THE ROLE OF OXYGEN IN PHOTORESPIRATION

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ABSTRACT

THE ROLE OF OXYGEN IN PHOTORESPIRATION

By

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The role of oxygen in photorespiration, in the inhibition of net photosynthetic CO_2 fixation, the Warburg effect, and in the synthesis and metabolism of glycolate has been investigated. Oxygen consumption occurs during P-glycolate formation in chloroplasts and glycolate oxidation in peroxisomes.

Glycolate oxidase (E.C. 1.1.3.1) was purified to homogeneity from the peroxisomes of spinach leaves. Glycolate oxidase catalysed the oxidation of glycolate to glyoxylate, glyoxylate to oxalate and L-lactate to pyruvate. The $K_M(O_2)$ for each of these three reactions was the same, namely, about 1.9×10^{-4}M at 25^o, which suggests that oxygen reacts with the same intermediate in each reaction. The energy of activation (E_a) for each substrate with oxygen as the terminal electron acceptor was 11.5 Kcal. It was concluded that the overall reaction is dependent upon the steady state concentration of the reduced flavin enzyme complex.

The apoenzyme of glycolate oxidase was prepared by treatment with 1M KBr. The reactivation of the

apoenzyme with flavin mononucleotide (FMN) was followed spectrophotometrically.

In vivo and in vitro experiments with $^{18}O_2$ dealt with the second site of oxygen consumption in photorespiration, namely that involved with glycolate synthesis. The technique of combined gas chromatography - mass spectrometry was used. When detached spinach leaves were exposed to an atmosphere of 100% oxygen containing $^{18}O_2$, a single atom of oxygen-18 was rapidly incorporated into the carboxyl group of glycine and serine, the metabolic derivatives of glycolate. This incorporation occurred only in the light as a photorespiratory event. No label was incorporated into the hydroxyl group of serine which is derived from water. Under the same conditions, glycerate and phosphoglycerate did not become labeled. The data was consistent with glycolate formation by a two or a four electron oxidation of a sugar phosphate from the photosynthetic carbon cycle.

A large pool of erythronic (or threonic) acid was also identified but it did not become labeled either with oxygen-18 or with carbon-14 during a 10 min $^{14}CO_2$ photosynthesis - photorespiration experiment.

[U-^{14}C]-ribulose diphosphate was synthesized for use as a precursor of phosphoglycolate. In the presence of Mn ions and hydrogen peroxide, ribulose diphosphate was non-enzymatically oxidised to phosphoglycolate and phosphoglyceraldehyde. A previous report

that ribulose diphosphate carboxylase (E.C. 4.1.1.39) catalysed the oxidation of ribulose diphosphate by molecular oxygen to phosphoglycolate was confirmed by the use of [^{14}C]-ribulose diphosphate. The other product was proven to be phosphoglycerate.

A manometric assay to follow oxygen consumption by this reaction was developed. Spinach leaf ribulose diphosphate carboxylase was purified by two methods to homogeneity as revealed by polyacrylamide disc gel electrophoresis. The first method included $(NH_4)_2SO_4$ fractionation, zonal centrifugation in a sucrose density gradient and hydroxylapatite column chromatography. The second method included DEAE-cellulose column chromatography and zonal centrifugation. Both purified preparations catalysed the oxidation of ribulose diphosphate. This activity has been named ribulose diphosphate oxygenase. Ribulose diphosphate carboxylase co-purified with ribulose diphosphate oxygenase. Several lines of evidence were marshalled to evaluate the homogeneity of the purified preparation but it has not been unequivocally established that the two activities are associated with one and the same protein. During storage in ammonium sulfate the oxygenase was generally more stable than the carboxylase. Under these conditions ribulose diphosphate carboxylase underwent polymerisation.

Oxygen uptake by ribulose diphosphate oxygenase was a linear function of time and enzyme concentration.

The oxygen uptake depended upon the presence of ribulose diphosphate, Mg^{2+}, oxygen and enzyme. Enzyme which had been boiled for 2 min was inactive. The activity was stimulated by, but not absolutely dependent upon, the presence of dithiothreitol. This stimulation was not due to the oxidation of dithiothreitol. The pH optimum for oxygen uptake was about 9.3. The stoichiometry of oxygen consumption to ribulose diphosphate consumption was one to one. The oxygenase activity was specific for ribulose diphosphate. Ribulose-5-phosphate, ribulose, fructose-6-phosphate, fructose-1,6-diphosphate and 3-phosphoglycerate were inactive when tested with the standard manometric assay and/or by the use of ^{14}C labeled substrates. The absence of activity with 3-phosphoglycerate established that the reaction with ribulose diphosphate did not first proceed by carboxylation followed by oxidation of the resultant 3-phosphoglycerate. Under 100% oxygen the K_M for ribulose diphosphate was about 1.5×10^{-4}M.

The reaction products of ribulose diphosphate oxygenase were identified by gas chromatography - mass spectrometry of the trimethylsilyl derivates of phosphoglycolate and phosphoglycerate. Experiments with $^{18}O_2$ and $H_2{}^{18}O$ were performed with the enzyme system. The results were consistent with those obtained <u>in vivo</u>. Oxygenation of ribulose diphosphate proceeds with the incorporation of a single atom of oxygen, derived from

molecular oxygen, into the carboxyl group of phosphoglycolate. No incorporation occurred into phosphoglycerate. By experiments with $H_2{}^{18}O$, the carboxyl oxygen of phosphoglycerate was shown to be derived from water. The enzyme did not catalyse the exchange of the carboxyl oxygens of phosphoglycolate or phosphoglycerate with those of the medium. A mechanism consistent with these observations, involving the formation of a ribulose diphosphate peroxide intermediate, was proposed. The overall reaction is

$$\text{Ribulose-1,5-diphosphate} + O_2 \xrightarrow{Mg^{2+}} \begin{matrix} \text{2-Phosphoglycolate} \\ + \\ \text{3-Phosphoglycerate} \end{matrix}$$

Phosphoglycolate biosynthesis from ribulose diphosphate and its subsequent oxidative metabolism serves as a biochemical model to explain photorespiration.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ammediol	2-amino-2-methyl-1,3-propanediol
BSTFA	bis(trimethylsilyl)trifluoroacetamide
DCPIP	dichlorophenolindophenol
DEAE	diethylaminoethyl
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
α GPD	α glycerophosphate dehydrogenase
IAA	iodoacetamide
α KG	α-ketoglutarate
Me_3Si	trimethylsilyl
PCMB	para-chloromercuribenzoate
3-PGA	3-phosphoglycerate
POPOP	phenyl-oxazolylphenyl-oxazolylphenyl
PPO	2,5-diphenyloxazole
RuDP	ribulose-1,5-diphosphate
SDS	sodium dodecyl sulfate
TEAE	triethylaminoethyl
THFA	tetrahydrofolic acid
TPI	triose phosphate isomerase
Tris	tris(hydroxymethyl)amino methane

INTRODUCTION

In 1920 the late Otto Warburg observed that oxygen inhibited net photosynthetic CO_2 fixation in Chlorella. This phenomenon, now referred to as the Warburg effect, has been observed in a wide variety of photosynthetic tissues. The Warburg effect can be explained, at least in large part, by photorespiration, the light dependent uptake of O_2 and release of CO_2 that occurs in the photosynthetic tissues of all higher plants. Photorespiration is the physiological manifestation of the glycolate pathway of metabolism, which has been the subject of extensive investigations by Tolbert, Zelitch, Bassham, Whittingham and their associates. The glycolate pathway is an important adjunct to the photosynthetic carbon reduction cycle and estimates of the quantity of carbon flowing through this pathway under natural conditions range from 30 to 90% of the total carbon fixed during photosynthesis. The function of the glycolate pathway is at present obscure. Whatever the function, it results in the uptake of O_2 and release of CO_2, thus decreasing the efficiency of photosynthesis and ultimately of growth.

This research reported in this thesis has

concentrated on the role of oxygen in photorespiration. Chapter I reports some modest and rather diverse observations on one of the enzymes involved in photorespiratory O_2 uptake, glycolate oxidase. Chapter II reports the discovery of a second site of oxygen consumption, that involved in the synthesis of glycolate. The succeeding chapters describe the research which arose out of the results reported in Chapter II and also from the report Bowes et al., (1971) that ribulose diphosphate carboxylase catalysed the oxidation of ribulose diphosphate to P-glycolate and P-glycerate.

LITERATURE REVIEW

The C-3 Photosynthetic Carbon Reduction Cycle

Since its enunciation by Calvin and his associates in the mid-1950's, the C-3 photosynthetic carbon reduction cycle has been the subject of several books (Calvin and Bassham, 1962 and Zelitch, 1971), symposia (Tolbert, 1963; San Pietro *et al.*, 1967; Goodwin, 1967 and Hatch *et al.*, 1970) and reviews (Stiller, 1962; Bassham, 1964; Gibbs, 1967 and Walker and Crofts, 1970). Although a number of nagging questions remain unanswered, there has been no major change in the overall scheme. The pathway is ubiquitous in all higher plants and algae. It is quantitatively the most important carbon dioxide fixing, gluconeogenic system.

The C-4 Dicarboxylic Acid Pathway

The discovery of the C-4 dicarboxylic acid pathway (see review by Hatch and Slack, 1970) was accompanied by speculation that this might represent an altogether different pathway of CO_2 fixation. Although this pathway is undoubtedly important, subsequent work (Björkman and Gauhl, 1969; Moss and Rasmussen, 1969; Slack *et al.*, 1969 and Hatch, 1971) has indicated that

the most probable function of the C-4 dicarboxylic acid pathway is that of a CO_2 concentrating mechanism. Thus, CO_2 is initially fixed as malate, oxalacetate or aspartate in the mesophyll cells of C-4 plants. These acids are then transported to the bundle sheath cells where they undergo decarboxylation, the CO_2 released being efficiently re-fixed by the classic C-3 photosynthetic carbon reduction cycle.

The Warburg Effect

Warburg (1920) first observed that oxygen inhibits photosynthetic CO_2 fixation in Chlorella. This phenomenon has subsequently been observed in a wide variety of algae and higher plants, (see review by Turner and Brittain, 1962) and isolated chloroplasts (Arnon *et al.*, 1954 and Ellyard and Gibbs, 1969). The investigations of Gaffron (1940), McAlister and Myers (1940), Tamiya and Huzisige (1949) and Turner *et al.*, (1956), established a number of general features concerning the phenomenon.

(a) The inhibition affects not only the net CO_2 uptake but also the net oxygen output.

(b) The magnitude of the inhibition is markedly dependent upon the light intensity, the CO_2 concentration and the oxygen concentration. In general, inhibition is greatest under saturating light intensities, limiting CO_2 concentrations and high oxygen concentrations.

The inhibition can be substantially reduced by saturating concentrations of CO_2.

(c) The inhibition is reversible, thus distinguishing it from irreversible photo-oxidation phenomena.

(d) The inhibition is not simply due to an increase in the rate of "dark" (mitochondrial) respiration, since this process is saturated by about 2% oxygen, whereas the Warburg effect continues to increase between 20 and 100% oxygen.

Photorespiration

Photorespiration is defined as the light dependent uptake of oxygen and release of CO_2 that occurs in the photosynthetic tissues of all higher plants. The subject has been comprehensively reviewed by Jackson and Volk (1970).

Measurements of the rate of photorespiration are based upon a variety of indirect methods. Owing to the internal re-cycling of the gases involved, these measurements all underestimate the true rate of photorespiration. Nevertheless, it is clear that significant changes in the respiratory processes occur upon illumination such that under certain conditions the rate of photorespiration may be a significant fraction of the rate of photosynthesis.

Higher plants fall into two classes based upon their respiratory responses to illumination:

(a) C-3 Plants: These plants fix CO_2 primarily by means of the C-3 photosynthetic carbon reduction cycle. They are capable of high rates of photorespiration, whether this is measured by CO_2 release or by oxygen uptake. Their CO_2 compensation point* is high, between 40 - 50 ppm.

(b) C-4 Plants: These plants possess the C-4 dicarboxylic acid pathway in addition to the C-3 photosynthetic carbon reduction cycle, as outlined in page 1. Due to extremely efficient CO_2 fixation, it has proven difficult to demonstrate photorespiratory CO_2 release from these plants. However, Jackson and Volk (1969) have demonstrated unequivocally that increased oxygen uptake does indeed occur in these plants. These plants are further characterized by their low CO_2 compensation points, from 1 to 5 ppm.

Whatever the method used to measure photorespiration, the results lead to the same conclusion, namely, that photorespiration exceeds that occurring in the dark. A large number of investigations have been conducted in which the effects of light intensity, wavelength, CO_2 concentration, oxygen concentration and temperature have been documented (see review by

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*The CO_2 compensation point is the external concentration of CO_2 at which there is no net uptake or release of CO_2.

Jackson and Volk, 1970). From these results it is evident that there exists a very close connection between photosynthesis and photorespiration. In general, the conditions required to demonstrate a maximum Warburg effect are those now known to promote photorespiration; i.e., saturating light intensities, limiting CO_2 concentrations and high oxygen concentrations. The Warburg effect is therefore in large part due to photorespiration.

The substrate pools for photorespiration and dark respiration are clearly different. Goldsworthy (1966) has shown that, following a period of photosynthesis in [^{14}C] CO_2, the specific activity of the CO_2 subsequently released into CO_2 free air in the light is 1.5 times that released in the dark. Clearly photorespiration makes greater use of recently fixed photosynthate than does dark respiration. By performing similar experiments Zelitch (1966) has shown that the immediate substrate pools for photorespiration are small and rapidly turned over.

The Role of Glycolate in Photorespiration

Concurrent with the physiological investigations of photorespiration it was recognized that a considerable portion of the total carbon fixed during photosynthesis passed through the glycolate pathway. Under natural conditions, it has been estimated that 50 - 75% of the

total carbon fixed passes through the glycolate pathway (Zelitch, 1959 and Atkins et al., 1971). Furthermore, the results of Wilson and Calvin (1955), Bassham and Kirk (1962), Pritchard et al. (1962) and Tolbert (1963) have clearly established that the physiological conditions which enhance glycolate synthesis and metabolism are precisely those under which photorespiration and the Warburg effect become apparent. The close relationship between the Warburg effect, photorespiration and glycolate metabolism has been summarized by Gibbs (1969).

The elucidation of the glycolate pathway (Fig. 1) has been largely due to the work of Tolbert's group (Tolbert and Cohen, 1953; Richardson and Tolbert, 1961a; Rabson et al., 1962; Kearney and Tolbert, 1962; Jiminez et al., 1962; Orth et al., 1966; Hess and Tolbert, 1966, 1967; Chang and Tolbert, 1970 and Bruin et al., 1970). The finding that many of the enzymes associated with the glycolate pathway are located in the peroxisomal (microbody) fraction, distinct from both mitochondria and chloroplasts (Tolbert, 1971) has added a further dimension to our understanding of photorespiration and the glycolate pathway. The metabolism of glycolate by both higher plants and algae has been comprehensively reviewed on several occasions (Tolbert, 1963; Zelitch, 1964; Hess, 1966; Anderson, 1969; Bruin, 1969; Jackson and Volk, 1970 and Tolbert, 1971).

Examination of the glycolate pathway (Fig. 1)

Figure 1: The Glycolate Pathway. The enzymes involved are:

(1) P-glycolate phosphatase

(2) Glycolate oxidase

(3) Catalase

(4) Transaminase

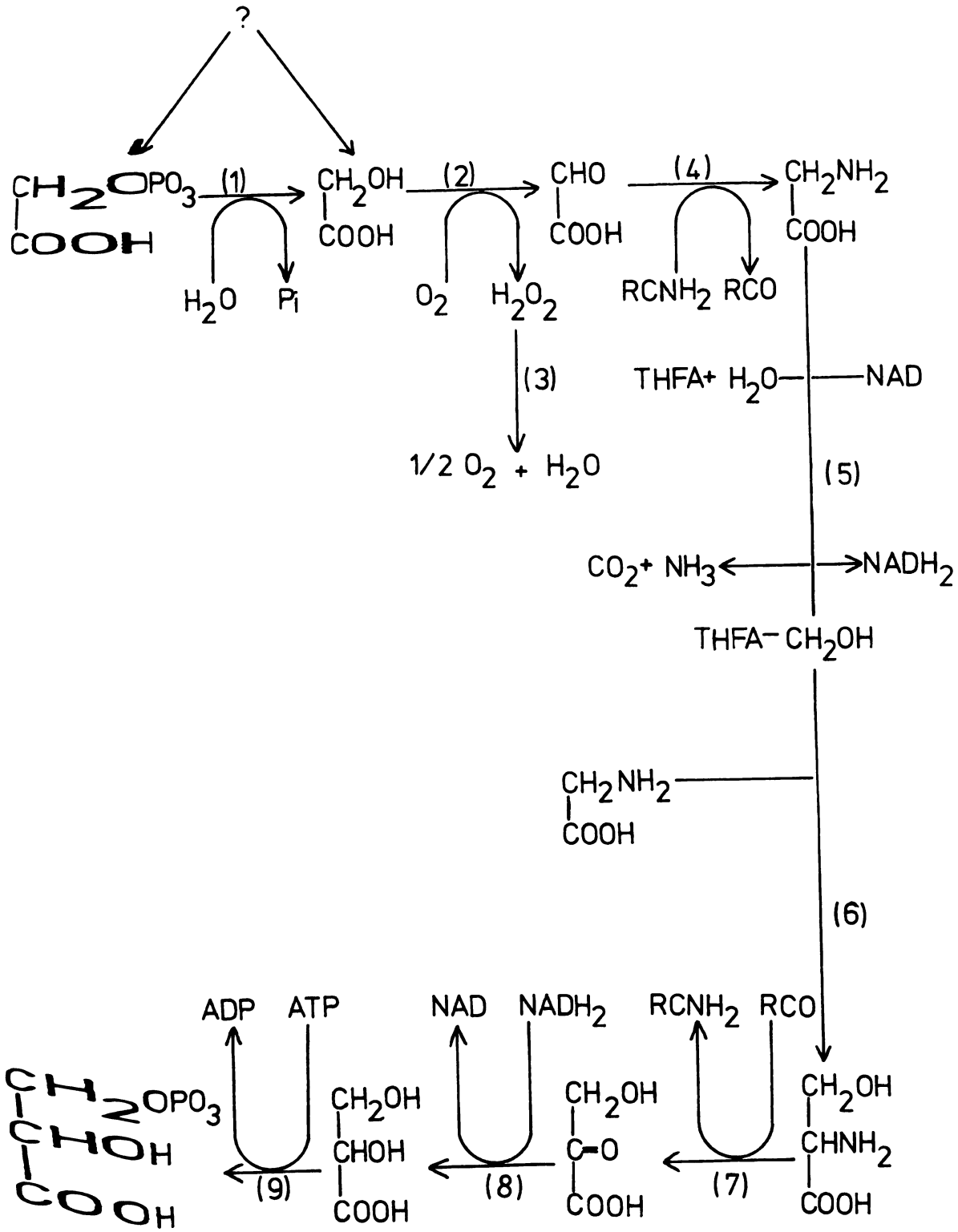
(5) Glycine decarboxylase

(6) Serine hydroxymethyl transferase

(7) Transaminase

(8) Hydroxypyruvate reductase

(9) Glycerate kinase


?
CH2OPO3
COOH
(1)
H2O
Pi
CH2OH
COOH
(2)
O2
H2O2
CHO
COOH
(4)
RCNH2
RCO
CH2NH2
COOH
(3)
1/2 O2 + H2O
THFA+ H2O
NAD
(5)
CO2+ NH3
NADH2
THFA- CH2OH
CH2NH2
COOH
(6)
ADP
ATP
(9)
CH2OPO3
CHOH
COOH
CH2OH
CHOH
COOH
NAD
NADH2
(8)
CH2OH
C=O
COOH
RCNH2
RCO
(7)
CH2OH
CHNH2
COOH

reveals the most probable sites of photorespiratory oxygen uptake and CO_2 release. Photorespiratory oxygen uptake is clearly at least in part associated with the oxidation of glycolate to glyoxylate, the reaction catalysed by glycolate oxidase. No other oxygen consuming, photorespiratory reactions are known, however. The oxidation of glyoxylate to oxalate, also catalysed by glycolate oxidase (Richardson and Tolbert, 1961b), is probably only of significance in nitrogen deficient plants.

In the presence of amino donors glyoxylate undergoes transamination to yield glycine. In turn, glycine is converted to serine by the action of glycine decarboxylase and serine hydroxymethyl transferase (Kisaki and Tolbert, 1969, 1970 and Kisaki et al., 1971). These in vitro studies and the in vivo studies of Cossins and Sinha (1966) and Marker and Whittingham, (1966) indicate that the carboxyl group of glycine is the most immediate source of photorespiratory CO_2 release.

Glycolate Biosynthesis

Benson and Calvin (1950) first observed that glycolate was among the most rapidly labeled compounds formed during $[^{14}C]CO_2$ photosynthesis by algae. Subsequently, Schou et al. (1952) demonstrated that the

glycolate so formed was uniformly labeled. However, despite considerable effort, the mechanism of glycolate synthesis has remained unsolved since then. In the intervening years the optimal conditions for glycolate synthesis were defined (Wilson and Calvin, 1955; Bassham and Kirk, 1962; Pritchard et al., 1962 and Tolbert, 1963). Synthesis of glycolate and of its metabolic derivatives, glycine and serine, was found to be enhanced by saturating light intensities, limiting CO_2 concentrations and high oxygen concentrations. In addition, alkaline pH's (9.0 and above) greatly enhanced the formation of glycolate, glycine and serine both in vivo (Orth et al., 1966) and in cell free chloroplast preparations (Dodd and Bidwell, 1971). The significance of this observation was not understood.

