlorophyll a/b ratio and products of $^{14}CO_2$ fixation by algae grown in blue or red light. Plant Physiol. 42: 1123-1130.

120. Hess, John L., N. E. Tolbert, and Lee M. Pike. 1967. Glycolate biosynthesis by Scenedesmus and Chlorella in the presence or absence of $NaHCO_3$. Planta (Berl.) 74: 278-285.

121. Hill, Robert L. and Louis Kanarek. 1964. The subunits of fumarase. Brookhaven Symp. Biol. 17: 80-94.

122. Hoagland, D. R. and D. I. Arnon. 1938. The water-culture method for growing plants without soil. Univ. Cal. Agr. Exp. Station Circular 347.

123. Hoch, George, Olga H. Owens, and Bessel Kok. 1963. Photosynthesis and respiration. Arch. Biochem. Biophys. 101: 171-180.

124. Horecker, B. L. and Arthur Kornberg. 1948. The extinction coefficients of the reduced band of pyridine nucleotides. J. Biol. Chem. 175: 385-390.

125. Jackson, William A. 1967. Comments on water and CO_2 transport in the photosynthetic process. In: Harvesting the Sun. Anthony San Pietro, Frances A. Greer, and Thomas J. Army, eds. Academic Press, New York and London, pp. 249-254.

126. Jagow, Rosemarie von, Manfred Kiese, and Werner Lenk. 1968. Hydroxylation of acetic acid to glycolic acid in rabbits. Biochim. Biophys. Acta 158 (1): 45-50.

127. Jencks, William P. 1962. Mechanisms of phosphate ester cleavage. Brookhaven Symp. Biol. 15: 134-153.

128. Jiang, Nai-Siang and D. P. Groth. 1962. Polycarboxylic acid activation of rat liver deoxyribose phosphate aldolase. J. Biol. Chem. 237: 3339-3341.

129. Johnson, L. Audrey and Marvin J. Seven. 1960. Observations on the in vivo stability of metal chelates. In: Metal-Binding in Medicine. Marvin J. Seven, ed. J. B. Lippincott Co., Philadelphia, Montreal, pp. 225-229.

130. Kaplan, N. O. 1964. Lactate dehydrogenase-structure and function. Brookhaven Symp. Biol. 17: 131-149.

131. Kearney, P. C. and N. E. Tolbert. 1962. Appearance of glycolate and related products of photosynthesis outside of chloroplasts. Arch. Biochem. Biophys. 98: 164-171.

132. Kok, Bessel. 1965. Photosynthesis: the path of energy. In: Plant Biochemistry. James Bonner and J. E. Varner, eds. Academic Press, New York and London, pp. 903-960.

133. Kolesnikov, P. A., E. I. Petrochenko, and S. V. Zore. 1958. Enzymatic reduction of quinone by glycolic acid. Dokl. Akad. Nauk. U.S.S.R. 123: 729.

134. Kolesnikov, P. A., E. I. Petrochenko, and S. V. Zore. 1960. The relation between glycolic acid oxidase and polyphenol oxidase. Fiziol. Rastenii (Transl.) 6: 607-611.

135. Kornberg. H. L. 1966. The role and control of the glyoxylate cycle in Escherichia coli. Biochem. J. 99: 1-11.

136. Kornberg, Arthur and B. L. Horecker. 1953. Triphosphopyridine nucleotide. Biochem. Prep. 3: 24-28.

137. Krebs, H. A. 1953. The equilibrium constants of the fumarase and aconitase systems. Biochem. J. 54: 78-82.

138. Krebs, H. A. and Olga Holzach. 1952. The conversion of citrate into cis-aconitate and isocitrate in the presence of aconitase. Biochem. J. 52: 527-528.

139. Krebs, H. A. and J. M. Lowenstein. 1960. The tricarboxylic acid cycle. In: Metabolic Pathways, Vol. I. Second Edition. David M. Greenberg, ed. Academic Press, New York and London, Chapter 4, pp. 129-203.