The labelling of P-glycolate during $[^{14}C]CO_2$ photosynthesis has been demonstrated (Benson et al., 1952). Furthermore, Richardson and Tolbert (1961a) have described a specific phosphatase, located in the chloroplast, which catalyses the hydrolysis of P-glycolate to glycolate. Thus, in vivo, glycolate may arise from P-glycolate. However, whether it does so exclusively is not clear.

Two radically different mechanisms have been proposed to account for the formation of uniformly labelled glycolate during photosynthetic $[^{14}C]CO_2$ fixation. One, proposed by Tanner et al., (1960);

Stiller, (1962) and Zelitch (1965) suggests that glycolate arises by means of a hitherto undiscovered reductive condensation of two molecules of CO_2 or of CO_2 with some C-1 acceptor compound. Zelitch (1965) based this proposal on his observation that after "short times" (2 minutes) of $[^{14}C]CO_2$ photosynthesis by tobacco leaf discs, the specific activity of glycolate was greater than that of 3-P-glycerate. However, his methods were such that the 3-P-glycerate could well have become considerably diluted with ^{12}C before the leaf discs were finally killed. Similar $[^{14}C]CO_2$ labelling experiments by Hess and Tolbert (1966) and Coombs and Whittingham (1966) did not substantiate Zelitch's findings. These experiments tend to discount this first proposed mechanism and, instead lend support to the second proposed mechanism of glycolate synthesis. This proposal, stated in various forms, suggests that glycolate or P-glycolate or both arise as the result of the oxidation of one or more intermediates of the C-3 photosynthetic carbon reduction cycle (Wilson and Calvin, 1955; Bassham and Kirk, 1962; Tolbert, 1963; Coombs and Whittingham, 1966 and Gibbs, 1969). These proposed mechanisms have one point in common, namely, that glycolate or P-glycolate is derived from carbon atoms one and two of one or more of the ketose sugar phosphates. The labelling pattern of the C-3 photosynthetic carbon reduction cycle is consistent

with such a proposal. Carbon atoms one and two of the ketose sugar phosphates become and remain uniformly labelled during [^{14}C]CO_2 photosynthesis. The lag period observed during [^{14}C]CO_2 photosynthesis before glycolate becomes labelled is also consistent with these proposed mechanisms.

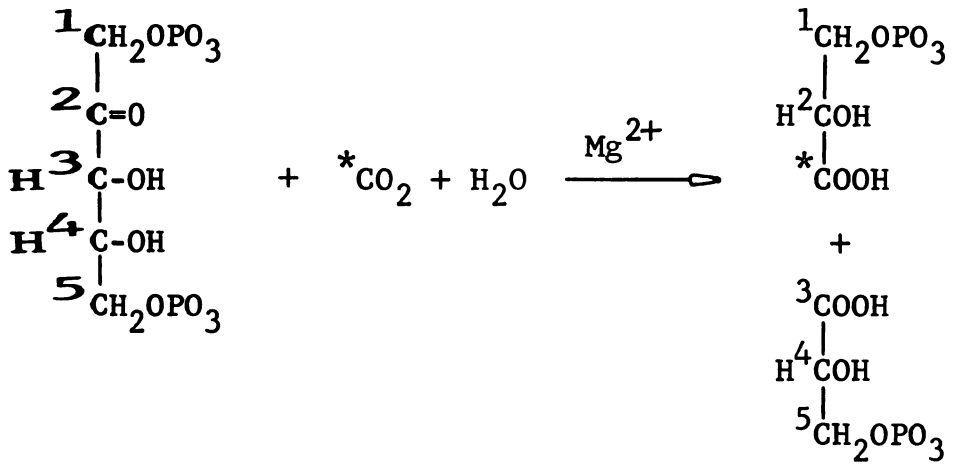
Wilson and Calvin (1955) first suggested that glycolate was formed as the result of the oxidation of the "active glycolaldehyde" formed as an intermediate in the transketolase reaction. More recently it has been further suggested that the oxidant in this process, *in vivo*, is hydrogen peroxide, generated from oxygen in a Mehler reaction from reduced ferredoxin (Coombs and Whittingham, 1966 and Gibbs, 1969). This proposed mechanism is based upon observations that the α, β-dihydroxyethylthiamine pyrophosphate is enzymatically oxidised to glycolate and thiamine pyrophosphate in the presence of such electron acceptors as ferricyanide (Bradbeer and Racker, 1961 and Holzer and Schroter, 1962) or 2,6-dichlorophenolindophenol (daFonseca-Wollheim *et al.*, 1962). Shain and Gibbs (1971) have studied the formation of glycolate from fructose-6-P in a reconstituted chloroplast system containing transketolase, NADP and ferredoxin. The formation of glycolate in this system was shown to be light dependent and enhanced by increasing the oxygen concentration. Addition of catalase markedly reduced the rate of

glycolate formation indicating the involvement of H_2O_2.

Attempts to determine the immediate precursor of glycolate or P-glycolate by feeding [^{14}C] labeled sugars have been suggestive but no conclusive. For example, Griffith and Byerrum (1959) observed a conversion of ribose-1-[^{14}C] to glycolate in tobacco leaves. However, the yield of glycolate was very low. Zelitch (1960) reported a 10% conversion of uniformly labelled [^{14}C] ribulose-5-phosphate to glycolate by spinach chloroplasts. However, interpretation of these results is made difficult by virtue of the rapidity with which the intermediates of the C-3 photosynthetic carbon reduction cycle become equilibrated with one another.

Ribulose Diphosphate Carboxylase

The properties of this enzyme have recently been reviewed (Kawashima and Wildman, 1970a and Wishnick and Lane, 1971). Ribulose diphosphate (RuDP) carboxylase (E.C. 4.1.1.39) catalyses the primary carboxylation reaction of the C-3 photosynthetic carbon reduction cycle, namely the essentially irreversible reaction of CO_2 with RuDP and water to yield 2 molecules of 3-P-glycerate.



The protein was first purified by Wildman and Bonner (1947) before its enzymatic properties became known. It was then referred to as "Fraction 1 Protein." It is now clear that Fraction 1 Protein and RuDP carboxylase are one and the same (see review of Kawashima and Wildman, 1970a). The enzyme is present in the leaves of all higher plants and may constitute up to 50% of the soluble protein of the leaf. The protein was first isolated as RuDP carboxylase by Weissbach et al. (1956) and since then has been the subject of over 100 publications.

Several molecular weight studies have been performed (Trown, 1965; Paulsen and Lane, 1966; Pon, 1967; Ridley et al., 1967 and Steer et al., 1968) which indicate that the molecular weight of the native enzyme is about 560,000 daltons. Measurements of the frictional coefficient, f/f_o range from 1.11 (Paulsen and Lane, 1966) to 1.35 (Steer et al., 1968) indicating

that the molecule is very nearly spherical. Electron microscope studies have confirmed this, the estimated diameter of the enzyme being about 100 Å (Trown, 1965; Steer et al., 1968).

SDS electrophoresis first revealed that the enzyme was composed of two distinctly different species of subunits. The ratio of the protein content of the large subunits to that of the small is about 77 : 23 (Rutner and Lane, 1967). The amino acid compositions of the two subunits are clearly different (Rutner and Lane, 1967 and Kawashima, 1969). Interestingly the amino acid composition of the large subunit is remarkably similar from species to species. Significant differences exist in the amino acid composition of the small subunit. Fingerprint analyses of the peptides obtained following trypsin digestion reveal that of the 20 peptides obtained from the large subunits of the tobacco and spinach enzymes, there were differences in only 2 or 3. In contrast, 9 out of the 15 peptides obtained from the small subunits were different (Kawashima and Wildman, 1971). The constancy of composition of the large subunit suggests that it contains the catalytic site. Claims that the large subunit retains carboxylase activity following dissociation in 4M urea (Sugiyama et al., 1969) remain to be confirmed.

The native enzyme may also be dissociated by

use of urea (Moon and Thompson, 1969) and alkaline conditions (pH's greater than 11.0) (Kawashima and Wildman, 1970). Estimates of the molecular weights of the subunits by SDS electrophoresis and gel filtration chromatography yield values from 52,000 to 56,000 for the large subunit and from 12,000 to 24,000 for the small subunit (Rutner and Lane, 1967; Moon and Thompson, 1969 and Kawashima and Wildman, 1970a). It is generally agreed that there are 8 large subunits but estimates of the number of small subunits range from 6 to 12. Models of the native enzyme have been constructed (Steer et al., 1968 and Kawashima and Wildman, 1970b) but in view of the uncertainty in the numbers of the different subunits, there is no compelling reason to favour one over the other.

Until recently there was no reason to suspect that RuDP carboxylase was a metalloenzyme. However, after demonstrating that cyanide is a potent inhibitor of the carboxylase reaction (Wishnick and Lane, 1969) it was subsequently reported that the native enzyme contains one g atom of copper (mostly copper II) per mole of enzyme (Wishnick et al., 1969). However, this copper does not appear to have any role in the carboxylase reaction, for the copper-free protein, obtained by treatment with cyanide, was found to be catalytically indistinguishable from the native enzyme with respect to the maximal rate of catalysis, the apparent K_m values

for all reaction components and the extent of inhibition by cyanide (Wishnick et al., 1970).

In view of the presence of bound copper, the enzyme might be expected to show some visible absorption properties. Ultraviolet and visible absorption spectra reveal no characteristic chromophoric groups that would distinguish the enzyme from most other proteins (Paulsen and Lane, 1966 and Kawashima and Wildman, 1970). However, simple calculations show that considerably greater protein concentrations (in the order of 100 mg/ml) would be required in order to see the visible absorption due to copper.

Using p-chloromercuribenzoate, some 90 SH groups per mole of enzyme have been detected (Sugiyama et al., 1968a). Calculations based on the molar ratio of cystine to the total amino acids and upon a molecular weight of 550,000 give similar values for the number of -SH groups. Thus, the titratable -SH groups fall in a range consistent with chemical analyses suggesting that S-S bonds do not exist in the native enzyme.

RuDP carboxylase activity is markedly dependent upon Mg^{2+}, though Mn^{2+} will substitute, albeit less effectively. In the presence of 0.01M Mg^{2+}, the pH optimum is near 7.8. However, decreasing the Mg^{2+} concentration results in a shift of the pH optimum towards more alkaline pH's (Bassham et al., 1968, Sugiyama et al., 1968b). The enzyme is highly specific for RuDP with a

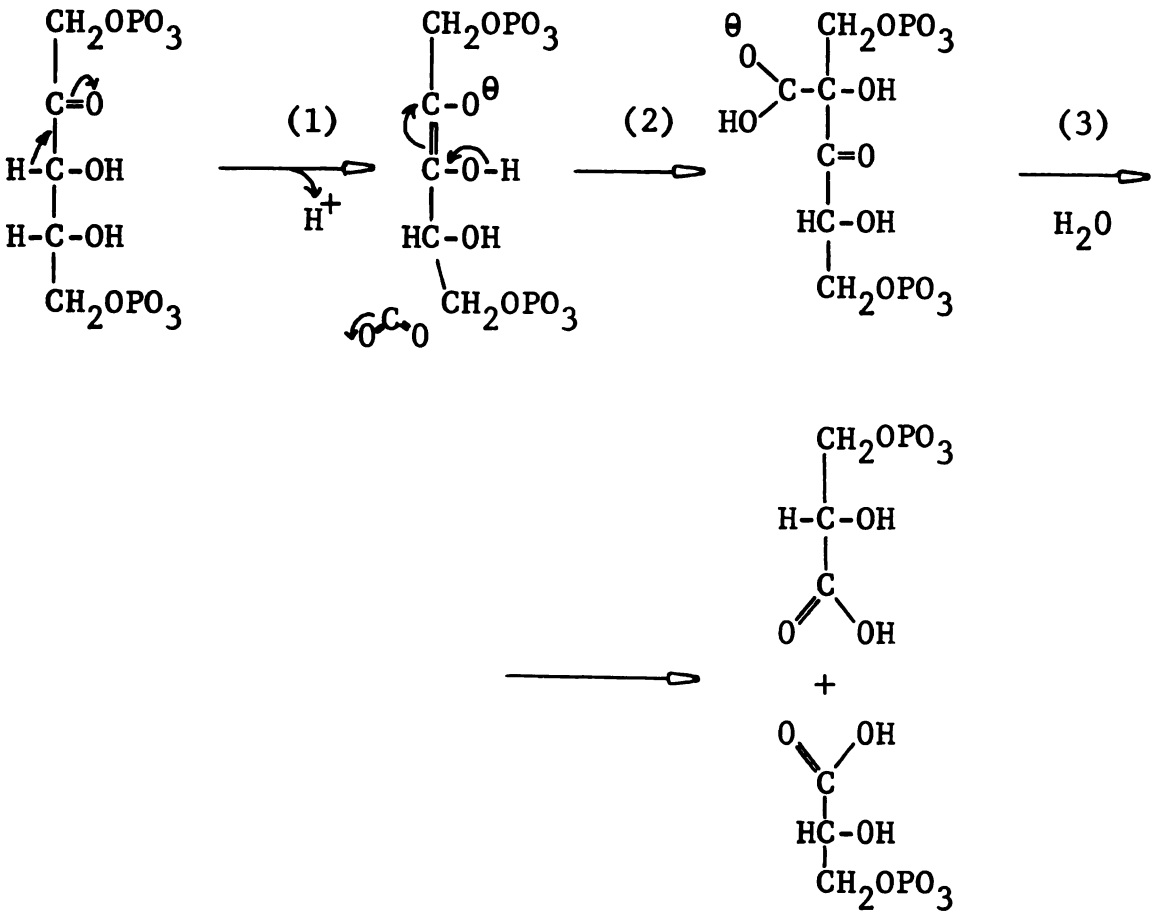
K_m of about 1 x 10^{-4}M. However, substrates such as fructose diphosphate have not been rigorously tested under a wide variety of conditions (Weissbach et al., 1956).

The affinity of the enzyme for CO_2 is very low. The K_m for bicarbonate is about 2 x 10^{-2}M (Paulsen and Lane, 1966). However, Cooper et al., (1969) have demonstrated that CO_2 rather than bicarbonate is the reactive species. Thus the K_m for CO_2 is about 4 x 10^{-4}M. The turnover number of the carboxylase is about 1300 moles CO_2 fixed per min per mole of enzyme at 30° (Paulsen and Lane, 1966). This low catalytic activity presents conceptual difficulties as to how the Calvin cycle could operate effectively at the known in vivo rates. The apparent K_m for CO_2 of intact leaves is about 9 x 10^{-6}M (equivalent to the concentration of CO_2 in equilibrium with air), while that for isolated, intact chloroplasts is about 1 x 10^{-5}M (Jensen and Bassham, 1968). In order to rationalize this discrepancy, suggestions have been made that purification of the enzyme results in the loss of some special activating factors (Wildner and Criddle, 1969 and Andersen et al., 1970) or that in vivo there exists some mechanism for concentrating CO_2 within the chloroplasts, possibly involving carbonic anhydrase (Graham et al., 1971). However, there is no convincing experimental evidence to support either hypothesis. Kinetic studies of the enzyme are usually conducted with

enzyme concentrations from 10 to 20 μg/ml. While this may be experimentally convenient it does not in any way correspond to the *in vivo* concentration of enzyme. It would be of interest to determine the reaction kinetics at concentrations of enzyme which more truely reflect the *in vivo* situation.

It has been known for some time that sulphydryl group blocking reagents such as PCMB and iodoacetamide (IAA) inactivate the carboxylase (Mayandon *et al.*, 1957 and Rabin and Trown, 1964). Trown and Rabin (1964) have suggested, on the basis of the protective effect of RuDP on the kinetics of inactivation by IAA, that two carboxylase -SH groups are involved in binding the RuDP. However, it is to be doubted that the protective effect of RuDP against alkylating reagents is specific for the catalytic site, since hexose mono- and di-phosphates also protected the enzyme from alkylation by IAA. Yet these compounds do not compete with RuDP for the catalytic site nor do they inhibit the enzymatic reaction. Furthermore, the finding that there are 8 large (catalytic ?) subunits and 8 binding sites for RuDP (Wishnick *et al.*, 1970) leads one to doubt the suggestion put forward by Trown and Rabin (1964).

The most often suggested mechanism of reaction of RuDP carboxylase is:



Thus, enolization (1) is followed by the attachment of CO_2 to the C-2 of RuDP (2) to form the 2-carboxy-3-keto intermediate which subsequently undergoes hydrolytic cleavage between C-2 and C-3 to yield 2 molecules of 3-PGA (3). The evidence to date supports this mechanism. Mullhofer and Rose (1965) demonstrated that carbon-carbon cleavage occurs between the C-2 and C-3 of RuDP. Therefore, carbon dioxide becomes attached to the C-2 of RuDP. Further, by performing the reaction in D_2O, they demonstrated that the deuterium becomes attached to the carbon that was originally the C-2 of RuDP. This result

eliminates the possibility of hydride transfer from the C-3 of RuDP to the developing C-2 of the 3-PGA.

The existence of the 2-carboxy-3-keto intermediate is inferred from the studies of Wishnick et al. (1970). They have reported that 2-carboxy-D-ribitol diphosphate, an analogue of the proposed intermediate, is an extremely effective inhibitor of the carboxylase reaction (K_i about 2×10^{-7}M). This inferrence is based on Wolfenden's (1969) proposal that unusually tight binding of an inhibitor might be characteristic of transition state analogues. Other apparent examples of this phenomenon have been reported; e.g., 2-phosphoglycolate for triose phosphate dehydrogenase (Wolfenden, 1969), oxalate for lactic dehydrogenase (Novoa et al., 1959) and pyrrole-2-carboxylate for proline racemase (Cardenale and Abeles, 1968).

Fiedler et al., (1967) have studied the carboxylase reaction using RuDP labeled with tritium in the C-3 position. In confirmation of the results reported by Mullhofer and Rose (1965) they found that, following reaction with CO_2, 98% of the radioactivity was associated with the medium. Enzymatic enolization reactions are frequently characterized by isotope exchange with medium protons. However Fiedler et al., (1967) reported that in the absence of CO_2, RuDP acquired no tritium when incubated in THO with the enzyme. They were unable to rule out the possibility that during enolization the

proton was prevented from exchanging by its tight binding to the enzyme. Examples of such tight binding of reaction protons derived from enolized substrates have been found in enzymatic isomerisation reactions (Wang et al., 1963 and Rose and O'Connell, 1961).

While the proposed mechanism would not predict a requirement for CO_2 for proton exchange at C-3, it is possible that CO_2 might be required to activate the protein in order to carry out the enolization step. The activation phenomena described by Pon et al., (1963) would be consistent with this interpretation. Nor would such an interpretation be without precedent. The enolisation of pyruvate catalysed by pyruvic kinase requires the other substrate, ATP. However, analogues of ATP, phosphate and arsenate are equally effective (Rose, 1960).

The experiments of Fiedler et al., (1967) with RuDP tritiated at C-3 revealed a marked isotope effect. The tritiated substrate reacted at 20% of the rate of the unlabeled substrate, possibly signifying that the enolisation step is rate determining for the overall reaction.

The role of Mg^{2+} or Mn^{2+} in the reaction has been studied by Wishnick et al., (1970). They reported that Mg^{2+} was not required in the binding of RuDP to the carboxylase. They further demonstrated formation of a dissociable enzyme-Mn^{2+} complex by measurements of

the effect of this species on the longitudinal nuclear magnetic relaxation rate of water protons. They suggested that Mg^{2+} was involved in the formation or stabilization of the 2-carboxy-3-keto intermediate. This suggestion was supported by the observation that Mg^{2+} increased the affinity of the enzyme for the intermediate analogue, 2-carboxy-D-ribitol diphosphate, as determined by gel filtration studies.

The interaction of RuDP carboxylase with oxygen was first proposed as early as 1949 by Tamiya and Huzisige, long before that path of carbon in photosynthesis or the central role of the carboxylase was realized. Based upon their extensive investigations of the physiological conditions required to demonstrate the Warburg effect and upon the effect of cyanide under these conditions, Tamiya and Huzisige (1949) proposed that there was a competition between oxygen and CO_2 for the carboxylating enzyme, and further, that cyanide acted upon this enzyme.

After examining the kinetics of photosynthesis and photorespiration Ogren and Bowes (1970) were lead to the conclusion that the rate limiting step in both processes was controlled by one and the same enzyme. They reported that RuDP carboxylase is inhibited by oxygen and that the inhibition was competitive with respect to CO_2. Following their own speculation they then demonstrated that oxygen will in fact substitute

for CO_2, the products of the reaction being 2-phosphoglycolate and, they presumed, 3-phosphoglycerate (Bowes et al., 1971).

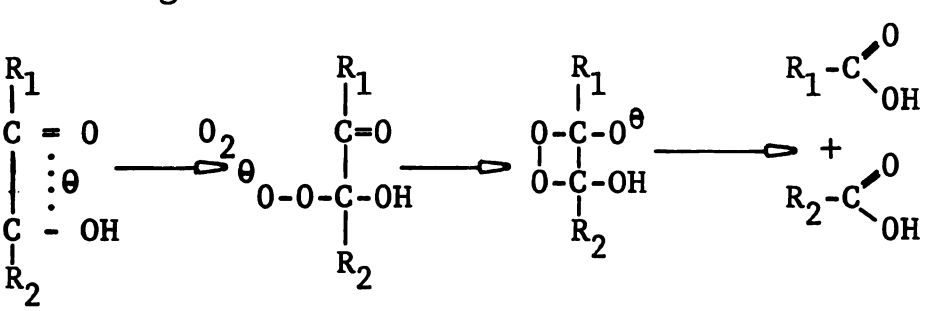
Oxidation of Carbohydrates and Related Compounds by Oxygen

The oxidation of carbohydrates and related compounds by oxygen and species derived from oxygen falls into two broad classes; (a) reactions with molecular oxygen in alkaline solutions and (b) free radical reactions involving the oxygen free radicals, the superoxide free radical, $HO_2\cdot$, and the hydroxyl free radical, $HO\cdot$. Most of the reported research is of a descriptive nature and little attention has been given to the rather complex mechanisms and kinetics. To my knowledge, the oxidation of sugar phosphates of biological interest by either class of reactions has not been reported.