140. Lanchantin, G. F., J. A. Friedmann, and D. W. Hart. 1967. Esterase and clotting activities derived from citrate activation of human prothrombin. J. Biol. Chem. 242: 2491-2501.

141. Laties, George G. 1967. The inhibition of citrate, isocitrate, and α-ketoglutarate oxidation in aged potato slices by γ-hydroxy-α-ketoglutarate. Phytochemistry 6: 181-185.

142. Layne, Ennis. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. In: Methods in Enzymology, Vol. III. Sidney P. Colowick and Nathan O. Kaplan, eds. Academic Press, New York, pp. 447-454.

143. Leech, Rachel M. 1966. Comparative biochemistry and comparative morphology of chloroplasts isolated by different methods. In: Biochemistry of Chloroplasts, Vol. I. T. W. Goodwin, ed. Academic Press, London and New York, pp. 65-74.

144. Lewis, Katharine F. and Sidney Weinhouse. 1957. Determination of glycolic, glyoxylic, and oxalic acids. In: Methods in Enzymology, Vol. III. Sidney P. Colowick and Mathan O. Kaplan, eds. Academic Press, New York, pp. 269-276.

145. Lineweaver, H. and D. Burk. 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56: 658.

146. Ljungdahl, Lars, Eckart Irion, and Harland G. Wood. 1965. Total synthesis of acetate from CO_2. I. Co-methylcobyric acid and Co-(methyl)-5-methoxybenzimidazolyl cobamide as intermediates with Clostridium thermoaceticum. Biochemistry 4: 2771-2780.

147. Lorber, Victor, M. F. Utter, Harry Rudney, and Margaret Cook. 1950. The enzymatic formation of citric acid studied with C^{14}-labelled oxalacetate. J. Biol. Chem. 185: 689-699.

148. Lowry, Oliver H., Nira J. Rosebrough, A. Lewis Farr, and Rose J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

149. Mahler, Henry R. and Eugene H. Cordes. 1966. Enzyme kinetics. In: Biological Chemistry. Harper and Row, New York and London, pp. 219-277.

150. Mahler, H. R. and Dorothee G. Elowe. 1954. Studies on metallo flavoproteins. II. The role of iron in diphosphopyridine nucleotide cytochrome c reductase. J. Biol. Chem. 210: 165-179.

151. Malachowski, R., M. Giedroye, and Z. Jerzmanowska. 1928. Untersuchungen über Aconitsäuren, II: Konstitution und Bildungsart der Aconitsäure-anhydride. Ber. 61B: 2525-2538.

152. Malachowski, R. and M. Maslowski. 1928. Untersuchungen über Aconitsäuren. I: Stereochemie der Aconitsäuren. Ber. 61B: 2521-2525.

153. Manton, I. 1966. Some possibly significant structural relations between chloroplasts and other cell components. In: Biochemistry of Chloroplasts, Vol. I. T. W. Goodwin, ed. Academic Press, London and New York, pp. 23-47.

154. Marsh, Herbert V., Jr., Jean M. Galmiche, and Martin Gibbs. 1965. Effect of light on the tricarboxylic acid cycle in Scenedesmus. Plant Physiol. 40: 1013-1022.

155. Martin, J. B. and D. M. Doty. 1949. Determination of inorganic phosphate. Modification of isobutyl alcohol procedure. Anal. Chem. 21: 965-967.

156. Martius, C. and F. Knoop. 1937. Der physiologische Abbau der Citronensäure. Vorläufige Mitteilung. Z. Physiol. Chem. 246: I-II.

157. Martius, C. and F. Lynen. 1950. Probleme des Citronensäurecyklus. In: Advances in Enzymology, Vol. X. F. F. Nord, ed. Interscience Publishers, Inc., New York and London, pp. 167-222.

158. Massey, V. 1953. The effects of inorganic anions on fumarase activity. Biochem. J. 53: 67-71.

159. Massey, V. 1953. Studies on fumarase. 4. The effects of inhibitors on fumarase activity. Biochem. J. 55: 172-177.

160. Milhaud, Gerard, A. A. Benson, and Melvin Calvin. 1956. Metabolism of pyruvic acid-2-C^{14} and hydroxy pyruvic acid-2-C^{14} in algae. J. Biol. Chem. 218: 599-606.

161. Miller, R. M., Meyer, C. M., and H. A. Tanner. 1963. Glycolate excretion and uptake by Chlorella. Plant Physiol. 38: 184-188.

162. Morrison, J. F. 1954. The purification of aconitase. Biochem. J. 56: 99-105.

163. Morrison, J. F. 1954. The activation of aconitase by ferrous ions and reducing agents. Biochem. J. 58: 685-692.

164. Morrison, J. F. 1954. The kinetics of the reactions catalyzed by aconitase. Australian J. Exp. Biol. Med. Sci. 32: 867-876.

165. Morrison, J. F. 1954. The influence of buffers on the pH optimum of aconitase. Australian J. Exp. Biol. Med. Sci. 32: 877-884.

166. Morton, Robert K. 1955. Methods of extraction of enzymes from animal tissues. In: Methods in Enzymology, Vol. I. Sidney P. Colowick and Nathan O. Kaplan, eds. Academic Press, New York, pp. 25-51.

167. Mudd, J. B. 1967. Fat metabolism in plants. Ann. Rev. Plant Physiol. 18: 229-252.

168. Mukerji, S. K. and Irwin P. Ting. 1968. Intracellular localization of CO_2 metabolism enzymes in Cactus phylloclades. Phytochemistry 7: 903-911.

169. Neilson, Nora E. 1955. The aconitase of Aspergillus niger. Biochim. Biophys. Acta 17: 139-140.

170. Neilson, Nora E. 1956. The presence of aconitase and "aconitic hydrase" in Aspergillus niger. J. Bacteriol. 71: 356-361.

171. Neilson, Nora E. 1962. Aconitic hydrase from Aspergillus niger. In: Methods in Enzymology, Vol. V. Sidney P. Colowick and Nathan O. Kaplan, eds. Academic Press, New York and London, pp. 614-616.

172. Nigam, S. N. and W. B. McConnell. 1962. Studies on wheat plants using carbon-14 compounds. XVIII. Utilization of pyruvate-3-^{14}C by wheat seedlings. Can. J. Biochem. and Physiol. 40: 1452-1454.

173. Noguchi, Masao and Einosuke Tamaki. 1962. Studies on nitrogen metabolism in tobacco plants. A. Part II. Diurnal variation in the amino acid composition of tobacco leaves. Arch. Biochem. Biophys. 98: 197-205.

174. Noguchi, Masao, Kyoko Yamamoto, Shigenobu Mizusaki, Takako Takahara, and Einosuke Tamaki. 1963. Studies on nitrogen metabolism in tobacco plants. A. Part IV. Diurnal variations in the keto acid content of tobacco leaves. Sci. Reports of Central Research Institute, Japan Monopoly Corporation, No. 105: 153-155.

175. Nordlie, Robert C. and Philip T. Johns. 1968. The inhibition of microsomal glucose-6-phosphatase by metal-binding agents. Biochemistry 7: 1473.

176. Nordlie, Robert C. and David G. Lygre. 1966. The inhibition by citrate of inorganic pyrophosphate-glucose phosphotransferase and glucose-6-phosphatase. J. Biol. Chem. 241: 3136-3141.

177. Ogren, William L. and David W. Krogmann. 1965. Studies on pyridine nucleotides in photosynthetic tissue. Concentrations, interconversions, and distribution. J. Biol. Chem. 240: 4603-4608.