(a) Reactions with molecular oxygen in alkaline solution: In the presence of strong alkali, sugars undergo complex transformations which depend upon the conditions of temperature, alkali concentration and the presence or absence of metal ion catalysts. Nef (1914) observed that in the presence of oxygen, alkaline solutions of glucose and fructose could be oxidised to yield a mixture of monocarboxylic acids. Formic acid and arabonic acid were the principal products but significant quantities of glycolic, glyceric and erythronic acids were also formed. Since glucose and fructose yielded the same oxidation products, it was clear that the hexoses first

underwent a Lobry de Bruyn-Alberda van Ekenstein transformation to yield the various enediols. Nef (1914) proposed that these were then split by oxygen into acids containing one to five carbon atoms. Thus, oxidation of the 1,2-enediol was thought to produce formic and arabonic acids and so on. This is illustrated in Fig. 2. The relative quantities of the various acids formed is then a reflection of the steady state concentration of each of the enediols.

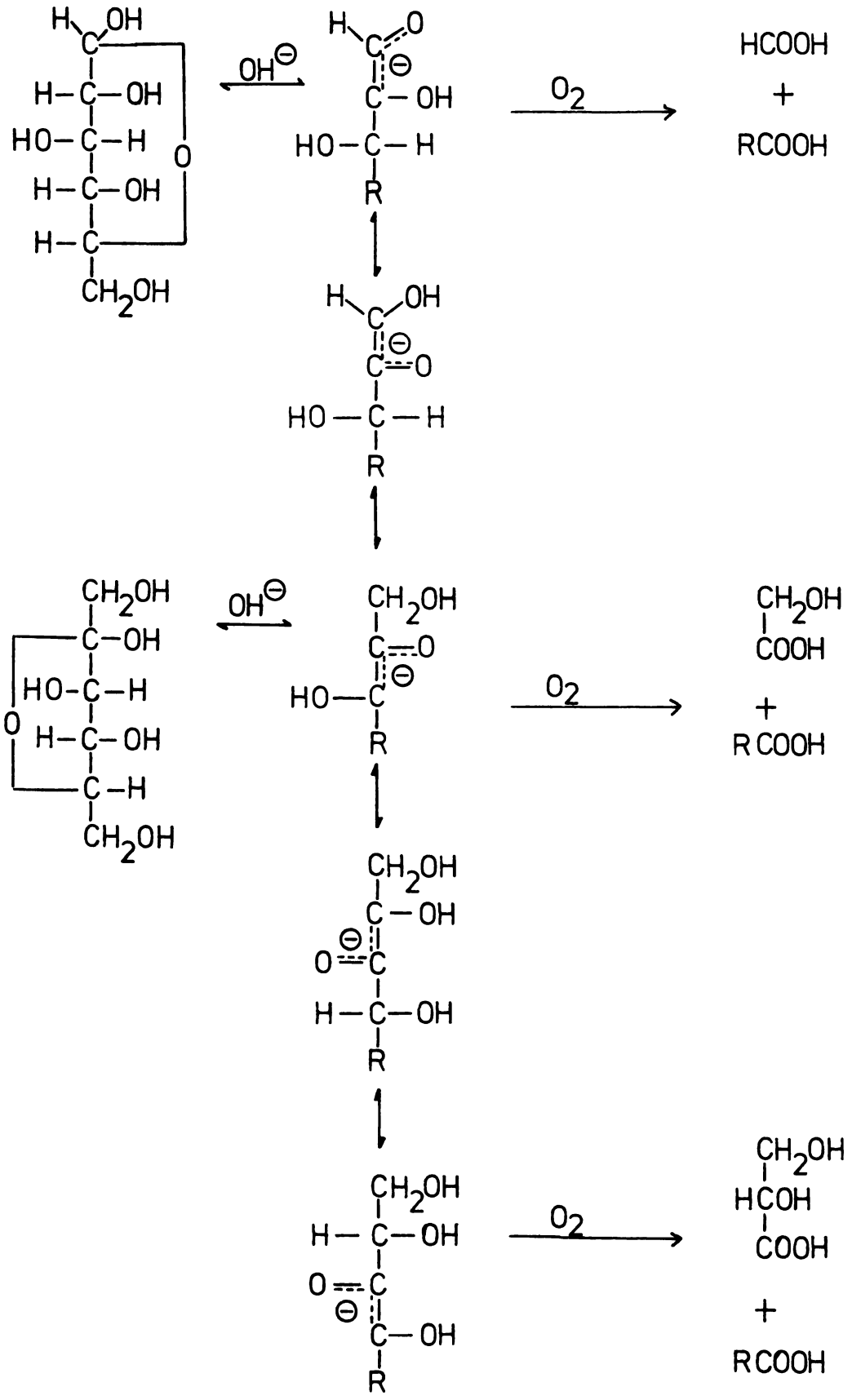
During a kinetic analysis of these reactions, Bamford et al., (1950a,b) demonstrated the intermediate formation of a peroxide. Thus, they and subsequent investigators (Dubourg and Naffa, 1959 and DeWitt and Kuster, 1971) have represented the reaction of oxygen with the enediol or enolate anion as proceeding by the following mechanism:



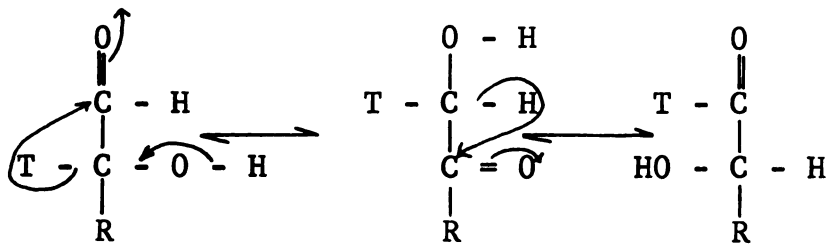
(I)

However, the mechanism may be more complex. Gleason and Barker (1971a,b) have found that the formic acid, produced from ribose-2-(^{3}H) by the action of oxygen, contains a substantial proportion of the label. They have proposed two concurrent mechanisms (i)

Figure 2: The reaction of glucose and fructose with molecular oxygen in alkaline solution.

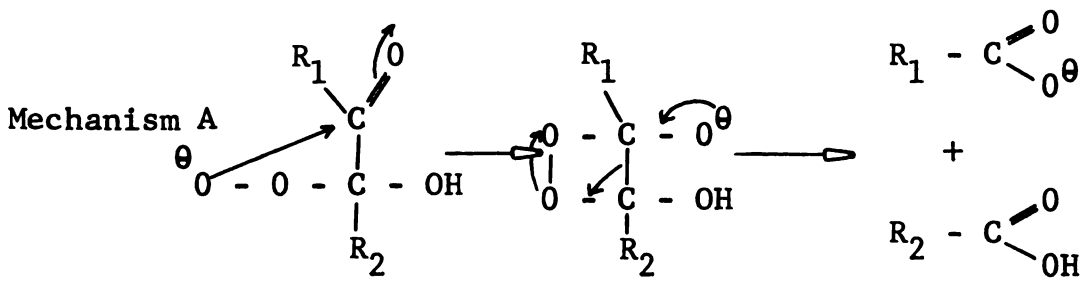

OH⊖
O2
HCOOH
+
RCOOH
OH⊖
O2
CH2OH
COOH
+
RCOOH
O2
CH2OH
HCOH
COOH
+
RCOOH

enolization and oxidation and (ii) hydride transfer of the C-2 hydrogen atom to C-1, followed by enolization of the resulting ketone and oxidation of the enediol.



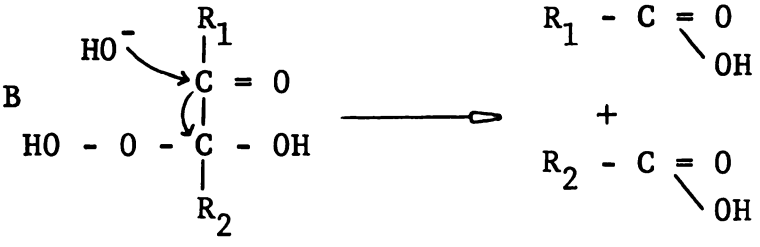
One may also conceive a free radical mechanism leading to the formation of the glycosulose peroxide (I) which would be consistent with the tritium labeling experiments of Gleason and Barker. However, Bamford et al., (1950a,b) were unable to accelerate the reaction by means of free - radical initiators, thus favoring an ionic mechanism.

The mechanism of decomposition of the glycosulose peroxide (I) intermediate to yield the two carboxylic acids is unknown. Conceivably, the peroxide could decompose intramolecularly by a concerted mechanism, perhaps involving a cyclic peroxide (Mechanism A).



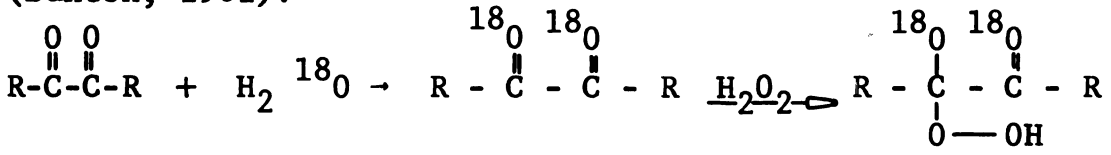
Alternatively the peroxide could decompose by the addition and elimination of a hydroxyl ion (Mechanism B).

Mechanism B

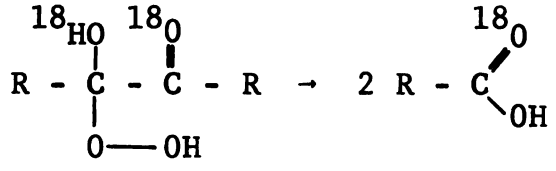


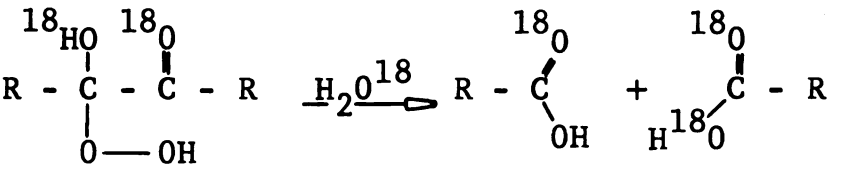
Since the mechanism of decomposition is of importance in the interpretation of some of the experimental results, a discussion of more completely understood analogous reactions is of interest.

Hydrogen peroxide will cleave α-diketones to form carboxylic acids (Bunton, 1961). The first step in this reaction is a nucleophilic attack upon the carbonyl group to form the peroxide intermediate shown below. The structure of this peroxide intermediate is strikingly similar to the proposed peroxide intermediate in the alkaline oxidation of sugars. By using $H_2{}^{18}O$ it has been possible to distinguish between two possible mechanisms of decomposition of the peroxide intermediate (Bunton, 1961).



Mechanism I

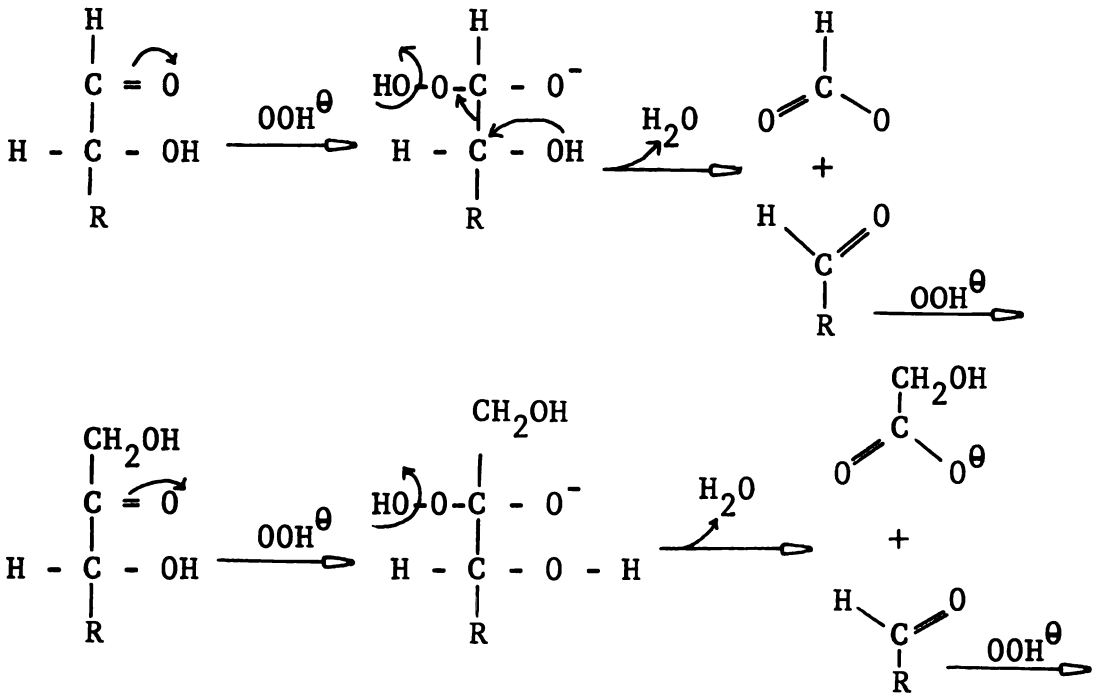


Mechanism II 

Mechanism I, analogous to reaction pathway A in the oxidation of sugars, proceeds intramolecularly and leads to a 50% enrichment of the carboxyl oxygens of the resultant acids. Mechanism II, involving an attack by water leads to a predicted 75% enrichment. The experimental data indicates that Mechanism II is operative.

Returning to the alkaline oxidation of sugars, one would therefore predict on the basis of this analogy, that only one atom of oxygen will be incorporated into the resultant carboxylic acids from molecular oxygen; i.e. the decomposition of the glycosulose peroxide involves the addition and elimination of a hydroxide ion.

(b) Free Radical Reactions: The reactions of carbohydrates with hydrogen peroxide have been reviewed by Moody (1964). Owing to secondary reactions and the effects of metal ion free radical catalysts, the nature of these reactions is poorly understood. In the absence of metal ion catalysts, the reaction probably proceeds by an ionic mechanism.



However, in the presence of redox metal ions, it is clear that oxidation occurs by free radical mechanisms. The nature of the reaction depends upon the peroxide concentration and the nature of the metal ion. In the first instance, the oxygen free radical formed depends upon the oxidation number of the initial cation. Thus

$$X^{m+} + H_2O_2 \rightarrow X^{(m+1)+} + OH^- + OH^{\cdot}$$

$$Y^{n+} + H_2O_2 \rightarrow N^{(n-1)+} + H^+ + HO_2^{\cdot}$$

In addition radicals may arise by secondary processes

$$OH^{\cdot} + H_2O_2 \rightarrow H_2O_2 + HO_2^{\cdot}$$

$$HO_2^{\cdot} + H_2O_2 \rightarrow H_2O + OH^{\cdot} + O_2$$

Both the hydroxyl free radical, $OH^{\cdot}$ and the superoxide free radical, $HO_2^{\cdot}$ are powerful oxidizing agents. The diversity and relative quantity of the products formed make it clear that there are several primary and secondary oxidations.

Oxygenases

The nature of oxygenases, enzymes which catalyse oxygen-fixation reactions, have been reviewed extensively (Hayaishi, 1962; King et al., 1964; Mason, 1965; Bloch and Hayaishi, 1966; Guroff et al., (1967); Hayaishi and Nozaki, 1969 and Hamilton, 1969). Several sub-classes of oxygenases may be recognized.

(i) Dioxygenases catalyse the incorporation of both atoms of molecular oxygen into a molecule of substrate.

$$S + {}^{18}O_2 \rightarrow S^{18}O_2$$

(ii) Internal Monoxygenases catalyse the incorporation of a single atom of oxygen into the substrate, the other atom of oxygen being reduced to water by electrons derived from the substrate itself.

$$SH_2 + {}^{18}O_2 \rightarrow S^{18}O + H_2{}^{18}O$$

(iii) External Monoxygenases catalyse the incorporation of a single atom of oxygen into the substrate, the other atom of oxygen being reduced to water by electrons derived from an additional electron donor.

$$S + AH_2 + {}^{18}O_2 \rightarrow S^{18}O + A + H_2{}^{18}O$$

(iv) α-Ketogluterate - dependent Oxygenases catalyse the incorporation of one atom of oxygen into the substrate concomitantly decarboxylating α-keto-glutarate (α-KG) to succinate with the incorporation of the second atom of oxygen into the substrate (Holme et al., 1971).

$$S + \alpha KG + {}^{18}O_2 \rightarrow S^{18}O + CO_2 + \text{succinate} - {}^{18}O$$

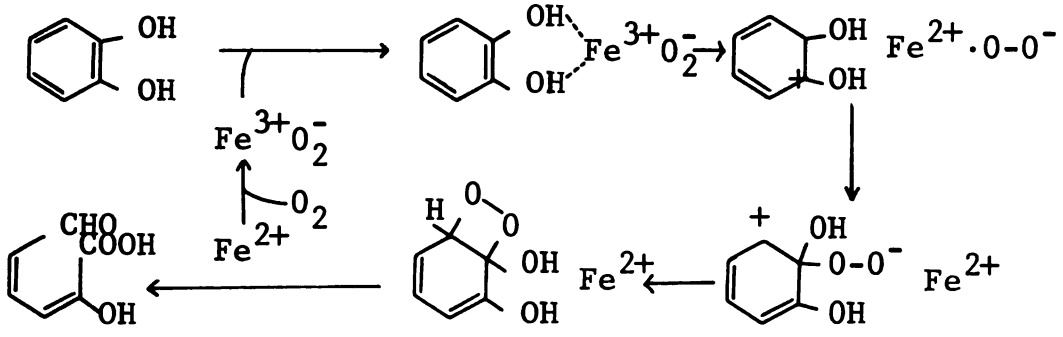
With the notable exception of lipoxygenase, oxygenases have been found to contain at least one additional component besides the protein moiety. The dioxygenases generally contain either ferrous or ferric iron as the sole cofactor, although in the case of tryptophan dioxygenase the iron is complexed in the form of a heme. The monoxygenases are a more diverse class with respect to the nature of the cofactor(s). Some such as lysine monoxygenase and imidazoleacetate monoxygenase appear not to contain any metal components but instead contain organic cofactors, in this case flavin adenine nucleotide. Others, such as dopamine β-hydroxylase contain copper and no organic cofactor. More complicated electron transfer systems exist such as the microsomal cytochrome P-450 hydroxylating system.

Studies on the mechanism of oxygenase action have concentrated on elucidating the nature of "active

oxygen" and the mechanism by which it is formed. Recent evidence (Hirata and Hayaishi, 1971 and Strobel and Coon, 1971) has implicated the superoxide radical in the mechanism of tryptophan dioxygenase and the hydroxylation reactions catalysed by cytochrome P-450. Both enzymes contain heme iron. The work of Knowles et al. (1969) and Massey et al. (1969) has established that reduced flavins are also capable of generating superoxide radicals upon interaction with molecular oxygen. In addition a number of biologically active reducing agents generate radicals in the presence of oxygen, e.g. various sulphydryl reagents (Sosnovsky and Zaret, 1970), reduced ferredoxin (Misra and Fridovich, 1971) and reduced quinones (Misra and Fridovich, 1972). There is little question then that superoxide radicals are produced in biological systems. Indeed, an enzyme, superoxide dismutase (erythrocuprein) has recently been described which catalyses the dismutation of superoxide (McCord and Fridovich, 1969).

$$.O_2^- + .O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$$

However, the concept of superoxide involvement in oxygenase reactions is not new, (see Hayaishi, 1962), and schemes similar to that proposed by Mason (1965) for metapyrocatechase have been suggested for other oxygenases (Bloch and Hayaishi, 1966).



Despite the attractiveness of these proposals and the experimental observations, the ability to inhibit a given oxygenase reaction with superoxide dismutase (Hirata and Hayaishi, 1971 and Strobel and Coon, 1971) does not constitute unequivocal evidence that the superoxide radical is the reactive form of oxygen. Rather a cautious interpretation is required since secondary reactions (see p.33) could generate other radical species from superoxide.

A cursory glance at a listing of oxygenases reveals that for the most part the substrates are either aromatic or highly reduced. "In general, oxygen-rich compounds, such as carbohydrates, are not favorable substrates for oxygenases" (Hayaishi and Nozaki, 1969). In this respect rat kidney inositol oxygenase is exceptional. This enzyme catalyses the oxygenation of myo-inositol to D-glucuronate, with the incorporation of one atom of oxygen (Charalampous, 1960). This is an internal monoxygenase but the details of the mechanism

remain to be elucidated. Work to date has not distinguished between a dehydrogenation-oxygenation and a dehydration-oxygenation mechanism (Crandall, 1964).

CHAPTER I

STUDIES ON PEROXISOMAL GLYCOLATE OXIDASE

Introduction

The role of glycolate oxidase in photorespiratory oxygen uptake is now well established. However, although the enzyme has been studied with DCPIP as the electron acceptor by several investigators, little is known about the role of the natural electron acceptor, oxygen, in the reaction in its native state. Previous work in this laboratory (Baker and Tolbert, 1967) indicated that precipitation of the enzyme with ammonium sulfate in the course of purification of the enzyme altered its kinetic and spectral properties. Accordingly, in order to study the enzyme in a state that more probably reflects it in vivo activity, the enzyme was purified by first isolating the peroxisomes. The subsequent purification procedures avoided recourse to ammonium sulfate precipitation. This chapter reports this method of purifying glycolate oxidase, the reactivity of the enzyme towards oxygen and the preparation and reactivation of the apoenzyme.