178. Ogston, A. G. 1951. Interpretation of experiments on metabolic processes, using isotopic tracer elements. Nature 162: 963.

179. Ogston, A. G. 1951. Specificity of the enzyme aconitase. Nature 167: 693.

180. Ogur, Maurice, Lowell Coker, and Sylvia Ogur. 1964. Glutamate auxotrophs in Saccharomyces. I. The biochemical lesion in the glt_1 mutants. Biochem. Biophys. Res. Comm. 14: 193-197.

181. Ogur, M., A. Roshanmanesh, and S. Ogur. 1965. Tricarboxylic acid cycle mutants in Saccharomyces: comparison of independently derived mutants. Science 147: 1590.

182. Ongun, Alpaslan and C. R. Stocking. 1965. Effect of light and dark on the intracellular fate of photosynthetic products. Plant Physiol. 40: 825-831.

183. Orth, Gertrude M., N. E. Tolbert, and Eduardo Jimenez. 1966. Rate of glycolate formation during photosynthesis at high pH. Plant Physiol. 41: 143-147.

184. Pal, H. R. S. and P. S. Krishnan. 1961. Purification and properties of cis-aconitic decarboxylase. Arch. Mikrobiol. 39: 335-342.

185. Palmer, M. J. 1964. The relationship between iron and activity of aconitase purified from the leaves of mustard (Sinapis alba). Biochem. J. 92: 404-410.

186. Park, Jane Harting and D. E. Koshland, Jr. 1958. The hydrolytic activity of glyceraldehyde-3-phosphate dehydrogenase. J. Biol. Chem. 233: 986-990.

187. Payes, Benjamin and George G. Laties. 1963. The inhibition of several tricarboxylic acid cycle enzymes by γ-hydroxy-α-ketoglutarate. Biochem. Biophys. Res. Comm. 10: 460-466.

188. Pedersen, T. A., Martha Kirk, and J. A. Bassham. 1966. Inhibition of photophosphorylation and photosynthetic carbon cycle reactions by fatty acids and esters. Biochim. Biophys. Acta 112: 189-203.

189. Pedersen, T. A., Martha Kirk, and J. A. Bassham. 1966. Light-dark transients in levels of intermediate compounds during photosynthesis in air-adapted Chlorella. Physiol. Plantarum 19: 219-231.

190. Peterkofsky, A. and E. Racker. 1961. The reductive pentose phosphate cycle. III. Enzyme activities in cell-free extracts of photosynthetic organisms. Plant Physiol. 36: 409-414.

191. Peters, R. A. 1961. Further experiments on the inhibition of aconitase by enzymically prepared fluorocitric acid. Biochem. J. 79: 261-268.

192. Peterson, Elbert A. and Herbert A. Sober. 1962. Column chromatography of proteins: substituted celluloses. In: Methods in Enzymology, Vol. V. Sidney P. Colowick and Nathan O. Kaplan, eds. Academic Press, New York, pp. 3-27.

193. Pierpoint, W. S. 1963. The distribution of succinate dehydrogenase and malate dehydrogenase among components of tobacco-leaf extracts. Biochem. J. 88: 120-125.

194. Preiss, Jack, Martie Louise Biggs, and Elaine Greenberg. 1967. The effect of magnesium ion concentration on the pH optimum of the spinach leaf alkaline fructose diphosphatase. J. Biol. Chem. 242: 2292-2294.

195. Rabinowitch, Eugene I. 1951. Absolute maximum rates of photosynthesis of different plants. In: Photosynthesis, Vol. II. Part 1. Interscience Publishers, Inc., New York, pp. 990-991.

196. Racker, E. 1950. Spectrophotometric measurements of the enzymatic formation of fumaric and cis-aconitic acids. Biochim. Biophys. Acta 4: 211-214.

197. Racker, Efraim. 1965. Mechanisms in Bioenergetics. Academic Press, New York and London, pp. 89-92.

198. Rahatekar, H. I. and M. R. Raghauendra Rao. 1963. Cofactor requirements of aconitase from diverse sources. Enzymologia 25: 292-296.

199. Ranson, S. L. 1965. The plant acids. In: Plant Biochemistry. James Bonner and J. E. Varner, eds. Academic Press, New York and London, pp. 493-525.

200. Ranson, S. L. and M. Thomas. 1960. Crassulacean acid metabolism. Ann. Rev. Plant Physiol. 11: 81-110.

201. Richardson, K. E. and N. E. Tolbert. 1961. Oxidation of glyoxylic acid to oxalic acid by glycolic acid oxidase. J. Biol. Chem. 236: 1280-1284.

202. Richardson, K. E. and N. E. Tolbert. 1961. Phosphoglycolic acid phosphatase. J. Biol. Chem. 236: 1285-1290.

203. Rose, Irwin A. 1966. Mechanisms of enzyme action. Ann. Rev. Biochem. 35: 23-52.

204. Rose, Irwin A. and Edward L. O'Connell. 1967. Mechanism of aconitase action. I. The hydrogen transfer reaction. J. Biol. Chem. 242: 1870-1879.

205. Ruffo, A., E. Testa, Anna Adinolfi, and Giuseppina Pelizza. 1962. Control of the citric acid cycle by glyoxylate. Biochem. J. 85: 588-593.

206. Ruffo, A., E. Testa, A. Adinolfi, G. Pelizza, and R. Moratti. 1967. Control of the citric acid cycle by glyoxylate. Mechanism of the inhibition by oxalomalate and γ-hydroxy-α-oxoglutarate. Biochem. J. 103: 19-23.

207. Rutter, William J. 1964. Evolution of aldolase. Fed. Proc. 23: 1248.

208. Sakaguchi, Kin-ichirô and Teruhiko Beppu. 1959. Study of alloisocitric acid formation. Arch. Biochem. Biophys. 83: 131-140.

209. Salas, Maria L., E. Vinuela, Margarita Salas, and A. Sols. 1965. Citrate inhibition of phosphofructokinase and the Pasteur effect. Biochem. Biophys. Res. Comm. 19: 371-376.

210. Santarius, Kurt A. and Ulrich Heber. 1965. Changes in the intracellular levels of ATP, ADP, AMP, and P_i and regulatory function of the adenylate system in leaf cells during photosynthesis. Biochim. Biophys. Acta 102: 39-54.

211. Schachman, H. K. 1963. Considerations on the tertiary structure of proteins. Symp. Quant. Biol. 28: 409-430.

212. Schlesinger, Milton J. 1964. In vitro complementation and the subunit structure of E. coli alkaline phosphatase. Brookhaven Symp. Biol. 17: 66-76.

213. Schmidt, G. and M. Laskowski, Sr. 1961. Phosphate ester cleavage (survey). In: The Enzymes, Vol. 5. Second Edition. Paul D. Boyer, Henry Lardy, and Karl Myrback, eds. Academic Press, New York and London, pp. 3-35.

214. Schneider, Walter C. 1959. Mitochondrial metabolism. In: Advances in Enzymology, Vol. 21. F. F. Nord, ed. Interscience Publishers, Inc., New York, pp. 1-72.

215. Schramm, R. W. 1960. New solvents for paper chromatography of nonvolatile organic acids. Chem. Anal. (Warsaw) 5: 1055-1062.

216. Schroeder, Henry A. 1960. Possible relationships between trace metals and chronic diseases. In: Metal-Binding in Medicine. Marvin J. Seven, ed. J. B. Lippincott Co., Philadelphia, Montreal, pp. 59-67.

217. Searls, Robert L. and D. R. Sanadi. 1960. Evidence for a vicinal dithiol in dihydrothioctyl dehydrogenase. Biochem. Biophys. Res. Comm. 2: 189-192.