Materials

Spinach (Spinacia oleracea L.) was purchased from local markets or cultivated in a growth chamber. During the day period of 11 hours the light intensity was about 3,000 foot candles and the temperature was 21°. During the subsequent 13 hours of darkness, the temperature was 15°.

TEAE-cellulose was obtained from Serva Company and Sephadex-G25 (medium) from Pharmacia. Catalase was obtained from Worthington Biochemical Corp.

Methods

Protein Determination

Protein was determined by the method of Lowry et al., (1951), using bovine serum albumin as a standard.

Glycolate Oxidase Assays

(1) For routine purposes glycolate oxidase was assayed anaerobically by 2,6-dichlorophenolindophenol (DCPIP) reduction as previously described (Tolbert et al., 1968), except that cyanide was omitted. The DCPIP concentration in this reaction is not saturating. Thus, when an estimate of the maximum reaction rate was required, the assay was performed at several DCPIP concentrations, a Lineweaver-Burk plot constructed and the maximum reaction rate determined by extrapolation.

(2) The enzyme was also assayed by use of the oxygen electrode. The electrode was calibrated as described below. The complete reaction mixture contained the following components (in μmoles, unless otherwise stated): Tris (Cl) buffer, pH 8.5, 240; flavin mononucleotide (FMN), 0.10; sodium glycolate, 25 or lithium L-lactate, 31 or sodium glyoxylate, 84; 500 units catalase and glycolate oxidase in a total volume of 3.2 ml. The reaction mixture was flushed with 100% oxygen for 5 min prior to initiating the reaction with substrate. The temperature was as indicated in the text. The reaction was linear until about 35% of the oxygen had been consumed. The reaction was also proportional to enzyme concentration. One unit of enzyme activity is defined as the amount of enzyme required to consume one μmole of oxygen or reduce one μmole of DCPIP per min at 25°.

Calibration of the Oxygen Electrode

The method was based upon that described by Goldstein (1968) in which catalase was used to release stoichiometric amounts of oxygen from sodium perborate. Ten to fifty μl aliquots of freshly prepared 0.033M sodium perborate were added to 3.2 ml of 0.05M sodium phosphate, pH 7.0, containing 500 units catalase and the pen deflection recorded. The pen response was a linear function of the quantity of perborate added.

The response of the oxygen electrode to temperature was not a linear function. As the temperature increased, the pen response per se increased in an approximately Nernstian manner. However, this was in part offset by the decrease in the solubility of oxygen. Thus it was necessary to calibrate the electrode at the temperature at which it was to be used.

Isolation of Spinach Peroxisomes

Spinach peroxisomes were isolated by isopycnic sucrose density centrifugation in the 54 ml tubes of an SW 25.2 rotor as previously described (Tolbert et al., 1968) or in the B-29 or B-30 zonal rotor (Donaldson, 1971).

Purification of Glycolate Oxidase from Spinach Peroxisomes

The subsequent procedures, all performed at 4^{o}, were dependent upon the volume of the solution containing the peroxisomes. When the peroxisomes were isolated in the SW 25.2 rotor, Method A was used. When the peroxisomes were isolated in the zonal centrifuge, Method B was used.

Method A: The peroxisomes were broken by dilution with 3 volumes of 0.02M glycylglycine, pH 7.5, so that the final concentration of sucrose was 0.5M. The diluted solution was centrifuged at 144,000g for 60 min to remove membraneous material. From 5 to 10% of the activity was routinely found in the precipitate. The supernatant solution was dialysed against 50 volumes

0.005M Tris-Cl, pH 8.7 for 6 hours. The dialysate was reduced in volume to 2.9 ml by vacuum dialysis overnight. The concentrated solution was centrifuged at 14,000g for 20 min to remove the slight precipitate which had formed. The supernatant solution was applied to a 1 cm (i.d.) x 10 cm column of TEAE-cellulose equilibrated with 0.005M Tris-Cl, pH 8.7, and the enzyme which did not stick to the column, was washed through the column with this buffer.

Method B: The peroxisomes were broken by dilution with 3 volumes of 0.005M Tris-Cl, pH 8.7. The diluted solution was centrifuged at 144,000g for 60 min. The supernatant solution was applied to a 2.5 cm (i.d.) x 20 cm column of TEAE-cellulose, equilibrated with 0.005M Tris-Cl, pH 8.7, and the enzyme eluted with this buffer. The eluate containing the enzyme was concentrated by ultrafiltration using a PM 30 membrane (Amicon Corp.).

Polyacrylamide Gel Electrophoresis of Glycolate Oxidase

Polyacrylamide gel electrophoresis of the TEAE-cellulose purified glycolate oxidase was performed at pH 9.3 as described by Davies (1964) and at pH 7.0 as described by Williams and Reisfeld (1964). In both cases the gel concentration was 5.5%. Twenty to thirty μg of protein were applied. Following electrophoresis the gels were stained for protein with 0.5% (w/v) Amido-Schwartz in 7.5% (v/v) acetic acid for 2 hours and

destained in 7.5% (v/v) acetic acid. Other gels were stained for glycolate oxidase activity by incubation at 25° in the dark in a reaction solution containing 4.0 ml 0.1M Tris-Cl, pH 8.5, 0.25 ml 2M sodium glycolate, 0.01 mg phenazine methosulfate and 1.8 mg nitroblue tetrazolium. A control gel was incubated in the same reaction solution less glycolate.

Determination of the K_m for Oxygen

The K_m for oxygen for each of the three reactions catalysed by glycolate oxidase (glycolate to glyoxylate, glyoxylate to oxalate and L-lactate to pyruvate) was determined at 25° by analyses of the reaction progress curves rather than by measurement of initial reaction rates under different oxygen concentrations. Calculations showed that the organic acid concentrations were saturating throughout the course of the reactions. Increasing the concentration of the organic acids did not alter the progress of the reaction.

Determination of the Energies of Activation

The energies of activation for each of the three reactions catalysed by glycolate oxidase were determined by measuring the initial rate of oxygen consumption at various temperatures. Correction was made for the effect of temperature upon the pen response.

Preparation and Reactivation of the Apoenzyme of Glycolate Oxidase

The method of Massey and Curti (1966) was used with minor modifications. To a solution of glycolate oxidase, purified from spinach peroxisomes to the stage before TEAE-cellulose chromatography and containing about 2 units, was added an equal volume of 2M KBr in 0.1M sodium pyrophosphate, 3 x 10^{-3}M EDTA, pH 8.5. The solution was allowed to stand on ice for two hours before desalting on a 1 x 15 cm column of Sephadex G25. The apoenzyme was assayed by DCPIP reduction in the absence of FMN with concentration of enzyme sufficient to detect 1% holoenzyme.

The reactivation of the apoenzyme was followed anaerobically by DCPIP reduction as described except that the reaction was initiated by the addition of FMN. The concentration of FMN was determined spectrophotometrically at 450 nm using a value for the molar extinction coefficient of 1.22 x 10^{4} $cm^{-1}M^{-1}$ (Gibson *et al.*, 1962).

Results and Discussion

Purification of Glycolate Oxidase from Spinach Peroxisomes

The purification of glycolate oxidase from spinach peroxisomes by the two methods is presented in Table I. The biggest problem concerning both methods and one which was never satisfactorily overcome is the lability of the enzyme especially in dilute solution.

TABLE 1.--PURIFICATION OF GLYCOLATE OXIDASE FROM SPINACH PEROXISOMES.

METHOD A. Peroxisomes Isolated in SW 25.2 from 1.2 kg Spinach.

	mg Protein	Total activity μmoles min^{-1}	Specific activity μmoles $min^{-1}mg^{-1}$	% Yield[b]
PEROXISOMES	8.90	36.3	4.1	9.2
TEAE ELUATE	0.94	25.0	26.5[c]	6.3

METHOD B. Peroxisomes Isolated by Zonal Centrifugation from 3.2 kg Spinach.

	mg Protein	Total activity[a] μmoles min^{-1}	Specific activity[a] μmoles $min^{-1}mg^{-1}$	% Yield[b]
PEROXISOMES	74	276	3.7	32
TEAE ELUATE	11.2	129	11.5	15
CONCENTRATE	10.8	67	6.2	8

[a]Activity based on the extrapolated maximum rate of DCPIP reduction.

[b]The % yield was based upon the total enzyme activity in the crude homogenage.

[c]This preparation had an extrapolated maximum rate of oxygen consumption of 30 μmoles $min^{-1}mg^{-1}$.

The peroxisomes represent a localized high concentration of enzyme and as such the enzyme in about 2M sucrose lost only 10% of its activity over the period of three weeks. However, upon breakage of the peroxisomes by dilution of the sucrose, the enzyme lost activity very rapidly. The enzyme is considerably more stable when stored as an ammonium sulfate precipitate. Addition of thiol reagents, glycolate or bovine serum albumin did little to alter the rate of loss of activity. Attempts to concentrate the enzyme are hampered by the presence of the sucrose (initially about 2M) used to isolate the peroxisomes. Similar attempts to purify the enzyme from castor bean endosperm glyoxysomes (Shih and Breidenbach, 1971) indicate that it, too, is very labile.

Purification of the enzyme was aided by the knowledge that the isoelectric point of the enzyme is about 9.0 as measured by isoelectric focusing while the isoelectric points of the other known peroxisomal enzymes are all below 8.0 (Oeser and Tolbert, unpublished). Thus, column chromatography on TEAE-cellulose at pH 8.7 readily removed these contaminating proteins. Analyses of the eluate from the TEAE-cellulose column confirmed that none of the enzymes known to be in the peroxisome were present, except glycolate oxidase.

A specific activity of about 30 μmoles $min^{-1}mg^{-1}$ protein for the purified enzyme compares very favorably with the values of 5.3 and 9.9 reported for the

crystalline enzyme by Zelitch and Ochoa (1953) and Frigerio and Harbury (1958) respectively. These latter values should be multiplied 2 to 3 in order to account for the sub-saturating DCPIP concentration. Although the values in Table 1 do not appear to represent many fold purification it should be mentioned that the isolated peroxisomes represent about 1% of the cellular protein.

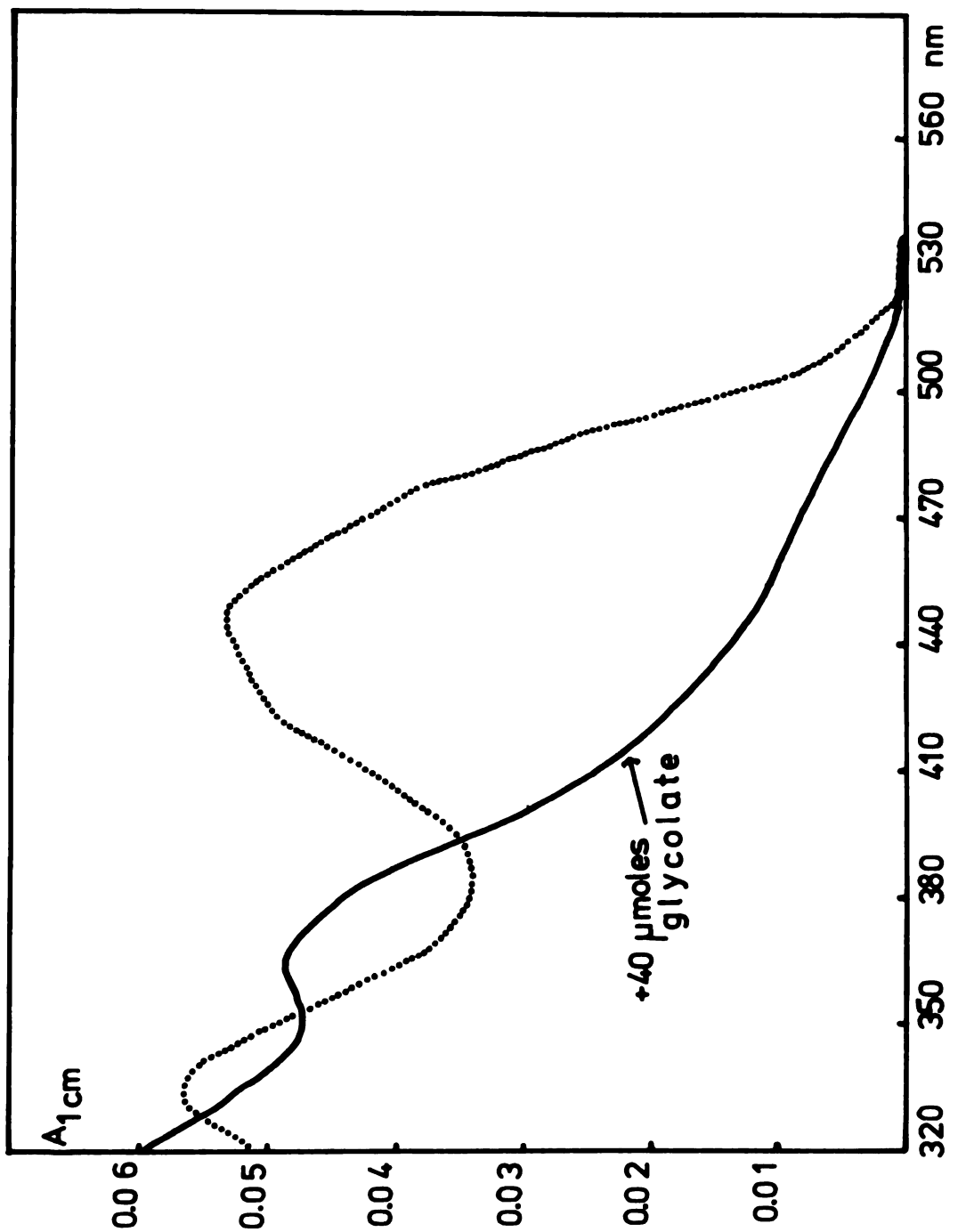
Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis of the purified enzyme at pH 7.0 for periods of time ranging from one to four hours as described in Methods section revealed the presence of a single band of protein which gave a positive reaction when tested for glycolate oxidase. After polyacrylamide gel electrophoresis at pH 9.3, no protein nor any glycolate oxidase activity could be detected on the gel, indicating that contaminating acidic proteins were below the level of detectability. Since glycolate oxidase has a pI of about 9.0, it would not be expected to migrate.

Visible Spectrum of Glycolate Oxidase

The visible spectrum of the purified enzyme was recorded (Fig. 3). The absorption at 450 nm was readily bleached by the addition of a large excess of sodium glycolate. The oxidized enzyme shows peaks at 335 nm and 448 nm with a pronounced shoulder at 420 nm. The

Figure 3. Visible spectrum of glycolate oxidase (0.31 mg/ml) in 0.005M Tris-Cl, pH 8.7, 25°.


A1cm
0.06
0.05
0.04
0.03
0.02
0.01
320
350
380
410
440
470
500
530
560 nm
+40 µmoles glycolate

position of the two peaks is in agreement with the spectrum recorded at pH 8.3 by Frigerio and Harbury (1958) but differs from that recorded at pH 7.4 by Zelitch and Ochoa (1953) who found peaks at 450 nm and about 365 nm. Free FMN has peaks at 370 nm and 450 nm at pH 7.0 (Hemmerlich et al., 1964). However, as the pH of the free FMN is raised from pH 9 to pH 12, the absorption at 375 nm decreases and a new peak develops at 335 nm. These changes have been attributed to the ionization of the 3-imino position in the isoalloxazine ring (Hemmerlich et al., 1964). Thus the differences in the spectra at pH 8.7 and pH 7.4 can be attributed to the effect of pH upon the FMN moiety. Subsequent to the termination of this study similar spectral properties were reported for the pig liver enzyme by Schuman and Massey (1971). Their study was considerably more comprehensive than that undertaken here. Interestingly the pig liver enzyme shows an even more pronounced shoulder at 420 nm. Investigations by Schuman and Massey (1971) have shown that the absorption at 420 nm is due to the presence of another chromophore besides FMN. The chemical nature of this chromophore remains to be identified.

Determination of the K_m for Oxygen

The K_m of glycolate oxidase for oxygen with each of the three substrates was determined as described in

the Methods section. Lineweaver-Burk plots (Fig. 4) indicate that the K_m (O_2) for each of the three reactions is the same, namely about 1.9 x 10^{-4}M oxygen at 25°. Air (20% oxygen) saturated water contains 2.6 x 10^{-4}M oxygen at 25°. Regular Michaelis-Menten plots (not shown) indicate that the enzyme is effectively saturated with oxygen at a concentration of about 8.0 x 10^{-4}M. This would correspond to an oxygen concentration in the gas phase of about 60%. This result is regarded as physiologically significant since in vivo measurements of photorespiration indicate that the overall process is not even saturated by 100% oxygen. Further discussion of the physiological significance of this finding is postponed until Chapter V.

The original purpose of these experiments was to gain an insight into the mechanism of action of glycolate oxidase. The fact that the K_m for oxygen is the same with each of the three substrates suggests that oxygen reacts with an intermediate which is either the same in each of the three reactions or so structurally similar that small differences in reactivity with oxygen cannot be detected.

Determination of the Energies of Activation

The Arrhenius energy of activation (E_a) was determined for each of the three reactions with oxygen as the terminal electron acceptor. The results (Fig. 5)

Figure 4. Lineweaver-Burk plot for the determination of the $K_m^{25^o}$ for oxygen of glycolate oxidase for each of the three substrates, glycolate (●——●), L-lactate (x——x) and glyoxylate (0——0). v is expressed as μmoles oxygen consumed min^{-1} 0.2 ml^{-1}. Oxygen electrode assay, 8 μg glycolate oxidase

K_m at 25^o = 0.19 mM 0_2

Air saturated water contains 0.26 mM 0_2 at 25^o.

$V_{max}^{25^o}$ = 30 μmoles/min/mg protein

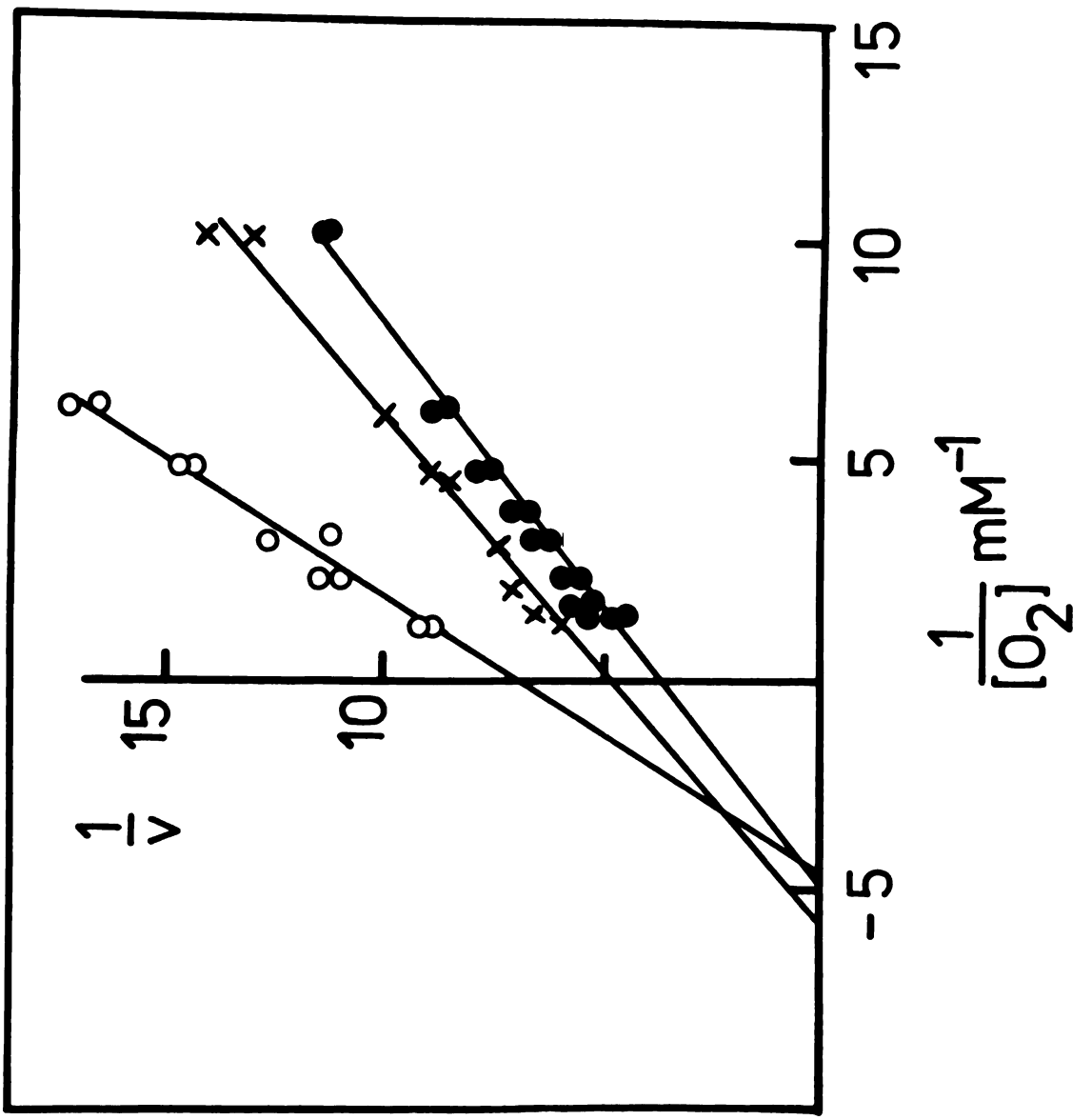
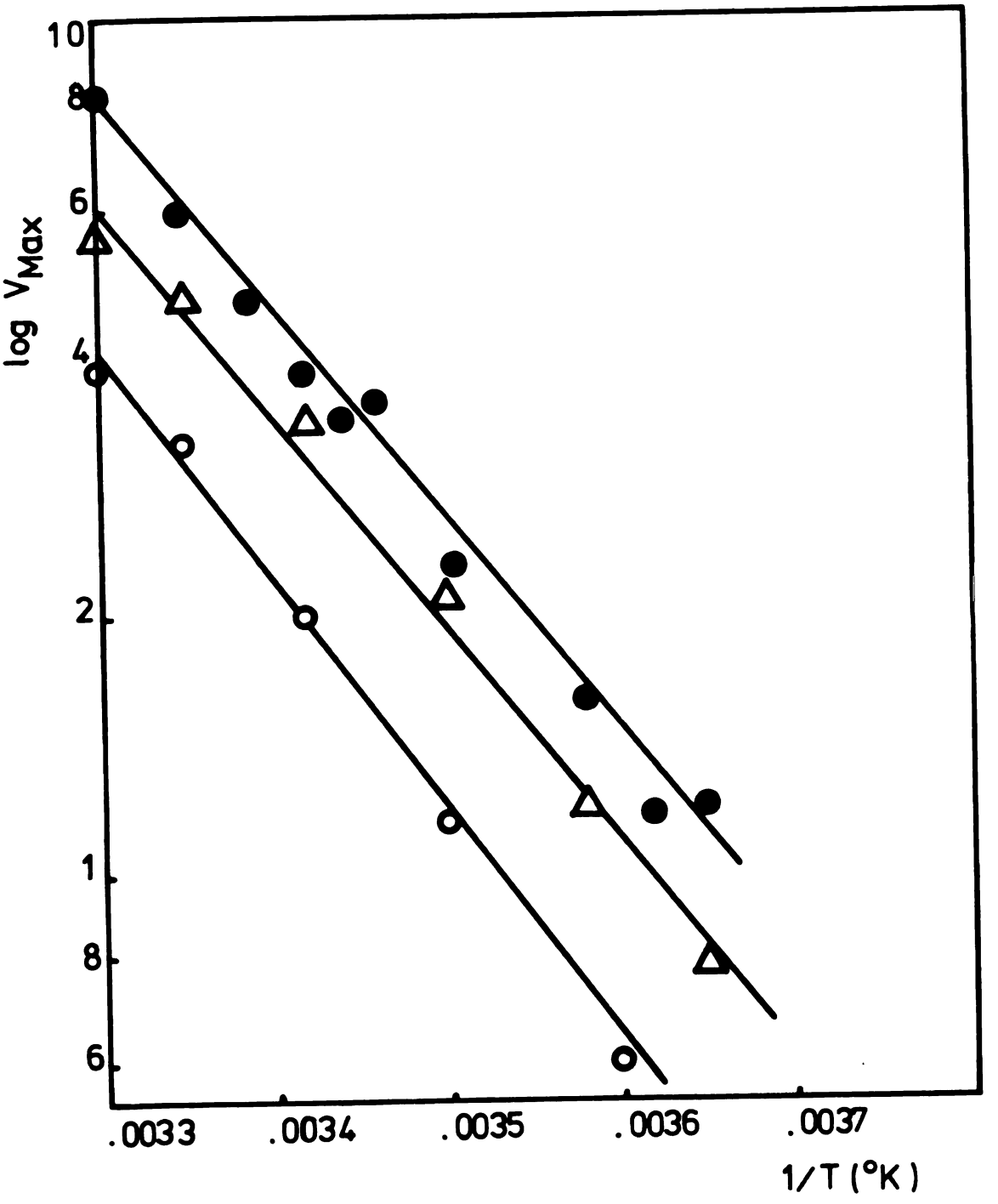