218. Sharma, N. N. and G. H. Bourne. 1964. Histochemical studies on the distribution of DPN and TPN diaphorases, β-glucuronidase, and some enzymes associated with the Krebs cycle in Trichomonas vaginalis. Histochemie 3: 487-494.

219. Siebert, Günther. 1965. Citrate and isocitrate determination with aconitase and isocitric dehydrogenase. In: Methods of Enzymatic Analysis. Second Printing, Revised. Hans-Ulrich Bergmeyer, ed. Verlag Chemie, GMBH, Weinheiml Bergstr., Academic Press, New York and London, pp. 318-323.

220. Sillen, Lars Gunnar and Arthur E. Martell. 1964. Stability Constants of Metal-Ion Complexes. Special Publication No. 17. London: The Chemical Society, Burlington House, W. 1, pp. 477-481.

221. Slack, C. R. 1968. The photoactivation of a phosphopyruvate synthase in leaves of Amaranthus palmeri. Biochem. Biophys. Res. Comm. 30: 483-488.

222. Slack, C. R. and M. D. Hatch. 1967. Comparative studies on the activity of carboxylases and other enzymes in relation to the new pathway of photosynthetic carbon dioxide fixation in tropical grasses. Biochem. J. 103: 660-665.

223. Speyer, Joseph F. and Sherman R. Dickman. 1956. On the mechanism of action of aconitase. J. Biol. Chem. 220: 193-208.

224. Stern, Joseph R. and Gunta Bambers. 1966. Glutamate biosynthesis in anaerobic bacteria. I. The citrate pathways of glutamate synthesis in Clostridium kluyveri. Biochemistry 5: 1113-1118.

225. Stern, Joseph R., C. S. Hegre, and Gunta Bambers. 1966. Glutamate biosynthesis in anaerobic bacteria. II. Stereospecificity of aconitase and citrate synthetase of Clostridium kluyveri. Biochemistry 5: 1119-1124.

226. Stiller, Mary. 1962. The path of carbon in photosynthesis. Ann. Rev. Plant Physiol. 13: 151-170.

227. Sugiyama, T., N. Nakayama, and T. Akazawa. Activation of spinach leaf ribulose-1,5-diphosphate carboxylase activities by magnesium ions. Biochem. Biophys. Res. Comm. 30 (2): 118-123.

228. Szulmajster, Jekisiel and Richard S. Hanson. 1964. Physiological control of sporulation in Bacillus subtilis. In: Spores III. A symposium held at Allerton Park, Illinois, pp. 162-173.

229. Tanner, Howard A., Thomas E. Brown, Clyde Eyster, and R. W. Treharne. 1960. A manganese dependent photosynthetic process. Biochem. Biophys. Res. Comm. 3: 205-210.

230. Thompson, C. M. and C. P. Whittingham. 1967. Intracellular localization of phosphoglycollate phosphatase and glyoxylate reductase. Biochim. Biophys. Acta 143: 642-644.

231. Thomson, John F., Sharron L. Nance, Karen J. Bush, and Patricia A. Szczepanik. 1966. Isotope and solvent effects of deuterium on aconitase. Arch. Biochem. Biophys. 117: 65-74.

232. Tolbert, N. E. 1958. Secretion of glycolic acid by chloroplasts. Brookhaven Symp. Biol. 11: 271-275.

233. Tolbert, N. E. 1963. Glycolate pathway. In: Photosynthetic Mechanisms of Green Plants. NAS-NRC Publication 1145, pp. 648-662.

234. Tolbert, N. E. and Marjorie S. Cohan. 1953. Products formed from glycolic acid in plants. J. Biol. Chem. 204: 649-654.

235. Tolbert, N. E. and J. L. Hess. 1966. The effect of hydroxymethanesulfonates on $^{14}CO_2$ photosynthesis by algae. J. Biol. Chem. 241: 5712-5715.

236. Tolbert, N. E., A. Oeser, T. Kisaki, R. H. Hageman, and R. K. Yamazaki. 1968. Peroxisomes from leaves with enzymes related to glycolate metabolism. Fed. Proc. 27 (No. 2): 344.

237. Tolbert, N. E., A. Oeser, T. Kisaki, R. H. Hageman, and R. K. Yamazaki. 1968. Peroxisomes from spinach leaves containing enzymes related to glycolate metabolism. J. Biol. Chem. 243: 5179-5184.

238. Tolbert, N. E. and L. P. Zill. 1956. Excretion of glycolic acid by algae during photosynthesis. J. Biol. Chem. 222: 895-906.

239. Tomizawa, Jun-Ichi. 1953. The mechanism of aconitase action. I. The steady-state analysis and the kinetic theory. J. Biochem. (Tokyo) 40: 339-349.

240. Tomizawa, Jun-Ichi. 1953. The mechanism of aconitase action. II. Some evidences supporting one enzyme and one activated complex theory and the comparative studies on fumarase action. J. Biochem. (Tokyo) 40: 351-359.

241. Tomizawa, Jun-Ichi. 1954. The mechanism of aconitase action. III. Kinetic analysis using DL-isocitric acid-2-C^{14}. J. Biochem. (Tokyo) 41: 567-575.

242. Treble, D. H., D. T. A. Lamport, and R. A. Peters. 1962. The inhibition of plant aconitate hydratase (aconitase) by fluorocitrate. Biochem. J. 85: 113-115.

243. Ullrich, Johannes. 1963. Phosphatase action on phosphoglycolic, 3-phosphoglyceric, and phosphoenol pyruvic acids in spinach chloroplast fragments in the presence and absence of high concentrations of methanol. Biochim. Biophys. Acta 71: 589-594.

244. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1964. Manometric Techniques. Fourth Edition. Burgess Publishing Company, Minneapolis, Minn., pp. 252-253.

245. Vagelos, Roy P., A. W. Alberts, and Donald B. Martin. 1963. Studies on the mechanism of activation of acetyl coenzyme A carboxylase by citrate. J. Biol. Chem. 238: 533-540.

246. Vallee, Bert L. 1960. Metal and enzyme interactions: correlation of composition, function, and structure. In: The Enzymes, Vol. 3. Paul D. Boyer, Henry Lardy, and Karl Myrbäck, eds. Academic Press, New York and London, pp. 225-276.

247. Van Wazer, John R. 1956. Principles of phosphorous chemistry. I. Some generalities concerning multiple bonding. J. Am. Chem. Soc. 78: 5709-5715.

248. Vickery, Hubert Bradford. 1962. A suggested new nomenclature for the isomers of isocitric acid. J. Biol. Chem. 237: 1739-1741.

249. Warburg, O. and W. Christian. 1941/1942. Isolierung und Kristallisation des Gärungsferments Enolase. Biochem. Z. 310: 384.

250. Warburg, Otto and Günter Krippahl. 1960. Glykolsäurebildung in Chlorella. Z. Naturforschung 15b: 197-199.

251. Warburg, Otto, Günter Krippahl, Klaus Jetschmann, and Arnold Lehmann. 1963. Chemie der Photosynthese. Z. Naturforschung 18: 837-844.

252. Webb, J. Leyden. 1963. Inhibition by reaction with substrate, coenzyme, or activator. In: Enzyme and Metabolic Inhibitors, Vol. I. Academic Press, New York and London, pp. 85-90.

253. Weimberg, Ralph. 1967. The effect of sodium chloride on the activity of a soluble malate dehydrogenase from pea seeds. J. Biol. Chem. 242: 3000-3006.

254. White, Abraham, Handler, Philip, and Smith, Emil L. 1964. Principles of Biochemistry. McGraw-Hill Book Company, New York, Toronto, London.