15
10
5
-5
$\frac{1}{[O_2]}$ mM^{-1}
15
10
$\frac{1}{v}$

Figure 5. Arrhenius plot for the catalytic activity of glycolate oxidase with oxygen and each of the three substrates, glycolate ●——●, L-lactate Δ——Δ and glyoxylate O——O. Slopes represent the lines of best fit. The calculated values for E_a are glycolate (11,500 cal.), L-lactate (11,200 cal.) and glyoxylate (12,400 cal.).


10
8
6
4
2
1
8
6
log V_{Max}
.0033
.0034
.0035
.0036
.0037
1/T (°K)

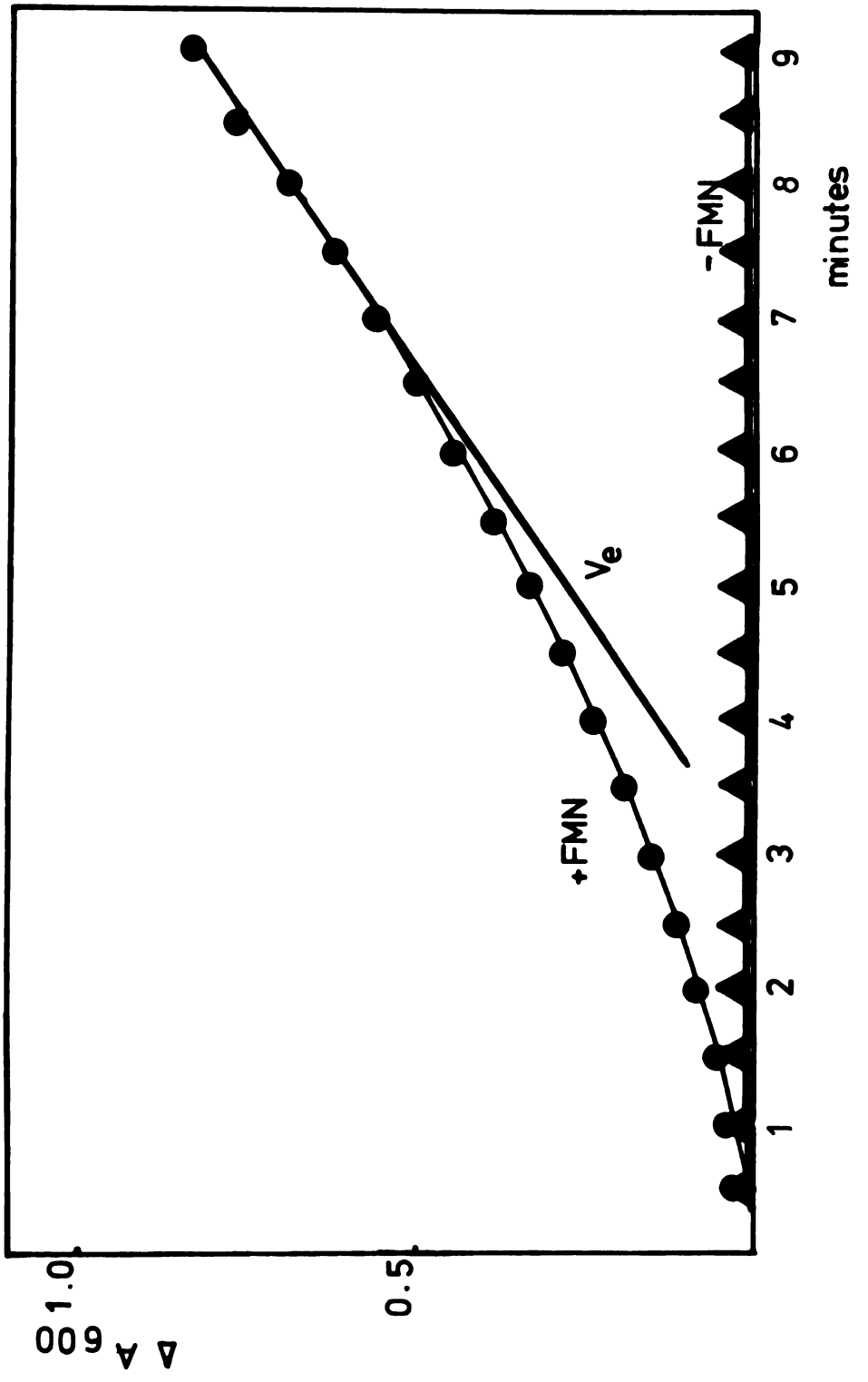
show that the values for E_a for each of the three reactions are close to being the same. Since the partial reaction common to all three overall reactions is the oxidation of the reduced flavin, this result suggests that the rate limiting step in each of the three reactions is the same, namely, the oxidation of the reduced flavin. However, the overall reaction rates for the oxidation of the three substrates are quite different. In order to be consistent with the results reported here it is necessary to conclude that the overall rate of reaction is dependent upon the steady-state concentration of the reduced flavin-enzyme complex. However, lacking further data, it does not seem profitable to speculate further about the steps involved in the mechanism.

Preparation and Reactivation of the Apoenzyme of Glycolate Oxidase

The apoenzyme of glycolate oxidase was prepared as described in the Methods section. The apoenzyme was completely inactive in the absence of FMN (Fig. 6). The recovery of active reconstituted holoenzyme was rather variable, ranging between 35 and 65%. The reason for this variability was not investigated. When the enzyme was dialysed against bromide for 12 hours as described by Massey and Curti (1966) in order to remove FMN only 10% of the activity was recovered.

The reactivation of the apoenzyme with FMN at 25^{o}

Figure 6. Reactivation of the apoenzyme of glycolate oxidase with FMN: T = 25°, FMN concentration = 2.50×10^{-6} M, 15 μg protein.


Δ A 600
1.0
0.5
+FMN
V_e
-FMN
1
2
3
4
5
6
7
8
9
minutes

did not take place immediately (Fig. 6). The order in which the reactants were added did not alter the progress of the reactivation, indicating that glycolate has no role in the reactivation process. Kinetic analysis of the reactivation process indicated that it was a first order event (Fig. 7). A family of curves similar to that obtained in Fig. 6 were obtained by varying the FMN concentration. Two kinetic constants may be obtained from such data. Firstly, the K_m for FMN can be calculated from the final steady state reaction rate (V_e) under different concentrations of FMN. A Lineweaver-Burk plot of the data obtained in this manner gives a value for the K_m for FMN of 4×10^{-6}M (Fig. 8). This agrees with the value of 3×10^{-6}M previously determined by Zelitch and Ochoa (1953). Analysis of the rate constants obtained at different FMN concentrations revealed that the reactivation process was not a simple one-step process. The simplest model for the reactivation would be of the type

$$E + FMN \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} {}^{*}E - FMN$$

where ${}^{*}E$ - FMN represents a catalytically active binary complex. In this case

$$k_{act} = k_{+1}[FMN] + k_{-1} \quad \text{(Strom } \underline{\text{et al}}\text{., 1971)}$$

and a plot of k_{act} versus [FMN] should yield a straight line with a slope of k_{+1} and an intercept on the ordinate

Figure 7. Reactivation of the apoenzyme of glycolate oxidase. First order plot of the results shown in Figure 6. V_t is the rate of DCPIP reduction at any time, t and V_e, the final linear rate of DCPIP reduction.

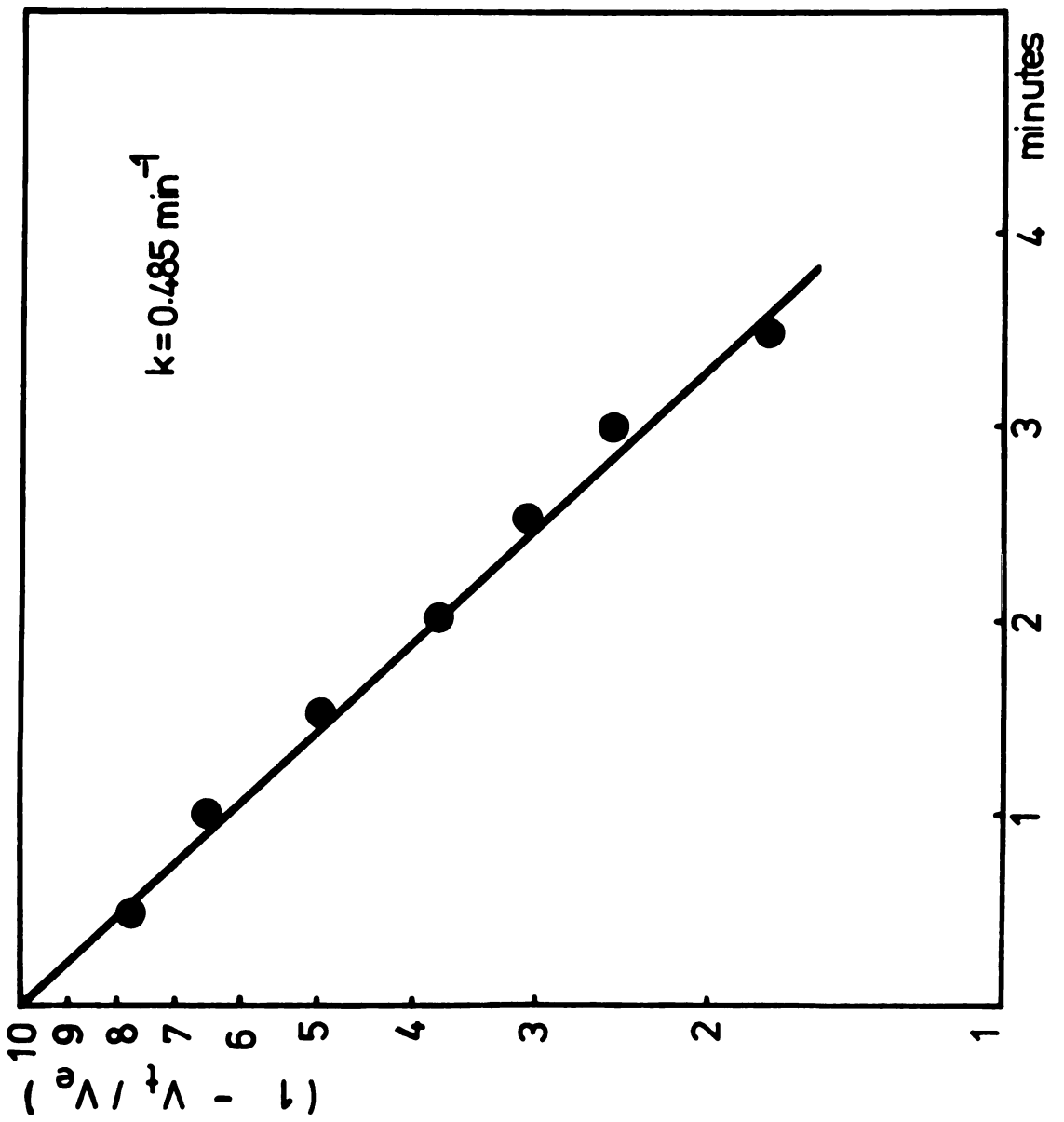
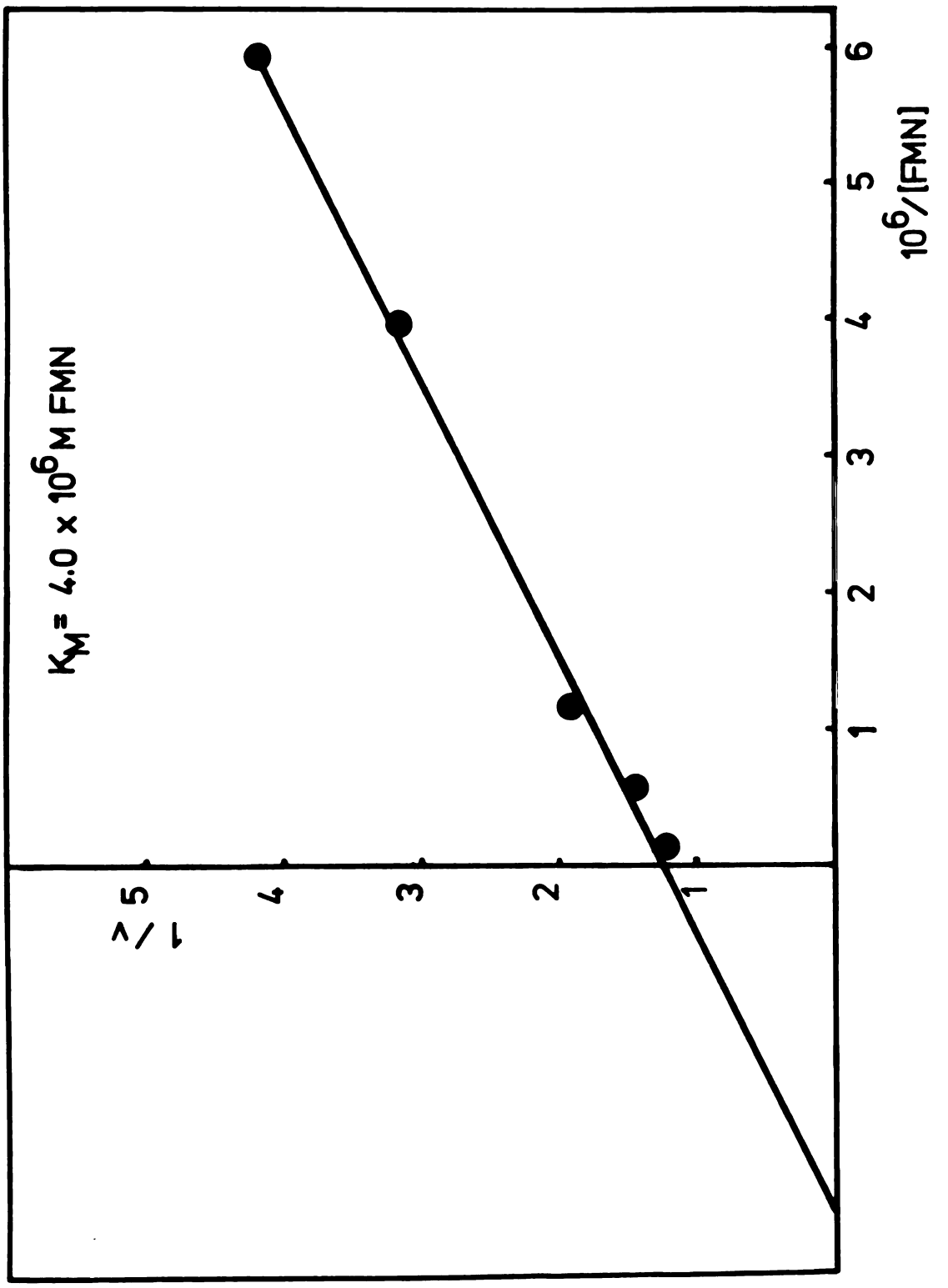

k=0.485 min^{-1}
$(1 - V_t / V_e)$
10
9
8
7
6
5
4
3
2
1
1
2
3
4
minutes

Figure 8. Lineweaver-Burk plot for the determination of the K_m for FMN. Standard anaerobic DCPIP reduction assay.


K_M= 4.0 x 10^6 M FMN
1/v
1
2
3
4
5
1
2
3
4
5
6
10^6/[FMN]

axis of k_{-1}. However, the plot of k_{act} versus [FMN] was hyperbolic, indicating that the activation process is not adequately described by the above model.

A two step mechanism involving the rapid formation of an E-FMN complex followed by a slow conversion of this complex to a catalytically active state may be considered.

$$E + FMN \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E - FMN \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E^* - FMN$$

In the case where $k_{+1}k_{+2} + k_{+1}k_{-2} \gg k_{-1}k_{-2}$ it can be shown (Strom et al., 1971) that

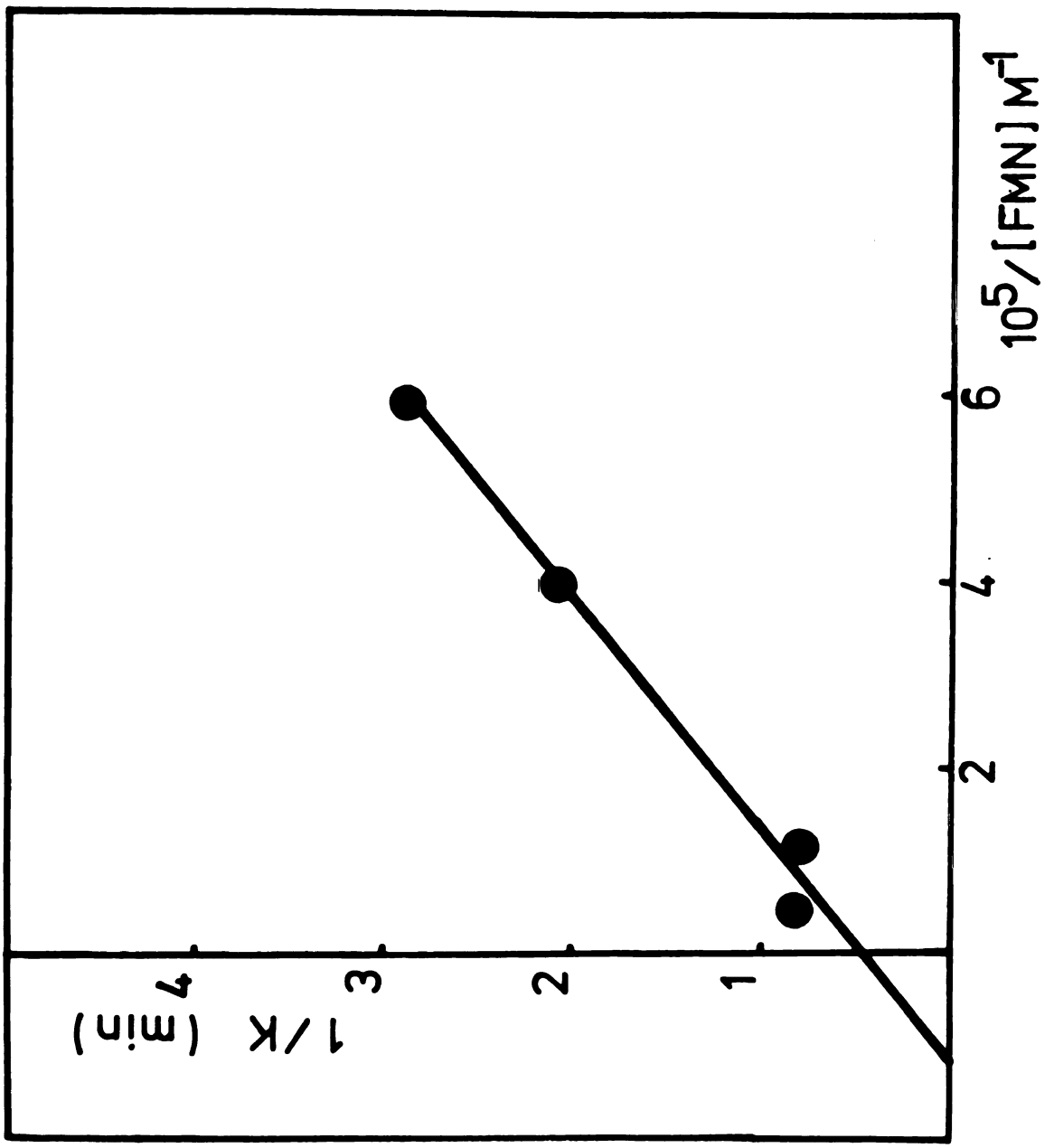
$$\frac{1}{k_{act}} = \frac{k_{-1} + k_{+2}}{k_{+1}(k_{+2}+k_{-2})} \cdot \frac{1}{[FMN]} + \frac{1}{k_{+2}+k_{-2}}$$

Here a double reciprocal plot of $1/k_{act}$ versus $1/[FMN]$ should yield a straight line. Fig. 9 shows that such an analysis of the data does yield a linear relationship. The following relationships were thus obtained:

$$k_{+2} + k_{-2} = 2.0 \text{ min}^{-1} \text{ and } k_{+1}/k_{-1}+k_{+2} = 2.4 \times 10^6 \text{ M}^{-1}$$

This kinetic analysis is consistent with the concept that the simple combination of the apoenzyme of glycolate oxidase with FMN is not sufficient for catalytic activity. This binary complex must then undergo an additional reaction (possibly a conformational transition) in order to become catalytically active. It is interesting that Massey and Curti (1966) have reported a similar phenomenon for the reactivation of the

Figure 9. Double reciprocal plot of the first order rate constants (obtained from the reactivation of the apoenzyme of glycolate oxidase by FMN) versus [FMN].