255. Whittingham, C. P., J. Coombs, and A. F. H. Marker. 1967. The role of glycollate in photosynthetic carbon fixation. In: Biochemistry of Chloroplasts, Vol. II. T. W. Goodwin, ed. Academic Press, New York and London, pp. 155-173.

256. Whittingham, C. P., R. G. Hiller, and M. Bermingham. 1963. The production of glycollate during photosynthesis. In: Photosynthetic Mechanisms of Green Plants. NAS-NRC Publication 1145, pp. 675-683.

257. Wilcox, Philip E., Charles Heidelberger, and Van R. Potter. 1950. Chemical preparation of asymmetrically labeled citric acid. J. Am. Chem. Soc. 72: 5019-5024.

258. Wilson, A. T. and M. Calvin. 1955. The photosynthetic cycle. CO_2 dependent transients. J. Am. Chem. Soc. 77: 5948-5957.

259. Wood, Norris P. 1966. Control of oxidation-reduction potential during purification. In: Methods in Enzymology, Vol. IX. Willis A. Wood, ed. Academic Press, New York and London, pp. 3-9.

260. Wood, W. A. 1966. Carbohydrate metabolism. Ann. Rev. Biochem. 35: 521-558.

261. Wu, Ray. 1966. Further analysis of the mode of inhibition and activation of Novikoff ascites tumor phosphofructokinase. J. Biol. Chem. 241: 4680-4685.

262. Yamazaki, R. K. and N. E. Tolbert. Malate dehydrogenase in leaf peroxisomes. (Submitted to Biochim. Biophys. Acta).

263. Yates, Richard A. and Arthur B. Pardee. 1956. Control of pyrimidine biosynthesis in Escherichia Coli by a feed-back mechanism. J. Biol. Chem. 221: 757-770.

264. Yielding, K. L. and G. M. Tomkins. 1962. Inhibition of glutamic dehydrogeanse by o-phenanthroline and its analogs. Biochim. Biophys. Acta 62: 327-331.

265. Yu, Y. L., N. E. Tolbert, and Gertrude M. Orth. 1964. Isolation and distribution of phosphoglycolate phosphatase. Plant Physiol. 39: 643-647.

266. Zelitch, Israel. 1955. The isolation and action of crystalline glyoxylic acid reductase from tobacco leaves. J. Biol. Chem. 216: 553-575.

267. Zelitch, I. 1957. α-Hydroxysulfonates as inhibitors of the enzymatic oxidation of glycolic and lactic acids. J. Biol. Chem. 224: 251.

268. Zelitch, I. 1958. The role of glycolic acid oxidase in the respiration of leaves. J. Biol. Chem. 233: 1299.

269. Zelitch, I. 1959. The relationship of glycolic acid to respiration and photosynthesis in tobacco leaves. J. Biol. Chem. 234: 3077.

270. Zelitch, Israel. 1964. Organic acids and respiration in photosynthetic tissues. Ann. Rev. Plant Physiol. 15: 121-142.

271. Zelitch, Isreal. 1965. The relation of glycolic acid synthesis to the primary photosynthetic carboxylation reaction in leaves. J. Biol. Chem. 240: 1869-1876.

272. Zelitch, Israel. 1966. Increased rate of net photosynthetic carbon dioxide uptake caused by the inhibition of glycolate oxidase. Plant Physiol. 41: 1623-1631.

273. Zelitch, Israel and G. A. Barber. 1960. Oxidative phosphorylation and glycolate oxidation by particles from spinach leaves. Plant Physiol. 35: 205-209.

274. Zelitch, Israel and D. A. Walker. 1964. The role of glycolic acid metabolism in opening of leaf stomata. Plant Physiol. 39: 856-862.

275. Zwarenstein, H. and V. Van der Schyff. 1967. Inhibition of phosphoglucomutase by citrate. Biochem. Biophys. Res. Comm. 26 (3): 372-375.