1/K (min)
1
2
3
4
2
4
6
$10^5/[FMN]\ M^{-1}$

apoenzyme of D-amino acid oxidase by FAD.

CHAPTER II

INCORPORATION OF MOLECULAR OXYGEN INTO GLYCINE AND SERINE DURING PHOTORESPIRATION IN SPINACH LEAVES

Introduction

The inhibition of net photosynthetic CO_2 fixation by oxygen, often referred to as the Warburg oxygen effect (Warburg, 1920), has been observed in a wide variety of algae and higher plants (Turner and Brittain, 1962) and isolated chloroplasts (Ellyard and Gibbs, 1969). It has become evident that this phenomenon is due to photorespiration; that is, the light dependent uptake of oxygen and release of CO_2 which is thought to be associated with the glycolate pathway of metabolism (Jackson and Volk, 1970). Photorespiration is especially evident under conditions of high light intensity, limiting CO_2 and high oxygen concentrations. In these circumstances a large part of the total carbon fixed during photosynthesis flows through the glycolate pathway (Tolbert, 1963). Recently, many of the enzymes of the glycolate pathway have been located in the peroxisomal (microbody) fraction, as distinct from both chloroplasts and mitochondria (Tolbert, 1971). The origin of phosphoglycolate and

glycolate is one of the most interesting problems in photosynthetic carbon metabolism and one which is of fundamental importance in photorespiration. Glycolate may arise from phosphoglycolate by the action of a specific phosphatase, which is located in the chloroplast (Richardson and Tolbert, 1961), but whether it does so exclusively is not clear. Two radically different mechanisms have been proposed to account for the formation of uniformly labelled glycolate during photosynthetic $[^{14}C]CO_2$ fixation. One, proposed by Tanner et al., (1960), Stiller (1962), and Zelitch (1965) suggests that glycolate arises by means of a hitherto undiscovered reductive condensation of two molecules of CO_2. However, the ^{14}C labelling experiments of Hess and Tolbert (1966) and Coombs and Whittingham (1966) tend to discount this possibility. The other mechanism, proposed in various forms, suggests that glycolate, phosphoglycolate or both are formed as the result of the oxidation of one or more intermediates of the photosynthetic carbon cycle (Wilson and Calvin, 1955; Bassham and Kirk, 1962; Tolbert, 1963; Coombs and Whittingham, 1966; and Gibbs, 1969). If the latter hypothesis is correct the nature of the oxidant is of considerable interest. To investigate the possibility that the oxidant is molecular oxygen, or is derived from it, experiments were performed in which detached spinach leaves were allowed to photorespire in an atmosphere

of [^{18}0] oxygen. This chapter concerns the incorporation of [^{18}0] into the products of the glycolate pathway, glycine and serine.

Materials

Spinach was grown as previously described (Chapter I, p. 40). Younger leaves, weighing about 2 gm and about 10 cm long, were harvested from mature plants immediately before use.

[^{18}0] Oxygen (93.5 atoms %) and [^{18}0]$H_2$0 (93 atoms %) were obtained from Miles Laboratories Inc., Elkhart, Indiana. Silylating reagents were obtained from Regis Chemical Co., Chicago, Illinois. Solvents were redistilled where necessary.

Methods

Photorespiration in [^{18}0] Oxygen

A spinach leaf was placed in a stoppered 2.2 cm (i.d.) x 10 cm test tube and the volume of the gas space determined by measuring the volume of water required to fill the tube completely. This volume was 31.0 $\pm$ 0.5 ml. The water was removed, except for the last 1 ml which covered the bottom of the petiole, and the leaf was allowed to photosynthesize in a stream of humidified air at 25^{o} while being illuminated from two sides with white light of about 4,000 foot candles. The

air entered and left the tube by means of syringe needles passing through the stopper. After about 40 min the system was flushed with 50 ml of 100% oxygen, followed immediately by an injection of 6 ml of isotopic oxygen. At various times after introduction of the isotope, the leaf was killed by filling the tube with boiling 90% (v/v) ethanol.

Extraction of glycine and serine.

After the leaf had remained in boiling 90% (v/v) ethanol for at least 3 min, the solution was decanted and the leaf further extracted for 3 min each with boiling 50% (v/v) ethanol and boiling absolute methanol. The extracts were combined and evaporated to dryness. The residue was dissolved in a minimum volume of chloroform-methanol-0.2 M formic acid (25:60:15 by vol.). The chloroform phase was removed and washed twice with 0.2 M formic acid. The aqueous phases were combined and washed twice with chloroform. Sufficient Dowex 50, H^+ form was added to the aqueous phase to render the supernatant solution colorless. This solution was removed and the resin was washed with 0.2 M formic acid. Glycine and serine were eluted from the resin with 1 M NH_4OH and the eluate was evaporated to dryness. The residue was dissolved in a minimum volume of water and applied as a streak to a 24 cm x 50 cm sheet of Whatman 3MM chromatography paper. Glycine and

serine markers were applied on both sides of the main streak. Chromatography was performed for 17 hr at 25° with n-butanol-propionic acid-water (10:5:7 by vol.) as solvent. The chromatogram was dried and thin strips were cut from both sides and from the middle. These strips were sprayed with ninhydrin solution (0.2% (w/v) in water-saturated n-butanol) and heated at 90° until color development was adequate. The area of the chromatogram containing both glycine and serine was located by comparison with the markers. Glycine and serine have very similar R_f values in this system. This area of the chromatogram was cut out and extracted with water. The resultant solution was evaporated to dryness in preparation for silylation.

Extraction of organic acids.

The supernatant solution plus washings from the Dowex 50 step were combined and evaporated to dryness. The residue was dissolved in 8 ml of 0.05 M NH_4OH, the pH of the resultant solution being about 9.4. This solution was incubated with approximately 3 units of alkaline phosphatase (Sigma Type VII) at 25° for at least 30 min and then applied to a 0.7 cm (i.d.) x 6 cm column of Dowex 1-acetate. The column was washed with water and the organic acids were eluted with 4 M acetic acid. This fraction was evaporated to dryness and the residue used to prepare the Me_3Si derivatives.

Preparation of Me_3Si derivatives.

The dried residues obtained as described above were suspended in 50 µl of acetonitrile and 50 µl of bis(trimethylsilyl)trifluoroacetamide containing 1% (v/v) trimethylchlorosilane under dry conditions and heated at 150° for 15 min to facilitate the silylation reaction. Standards containing glycine, serine, glycerate, malate and erythronate (1 µg/µl) were similarly prepared. Samples containing glycine were allowed to stand at room temperature for at least 48 hr before analysis to ensure the formation of $(Me_3Si)_3$ glycine (Bergstom et al., 1970).

Synthesis of [^{18}O] Glycine and [^{18}O] Serine

A mixture of glycylglycine (1.0 mg) and serylglycine (1.1 mg) was dissolved in 190 µl of 6 N HCl and 10 µl of [^{18}O]H_2O (93 atoms %). This solution was heated for 20 hr at 110° in a sealed vial.

Synthesis of [^{18}O] Glycerate

Calcium DL-glycerate (3.5 mg) was dissolved in 90 µl of 2N HCl and 10 µl of [^{18}O]H_2O (93 atoms %). This solution was heated at 90° for 17 hr in a sealed vial.

Isotope analysis

Aliquots (1 - 4 µl) of the above silylated samples were analyzed using an LKB-9000 combined gas chromatograph -

mass spectograph equipped with a 1.4 m x 3 mm (i.d.) silanized glass column packed with 3% (w/v) SE-30 on silanized Supelcoport (100-120 mesh, Supelco Inc., Bellefonte, Pa.). The column temperature was 100^{o} for glycine and serine and programmed at a rate of 5^{o}/min from 50^{o} to 200^{o} for the organic acids. The flow rate of the helium carrier gas was 30 ml/min. The temperature of the ion source was 290^{o} and the ionizing voltage 70 eV. Isotope incorporation was measured by the procedure of Thorpe and Sweeley (1967). The normal isotopic abundance in fragment ions was determined experimentally from the mass spectrum of the compound in question, rather than from probability theory based on empirical formula. This value (F) was determined by the ratio (P + 2) observed / P observed, where P and (P + 2) are the intensities of the ions at m/e = P and m/e = (P + 2) in the standards. In samples containing ^{18}O the following correction for normal isotopic abundance was made

$$(P + 2)_{corrected} = (P + 2)_{observed} - F \times P_{observed}$$

Finally, $$\%\ ^{18}O = \frac{(P + 2)_{corrected}}{P_{observed} + (P + 2)_{corrected}} \times 100\%$$

For most determinations the intensities of the various ions were determined by direct measurement of peak heights either from oscillographic recordings or from normalized bargraphs (Sweeley et al., 1970). When more accurate data were required for detection of any

possible incorporation into the hydroxyl group of serine, the accelerating voltage alternator unit (multiple ion analyzer) was used. This technique involves the continuous recording, at constant magnetic field, of the intensities of two ions formed by electron impact on the gas chromatographic effluent. The ions were carefully focused by manual setting of the magnetic field strength (for the ion at the lower m/e value) and by decreasing the accelerating voltage (for the ion at the higher m/e value) (Sweeley et al., 1966).

Results

Gas-Chromatography - Mass Spectrometry of Glycine and Serine

Owing to the small metabolic pool sizes of both phosphoglycolate and glycolate, it was necessary to investigate photorespiratory incorporation of [^{18}O] by analyzing the metabolic products of glycolate, glycine and serine. Both of these compounds accumulate in relatively large pools. In addition, the Me_3Si derivatives of glycolate, oxalate and pyruvate have nearly identical gas chromatographic retention times, emerging with the tail of the solvent front, such that a clean mass spectrum of $(Me_3Si)_2$ glycolate could not be obtained.

The Me_3Si derivatives of glycine and serine were formed, rather than the more commonly used

N-trifluoroacetyl-n-butyl esters, since the latter esterification would result in the loss of at least half of any ^{18}O incorporated into the carboxyl group.

$(Me_3Si)_3$ glycine and $(Me_3Si)_3$ serine were readily separated by gas chromatography from each other and from several other compounds present in the sample (Fig. 10). The mass spectra of authentic samples of $(Me_3Si)_3$ glycine and $(Me_3Si)_3$ serine were similar to those previously reported (Vandenheuvel and Cohen, 1970; Bergstrom, et al., 1970). The only ion in the mass spectrum of $(Me_3Si)_3$ glycine (Fig. 11a) suitable for measuring ^{18}O incorporation was that at m/e 276, which results from the loss of a methyl group from the parent compound and thus contains both carboxyl oxygen atoms. The spectrum of $(Me_3Si)_3$ serine (Fig. 11b) contains prominent ions at m/e 204 and m/e 218. The ion at m/e 204, which may be regarded as $Me_3Si\text{-}O\text{-}CH_2\ CH = {}^{+}NH - SiMe_3$ contains the hydroxyl oxygen only, while that at m/e 218, $Me_3Si - {}^{+}NH = CH - COO - SiMe_3$, contains only the carboxyl oxygen atoms.

Gas Chromatography - Mass Spectrometry of the Organic Acid Fraction

The Me_3Si derivatives of the organic acid fraction were prepared as described in the Methods section. The peaks in the gas chromatogram (Fig. 12) were identified by their mass spectra (Figs. 13a,b,c,d,e).

Comparison of authentic standards with regard

Figure 10. Gas chromatography of a silylated extract from spinach leaves containing glycine and serine. The column temperature was maintained at 100°. The tracing shown is the response of the total ion current detector. Mass spectra showed that peak I was $(Me_3Si)_3$ glycine and peak II was $(Me_3Si)_3$ serine. This tracing was obtained for a leaf exposed to 100% oxygen for 5 min in the light. Smaller peaks for glycine and serine were observed for shorter exposure times or when exposure occurred in the dark.

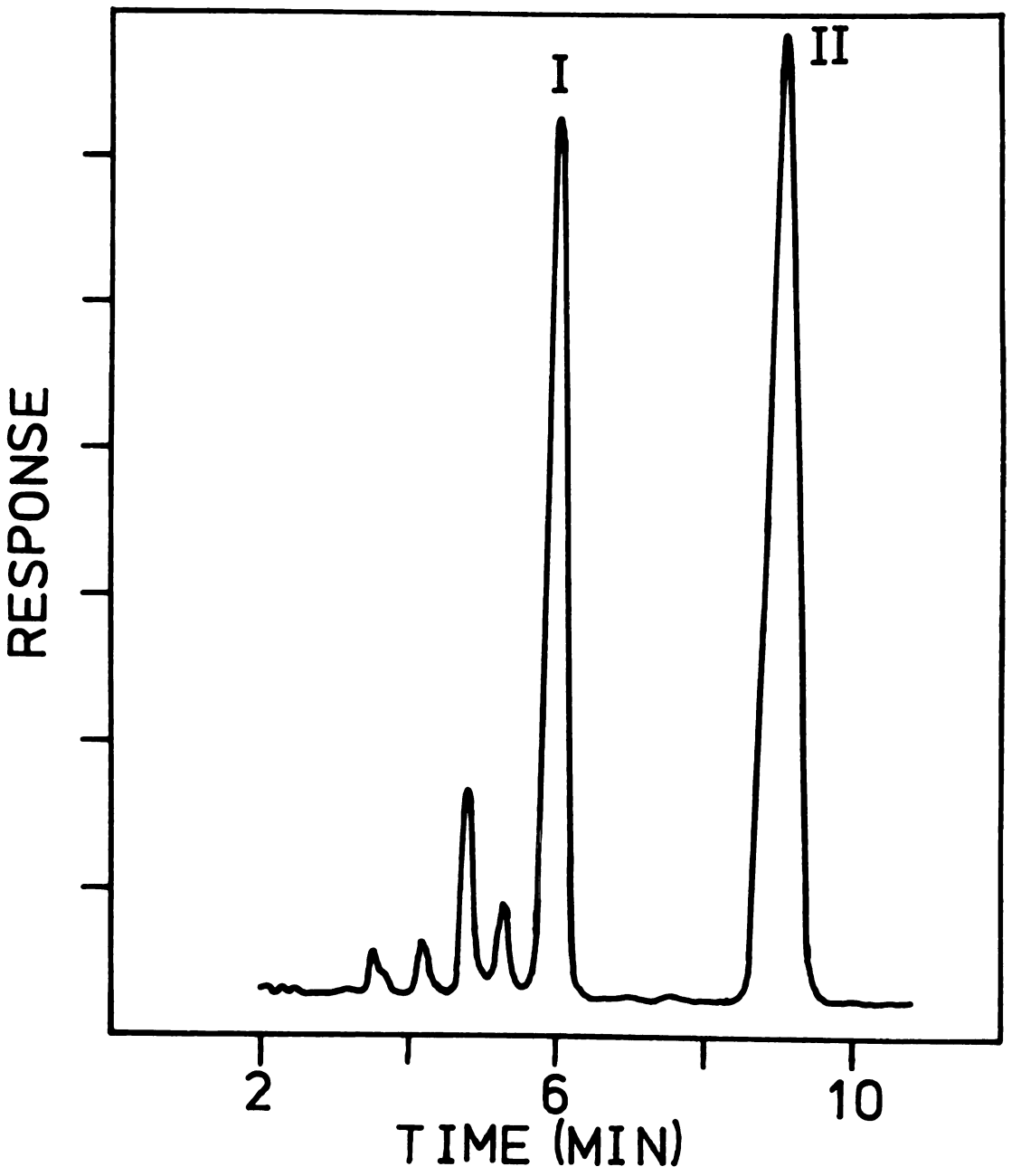
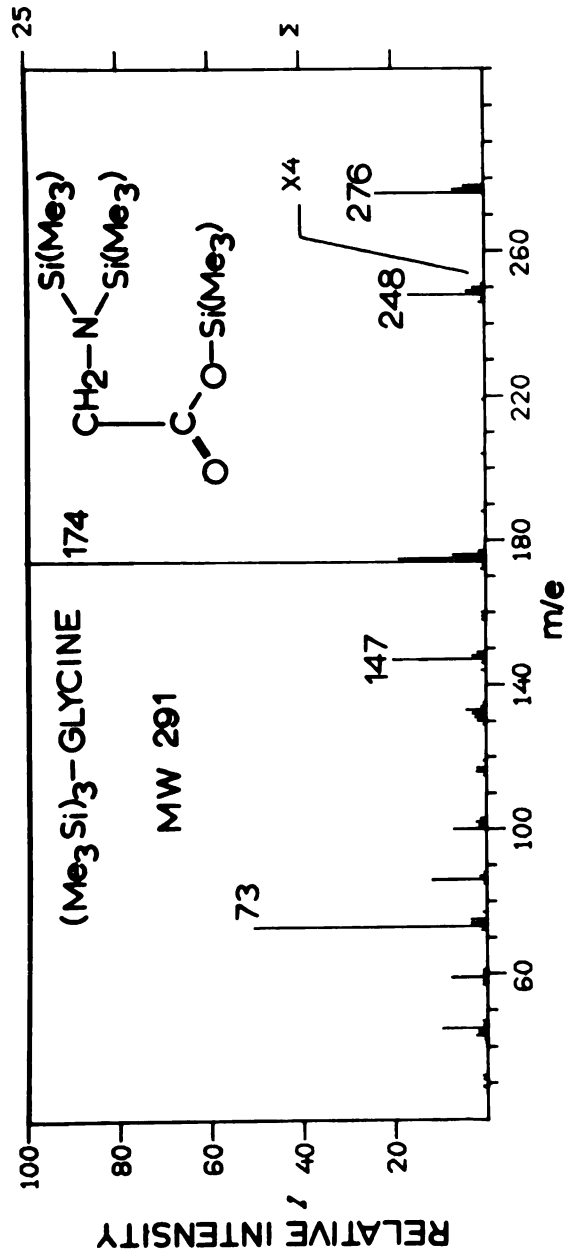

I
II
RESPONSE
2
6
10
TIME (MIN)

Figure 11 (a). Mass spectrum of $(Me_3Si)_3$ Glycine (70 eV.)

(b). Mass spectrum of $(Me_3Si)_3$ Serine (70 eV.)


(Me3Si)3-GLYCINE
MW 291
CH2-N(Si(Me3))-Si(Me3)
C(=O)-O-Si(Me3)
73
147
174
248
276
X4
RELATIVE INTENSITY
m/e

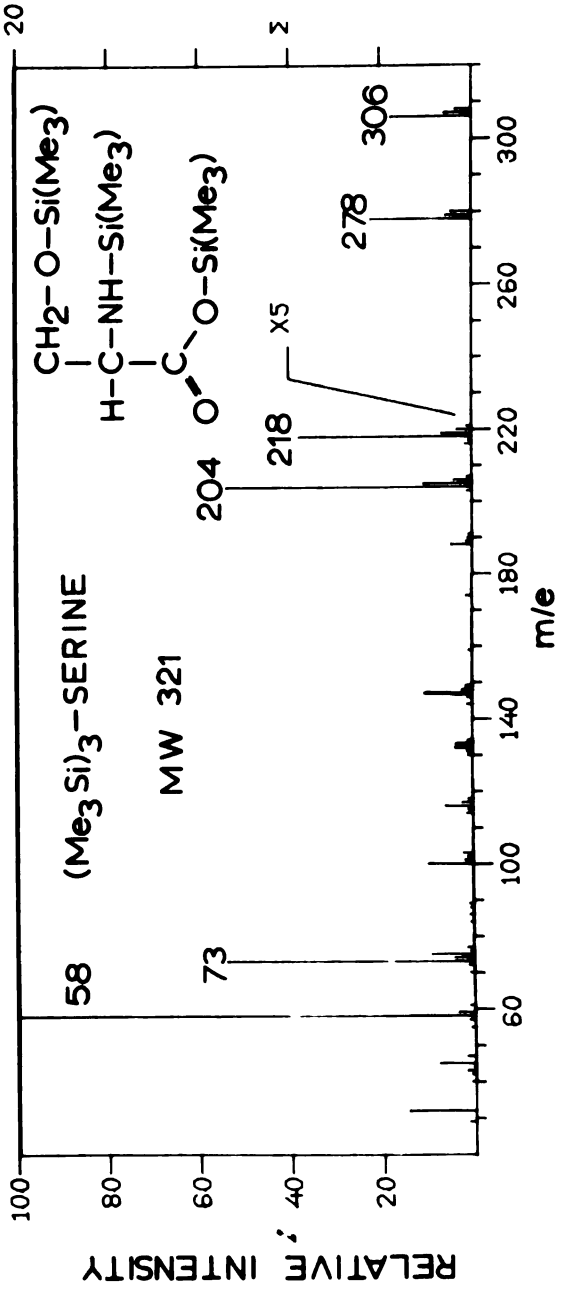

(Me3Si)3-SERINE
MW 321
CH2-O-Si(Me3)
H-C-NH-Si(Me3)
C(=O)-O-Si(Me3)
58
73
204
218
278
306
X5
RELATIVE INTENSITY
m/e

Figure 12. Gas chromatography of a silylated extract from spinach leaves containing organic acids. (——) response of total ion current detector, (----) column temperature. Subsequent mass spectrometry identified the various peaks as follows: I - $(Me_3Si)_2$ oxalate, II - $(Me_3Si)_3$ glycerate, III - $(Me_3Si)_2$ succinate, IV - $(Me_3Si)_3$ malate, V - $(Me_3Si)_4$ erythronate (or $(Me_3Si)_4$ threonate).

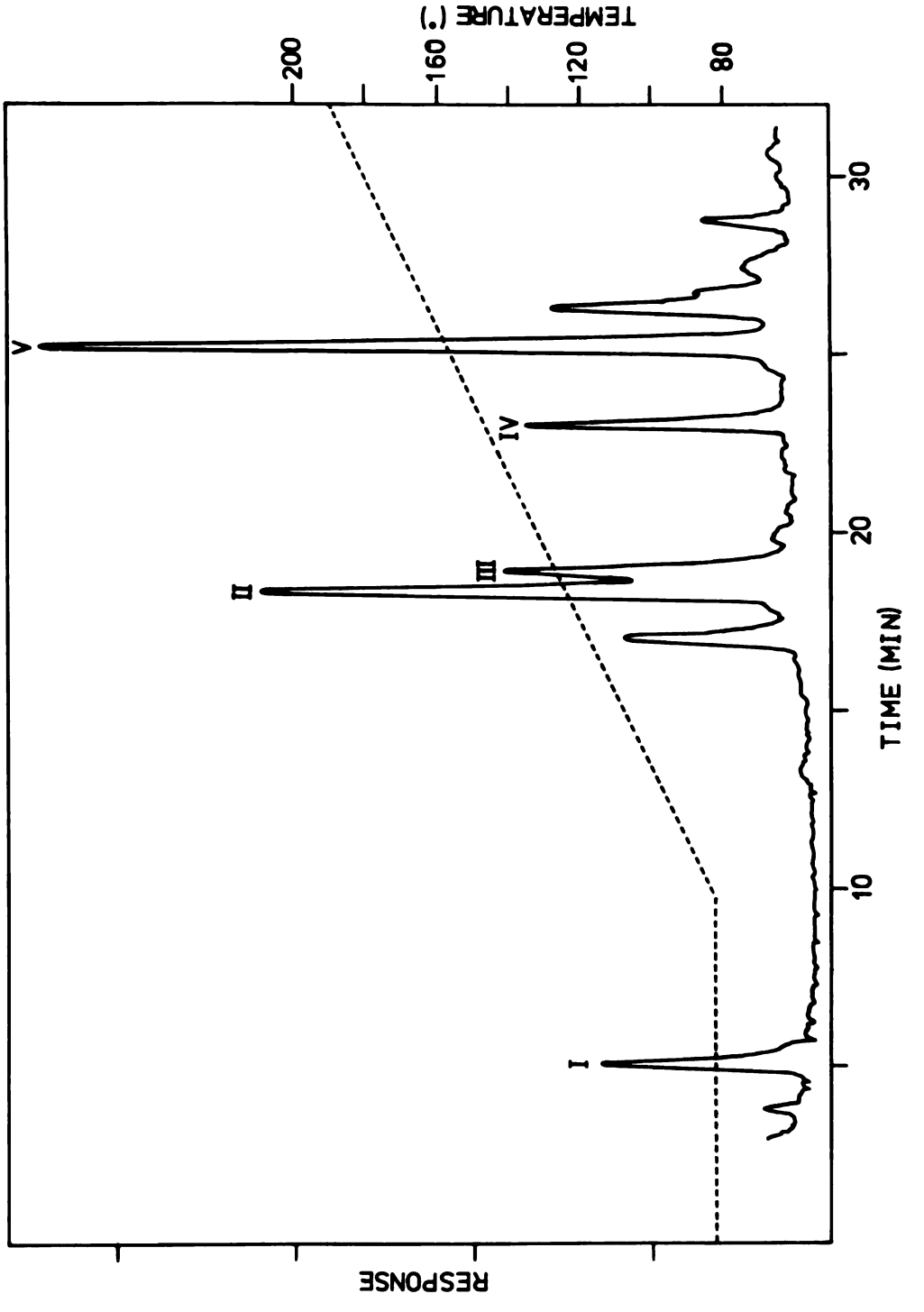

TEMPERATURE (°)
200
160
120
80
RESPONSE
TIME (MIN)
10
20
30
I
II
III
IV
V

Figure 13 (a) Mass spectrum of $(Me_3Si)_2$ oxalate (70 eV.)

(b) Mass spectrum of $(Me_3Si)_3$ glycerate (70 eV.)

(c) Mass spectrum of $(Me_3Si)_2$ succinate (70 eV.)

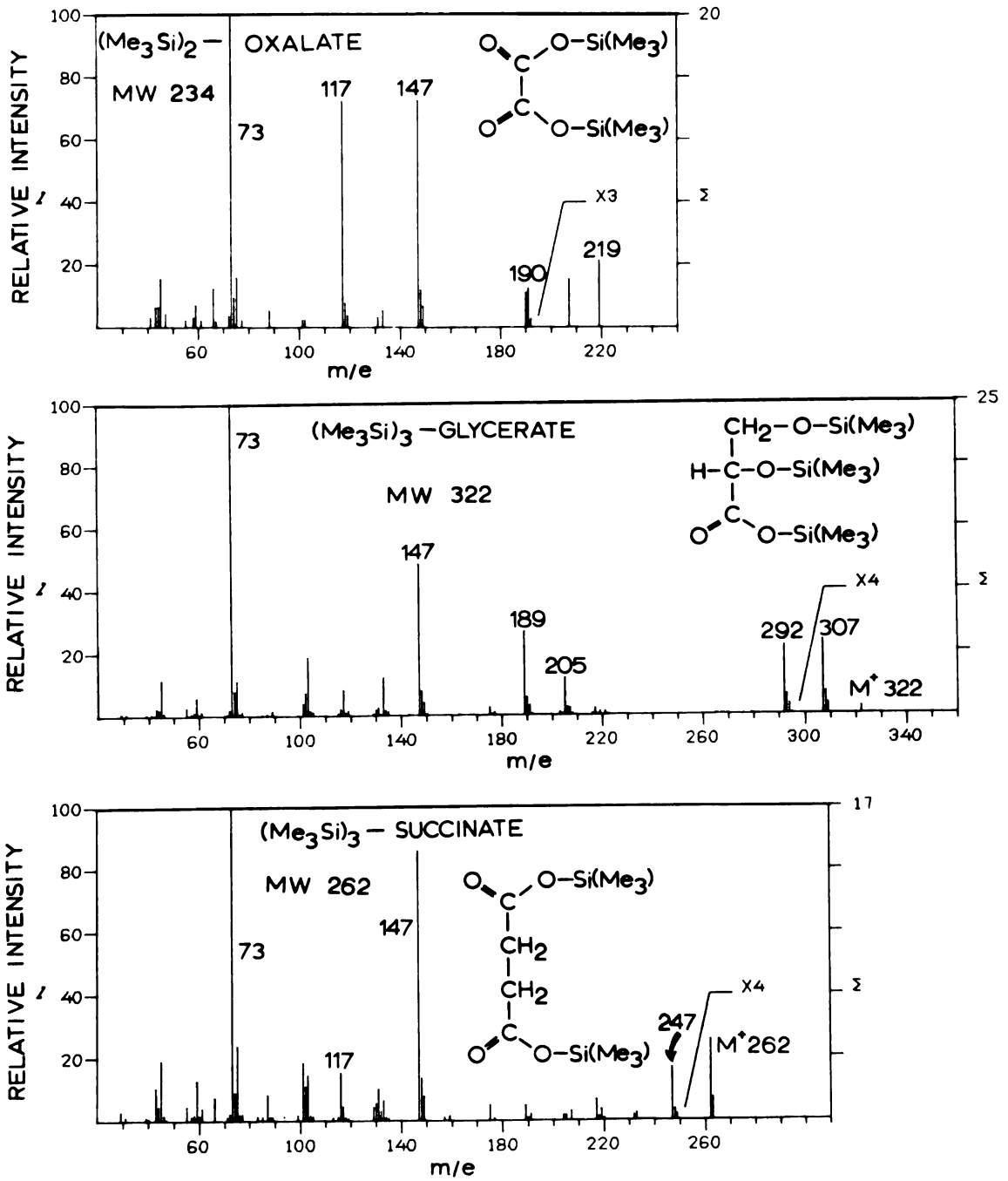
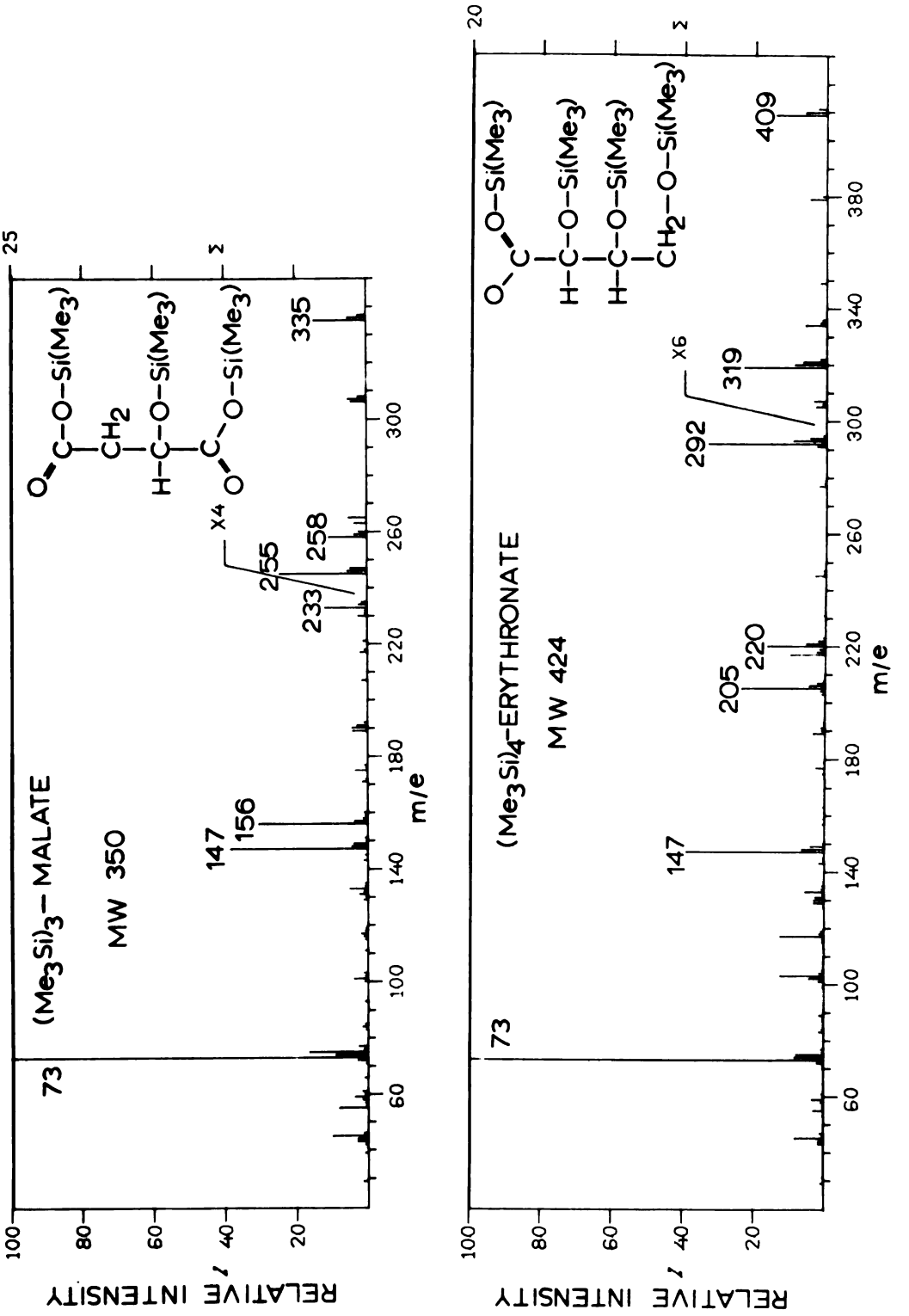

RELATIVE INTENSITY
100
80
60
40
20
I
20
Σ
(Me3Si)2 — OXALATE
MW 234
73
117
147
190
219
X3
O
C
O-Si(Me3)
C
O
O-Si(Me3)
60
100
140
180
220
m/e
RELATIVE INTENSITY
25
(Me3Si)3 —GLYCERATE
MW 322
73
147
189
205
292
307
X4
M+ 322
CH2—O-Si(Me3)
H—C—O-Si(Me3)
C
O
O-Si(Me3)
260
300
340
m/e
RELATIVE INTENSITY
17
(Me3Si)3 — SUCCINATE
MW 262
73
147
117
247
X4
M+262
O
C
O-Si(Me3)
CH2
CH2
C
O
O-Si(Me3)
m/e

Figure 13 (Continued)

(d) Mass spectrum of $(Me_3Si)_3$ malate (70 eV.)

(e) Mass spectrum of $(Me_3Si)_4$ erythronate (70 eV.)


(Me3Si)3– MALATE
MW 350
(Me3Si)4-ERYTHRONATE
MW 424
RELATIVE INTENSITY
m/e
73
147
156
205
220
233
255
258
292
319
335
409
X4
X6
Σ

to chromatographic retention time and mass spectra confirmed the identity of the various peaks. The major peaks were identified as the Me_3Si derivatives of oxalate, glycerate, succinate, malate and erythronate (or threonate). The small shoulder on the oxalate peak is probably due to glycolate.

Investigation of Extraction Procedures

To test whether any isotope incorporated into glycine or serine during the *in vivo* experiment might be wholly or partly lost during the subsequent extraction procedure, a sample containing both glycine and serine labeled with ^{18}O in the carboxyl groups was prepared and subjected to the same procedure used to isolate these amino acids from the spinach leaf. The results (Table 2) showed that no label was lost during the extraction procedure. It is interesting that in the case of serine the hydrolysis procedure resulted in an observed incorporation only marginally greater than the theoretical value expected if incorporation occurred only by hydrolysis of the peptide bond of serylglycine. In the case of glycine, the observed incorporation is greater than the theoretical value expected if incorporation occurred only by hydrolysis of glycylglycine, indicating, that some exchange occurred between the medium and the glycyl carboxyl oxygens of the dipeptides and/or of free glycine.

A similar control experiment was performed with

TABLE 2.--RETENTION OF ^{18}O DURING EXTRACTION PROCEDURES

Sample	^{18}O content (moles %)			
	Glycine	Serine		Glycerate
Before extraction	2.15 (1.61)[a]	4.85 (4.65)[a]	6.54[b]	6.54[c] (16.9)[d]
After extraction	2.63	4.76	6.54[b]	6.80[c]

Each value is the average of three or more measurements.

[a]The values in parenthesis are those predicted for incorporation of ^{18}O, assuming that incorporation occurs solely by hydrolysis of thepeptide bond.

[b]Values obtained by analysis of the ions at m/e 292 and 294.

[c]Values obtained by analysis of the ions at m/e 307 and 309.

[d]The value in parenthesis is that predicted for complete exchange of both oxygen atoms of the carboxyl group with the medium.

glycerate. Oxygen-18 labelled glycerate was prepared as described in the Methods section. This was then subjected to the extraction procedure for organic acids, omitting the Dowex-50 step and the alkaline phosphatase treatment. No label was lost during the extraction procedure (Table 2). The incorporation achieved was 39% of that predicted for complete exchange of both oxygen atoms of the carboxyl group of glycerate with the medium. It should be noted that these experiments do not eliminate the possibility that label is lost during the silylation process or during subsequent storage.

Incorporation of ^{18}O into Glycine and Serine

In Figures 14 and 15 the relevant regions of the mass spectra of the Me_3Si derivatives of authentic samples of glycine and serine are compared with similar spectra obtained for glycine and serine extracted from a spinach leaf which had been exposed to 100% oxygen containing 18.1 atoms % ^{18}O for 2 min in the light. In the case of the glycine spectra (Fig. 14), a comparison of the relative intensities of the ions at m/e 276 and m/e 278 clearly shows that ^{18}O has been incorporated into the carboxyl group. The absence of an ion at m/e 280 indicates that only one of the oxygen atoms of the carboxyl group has become labeled. For the serine spectra (Fig. 15), a similar comparison of the intensities

Figure 14. Comparison of part of the mass spectrum of authentic $(Me_3Si)_3$ glycine (a) with that of the same compound extracted from a leaf which had been exposed to 100% oxygen containing 18.1 atoms % ^{18}O for 2 min in the light (b).

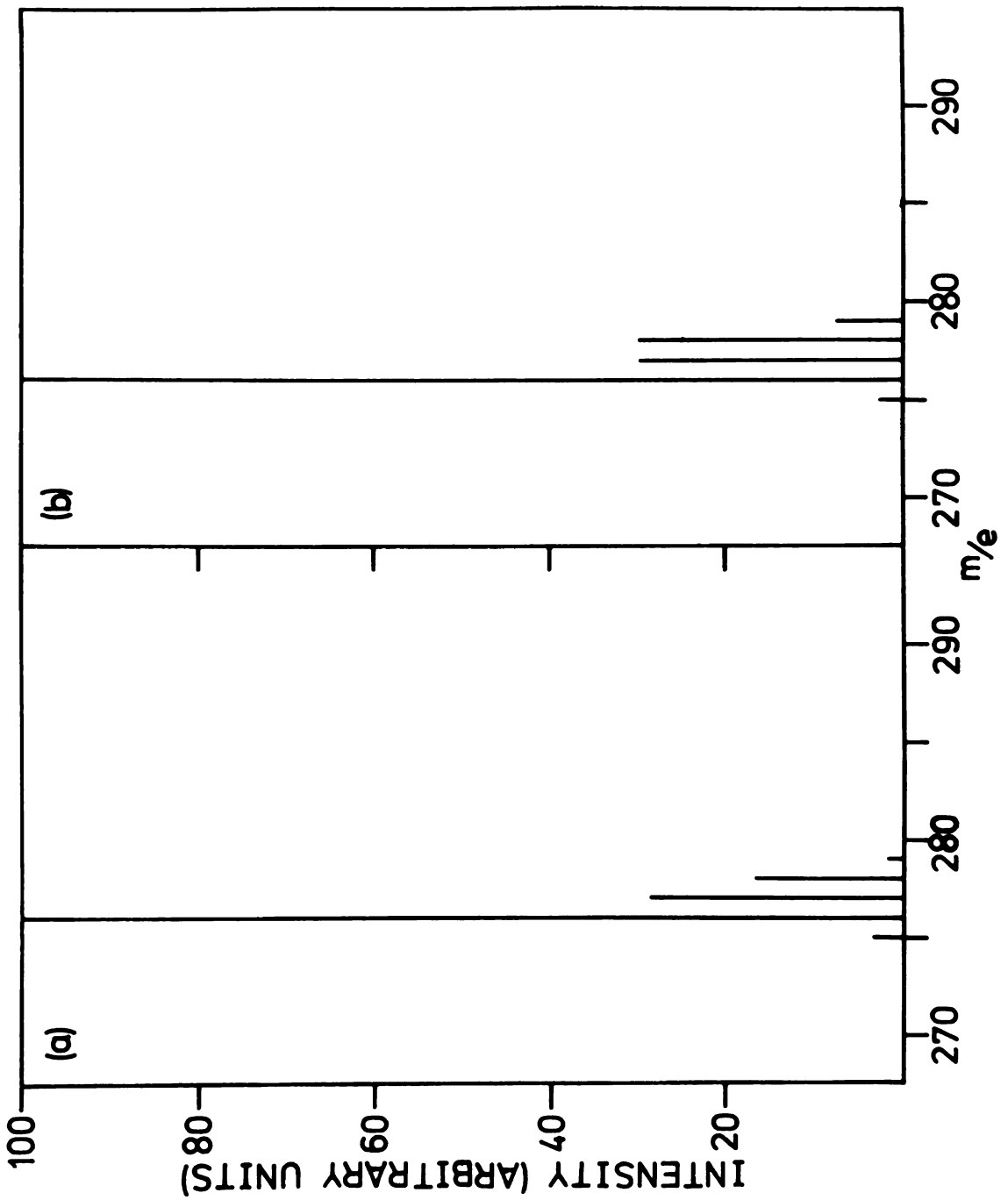
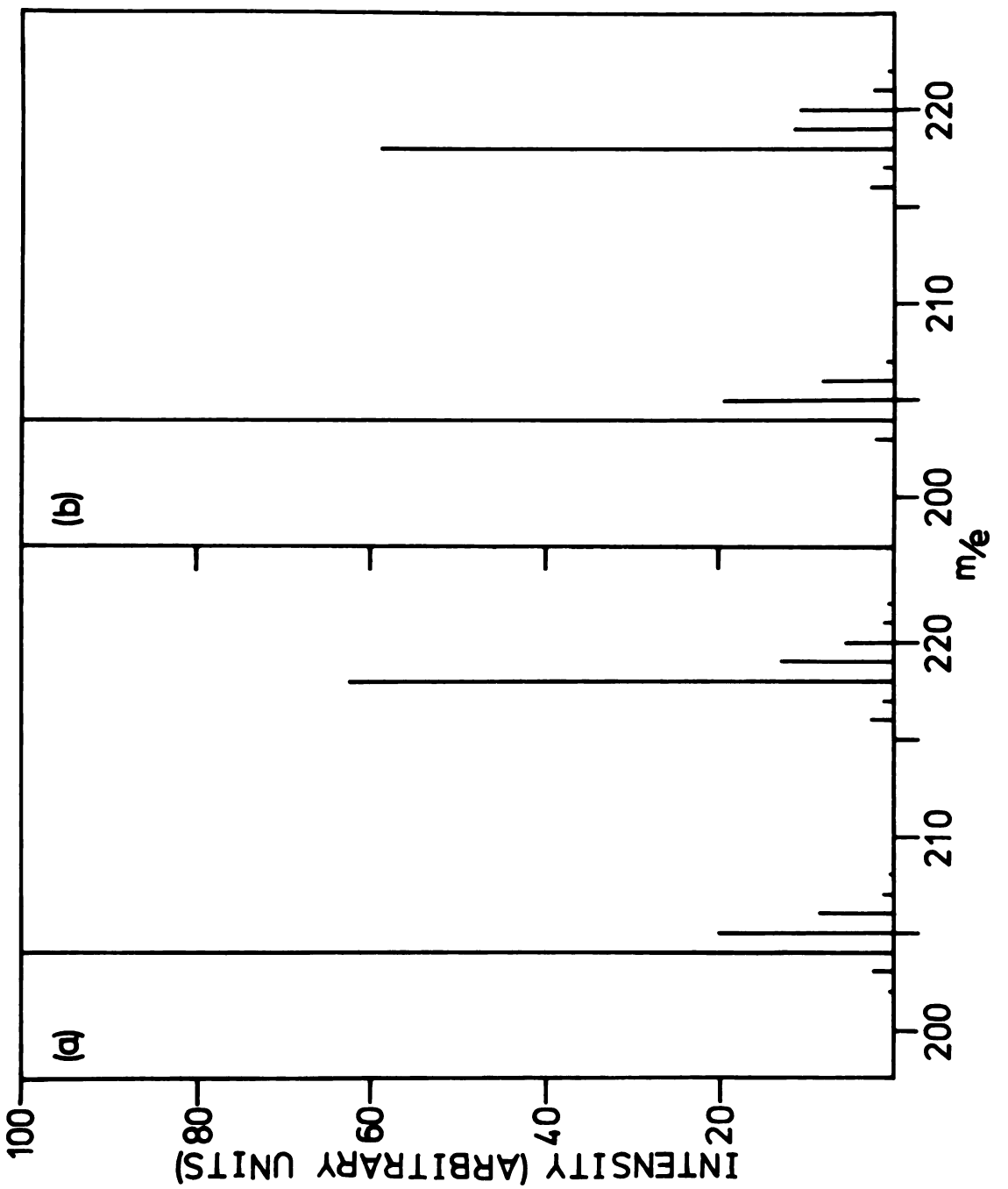

INTENSITY (ARBITRARY UNITS)
100
80
60
40
20
(a)
(b)
270
280
290
m/e

Figure 15. Comparison of part of the mass spectrum of authentic $(Me_3Si)_3$ serine (a) with that of the same compound extracted from a leaf which had been exposed to 100% oxygen containing 18.1 atoms % ^{18}O for 2 min in the light (b).


INTENSITY (ARBITRARY UNITS)
100
80
60
40
20
(a)
(b)
200
210
220
m/e

of the ions at m/e values 218, 220 and 222 shows that here also one, and only one, of the carboxyl oxygens has become labeled. Additonally, in the case of serine, a comparison of the intensities at m/e 204 and m/e 206 reveals that no incorporation into the hydroxyl group has occurred. More accurate data concerning the incorporation of ^{18}O into serine were obtained using the accelerating voltage alternator unit (multiple ion analyzer) of the mass spectrometer (Table 3). The greater sensitivity of this technique made it possible to be more certain that no incorporation into the hydroxyl group had occurred.

Experiments were performed in which the incorporation of ^{18}O into glycine and serine was followed as a function of time (Table 4). The leaf's pool of glycine became labeled more rapidly than did the pool of serine and the level of incorporation at saturation in moles %, was greater for glycine than for serine. In both cases this value was considerably less than the ^{18}O enrichment, in atoms %, of the oxygen supplied. Once again, no incorporation into the hydroxyl group of serine was observed. When the leaf was placed in darkness immediately before the labeled oxygen was supplied, insignificant incorporation into either amino acid occurred. It was apparent that a net synthesis of both glycine and serine occurred when the leaves were exposed to 100% oxygen in the light. Qualitative comparison of

TABLE 3.--INCORPORATION OF ^{18}O INTO SERINE.[a] DATA OBTAINED USING ACCELERATING VOLTAGE ALTERNATOR.

^{18}O Content (mole %)[b]	
Carboxyl Oxygens	Hydroxymethyl Oxygen
8.67 ± 0.29	-0.36 ± 0.31

[a]Serine extracted from a spinach leaf exposed to 100% oxygen containing 18.1 atoms % ^{18}O for 2 min in the light.

[b]Average of at least three measurements. The error quoted is the standard deviation.

TABLE 4.--INCORPORATION OF ^{18}O INTO GLYCINE AND SERINE AS A FUNCTION OF TIME.[a]

Time[b]	Incorporation of ^{18}O (moles %)		
		Serine	
	Glycine	Carboxyl	Hydroxymethyl
20 sec (light)	7.6[c]	6.0[c]	0.2
30 sec (light)	9.1	2.7	-0.2
2 min (light)	12.4	8.1	0
5 min (light)	12.1	7.9	0
5 min (dark)	0	0.2	0.2

[a]The gaseous phase was 100% oxygen containing 18.1 atoms % ^{18}O.

[b]A separate leaf was used for each time.

[c]A younger leaf, about 21 days from germination, was used.

the total ion current tracings (equivalent to gas chromatograms) for the samples in this experiment revealed that the leaf's content of these amino acids at least doubled during the course of the 5 min experiment. No increase was observed for the dark control.

The kinetics of incorporation of label into glycine and serine are dependent on the sizes of the pools which are associated with photorespiration and the activities of the glycolate-pathway enzymes.

These factors may vary with the age of the plant. In an experiment where a leaf from a young plant (about 21 days from germination) was exposed to [^{18}O] oxygen for 20 sec, the incorporation into the carboxyl group of serine was 6.0 moles %, a value higher than that obtained at 30 sec in the previous experiment where leaves from a fully mature plant were used. The incorporation into glycine, 7.6 moles %, was less than that observed previously at 30 sec.

Lack of Incorporation of ^{18}O into Glycerate and other Organic Acids

Organic acids, including phosphorylated organic acids, were extracted from a leaf that had been exposed to [^{18}O]oxygen for 5 min in the light. $(Me_3Si)_3$ glycerate was readily separated by gas chromatography from other compounds occurring in the extract (Fig. 12). Several of these compounds were identified by their mass spectra. In addition to $(Me_3Si)_3$ glycerate, the Me_3Si derivatives of oxalate, malate, succinate and erythronate (or threonate) were identified. Isotope analysis was conducted on glycerate, malate and erythronate. In no case was any incorporation of ^{18}O detected.

Discussion

To date, the only well documented reaction which consumes oxygen during photorespiration is the oxidation

catalyzed by glycolate oxidase. This flavin enzyme is known to produce hydrogen peroxide (Kenten and Mann, 1952; Zelitch and Ochoa, 1953). The reaction does not involve the carboxyl group of glycolate and should not lead to the incorporation of molecular oxygen. The enzymatic reactions leading to the formation of glycine and serine from glyoxylate are not known to involve oxygen. Therefore, the observed *in vivo* incorporation of ^{18}O supplied as molecular oxygen into the carboxyl groups of glycine and serine indicates that oxygen is involved in the photorespiratory process at a step earlier than glycolate oxidase. Therefore, oxygen, or a species derived from oxygen, is involved in the synthesis of glycolate and/or phosphoglycolate. Furthermore, this oxygen is incorporated into the carboxyl group in the process and subsequently appears in glycine and serine. The absence of incorporation in the dark confirms that the process is a photorespiratory event. The possibility that this incorporation could occur via $[^{18}O]H_2O$ formed in the leaf from $[^{18}O]$oxygen is eliminated by the observation that the hydroxyl group of serine did not become labelled. Since serine is formed from glycine in plant tissues by the combined action of glycine decarboxylase and serine hydroxymethyltransferase (Kisaki *et al.*, 1971), the hydroxyl oxygen atom must arise from water. Thus any labeling of water during the course of the experiment would be reflected

in the hydroxyl oxygen of serine.

The incorporation of isotopic oxygen occurred very rapidly. After as short a time as 30 sec, incorporation into glycine reached a value equal to 73% of the saturation level. However, the observed saturation levels of incorporation of ^{18}O into glycine and serine, 12.4 and 8.1 moles %, respectively, were below the theoretical maximum of 18.1 moles %. Control experiments indicated that ^{18}O was not lost by exchange during the extraction procedures. Some dilution of the isotopic oxygen with unlabeled oxygen formed in the photochemical act must surely have occurred. This would effectively decrease the maximum incorporation possible. However, such a dilution would have reduced the saturation level of incorporation into glycine and serine by equal amounts, which was not the case. An explanation which cannot be dismissed entirely is that there is more than one mechanism for the synthesis of glycolate, only one of which involves the incorporation of molecular oxygen. However, again it would be expected that the saturation level of incorporation into both amino acids would be reduced equally. A more likely explanation for the failure to reach the maximum theoretical incorporation lies in the possiblity that more than one pool of both glycine and serine might exist. Only that pool which is closely associated with photorespiration would become rapidly labelled with ^{18}O. The level of incorporation

at saturation would depend on the relative sizes of these pools. This explanation probably accounts for the higher rate of incorporation into serine observed when a leaf from an immature plant was used. Support for this interpretation comes from ^{14}C labelling experiments with Chlorella (Bassham et al., 1964) and with wheat leaves (Hellebust and Bidwell, 1963). There exist in both organisms pools of glycine and serine which are closely associated with photosynthetic carbon metabolism but which are not in rapid equilibrium with other pools of glycine and serine.

The aim of this study was to gain insight into the mechanism whereby an intermediate of the photosynthetic carbon reduction cycle is oxidized to glycolate and/or phosphoglycolate. As shown here, this oxidation proceeds with incorporation of oxygen into the carboxyl group. However, it was not possible to determine which form of oxygen was the reactive species. Thus in addition to oxygen itself, the reactive species might be the superoxide free radical, $O_2H\cdot$, hydrogen peroxide or the hydroxyl free radical, $OH\cdot$. Nor can the possibility that the reactive species was singlet oxygen be excluded. Indeed, the apparent requirement for light for incorporation of oxygen-18 could be interpreted as evidence that an "activated" form of oxygen was the reactive species.

Ogren and Bowes (1971) recently showed that oxygen is an inhibitor of ribulose diphosphate carboxylase and

that it is competitive in this respect with CO_2. They suggested that oxygen may substitute for CO_2 in the reaction of this enzyme so that ribulose diphosphate is oxidised to phosphoglycolate and phosphoglycerate. If such a four-electron oxidation occurred, and assuming that such an oxidation would occur with the incorporation of both atoms of oxygen, one would expect to find ^{18}O in phosphoglycerate. However, the analysis of the combined glycerate and phosphoglycerate pool did not detect any incorporation of ^{18}O. Again a control experiment ruled out the possibility of loss of incorporated isotope during the extraction procedure, suggesting that, in vivo, neither ribulose 1,5-diphosphate nor any phosphorylated pentose is subject to a four-electron oxidation by molecular oxygen to yield phosphoglycerate and phosphoglycolate or glycolate.

Gas chromatography of the acidic fraction extracted from spinach leaves showed that, in addition to such expected organic acids as glycerate, malate, succinate and oxalate, an unknown was present in large quantities (Peak V in Figure 12). This compound was identified by mass spectrometry as $(Me_3Si)_4$ erythronic (or threonic) acid. Since the extraction procedure involved treatment with alkaline phosphatase it is possible that this acid exists in vivo as 4-phosphoerythronate. It is also possible that this acid is an artifact formed during the extraction procedure. It

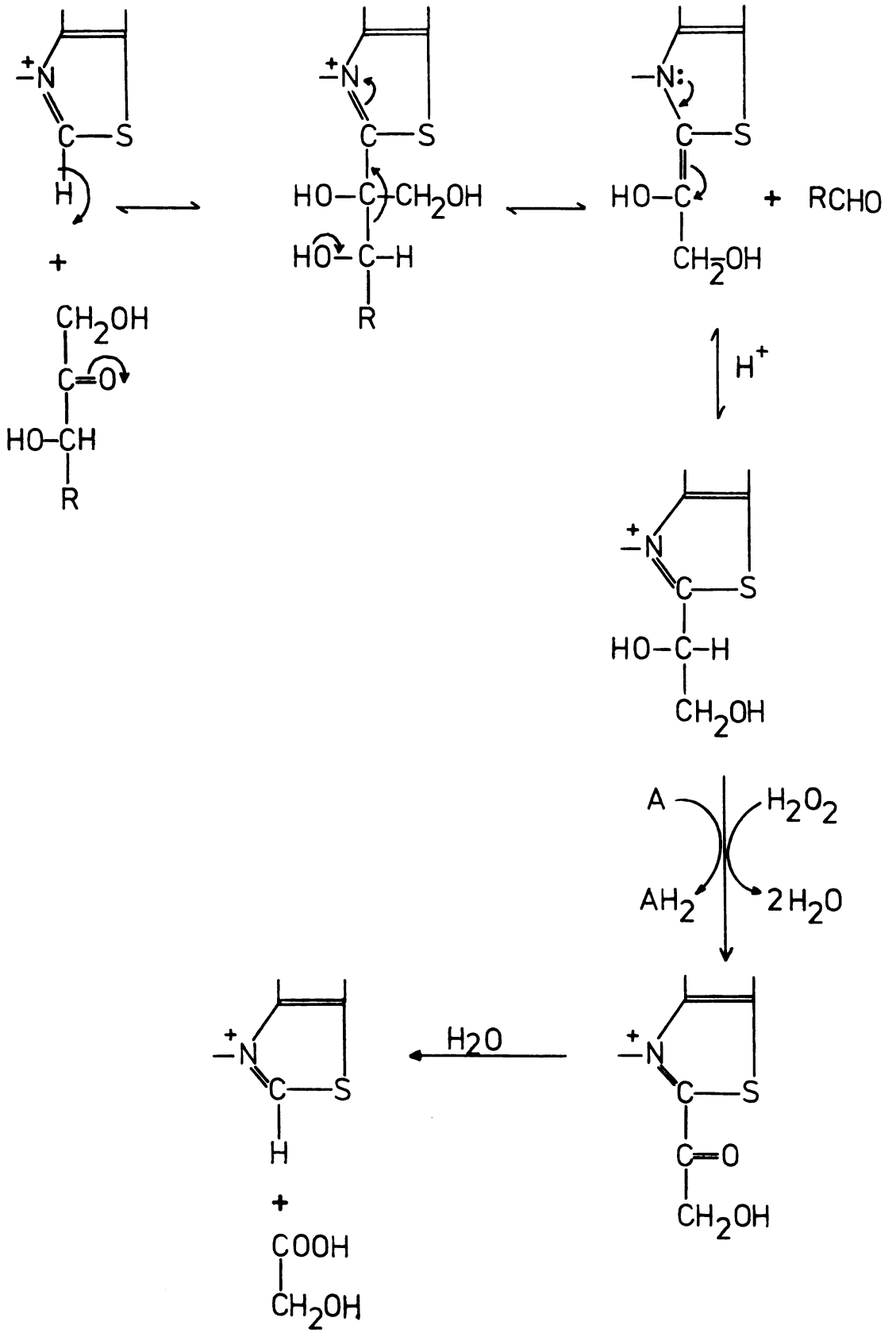
seems unlikely that a compound present in such large quantities could have escaped detection before now if it became labelled during [^{14}C]CO_2 fixation studies. Nothing is known of the formation or metabolism of erythronic acid or its phosphate ester. In any case, isotopic analysis failed to detect any incorporation of ^{18}O into erythronic acid, again suggesting that, in vivo, neither fructose 1,6-diphosphate nor any phosphorylated hexose is subject to a four-electron oxidation by molecular oxygen to yield phosphoerythronate and phosphoglycolate or glycolate.

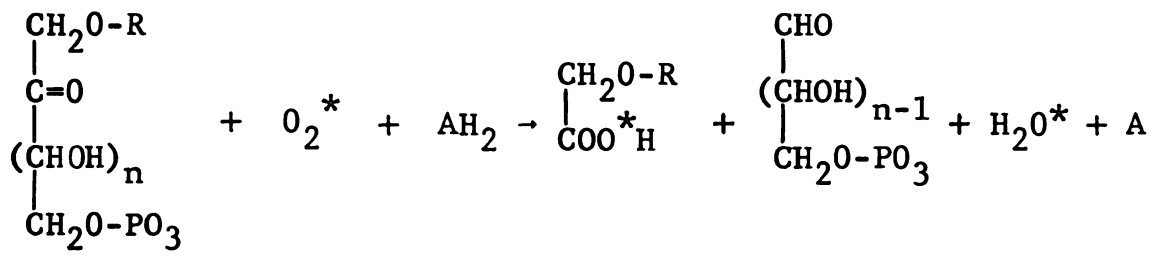
It has been suggested that glycolate is formed as a result of the oxidation of α,β-dihydroxyethylthiamine pyrophosphate formed as an intermediate in the transketolase reaction (Wilson and Calvin, 1955) and it has been further suggested that the oxidant in this process, in vivo, is hydrogen peroxide, generated from oxygen in a Mehler-type reaction with reduced ferredoxin (Coombs and Whittingham, 1966; Plaut and Gibbs, 1970). Indeed, Gibbs (1969) has stated that this reaction would result in the incorporation of an oxygen atom from hydrogen peroxide into the glycolate formed. Since there is no precedent for the formation of a phosphorylated thiamine pyrophosphate addition compound, this mechanism would not account for the formation of phosphoglycolate. The above proposal is based on observations that the α,β-dihydroxyethylthiamine pyrophosphate

is enzymatically oxidized to glycolate in the presence of such electron acceptors as ferricyanide (Bradbeer and Racker, 1961; Holzer and Schröter, 1962) or 2,6-dichlorophenolindophenol (daFonseca-Wollheim et al., 1962). The equation written for the hydrogen peroxide dependent reaction by both Coombs and Whittingham (1966) and by Plaut and Gibbs (1970), in which oxygen appears as a product, does not balance chemically. Assuming that hydrogen peroxide can indeed oxidize dihydroxyethylthiamine pyrophosphate, water rather than oxygen will be formed. In the oxidation of dihydroxyethylthiamine pyrophosphate by ferricyanide, it is thought that the ferricyanide first oxidizes the dihydroxyethylthiamine pyrophosphate to the α-keto derivative which then undergoes spontaneous hydrolysis to yield glycolate and thiamine pyrophosphate (Holzer and Schröter, 1962). If hydrogen peroxide were to act in a similar manner, that is, as an electron acceptor as it does in the peroxidase reaction, the carboxyl oxygen of the glycolate so formed would come from water, not from hydrogen peroxide. For clarity this is shown in Figure 16. For these reasons this mechanism cannot account for the incorporation of ^{18}O into glycolate.

A hypothesis which is consistent with experimental results involves the following reaction which is a two-electron oxidation.

Figure 16. The mechanism of action of transketolase (Mahler and Cordes, 1966) and the proposed mechanism of oxidation of α,β-dihydroxyethyl thiamine pyrophosphate to glycolate and thiamine pyrophosphate.


+
HO–C–CH2OH
HO–C–H
R
HO–C
CH2OH
+ RCHO
CH2OH
C=O
HO–CH
R
H+
HO–C–H
CH2OH
A
H2O2
AH2
2H2O
H2O
H
+
COOH
CH2OH
C=O
CH2OH



R = H or PO_3 and n = 2, 3, or 4.

The reductant, AH_2, might be reduced ferredoxin or $NADPH_2$. A special case of the reaction could involve a preliminary Mehler-type reaction between the reductant and oxygen to produce labelled hydrogen peroxide. This species could then perform the oxidation. The substrate could be any phosphorylated ketose intermediate of the photosynthetic carbon cycle. The products would be (phospho)glycolate and an aldose phosphate which could be glyceraldehyde 3-phosphate, erythrose 4-phosphate or ribose 5-phosphate. The aldehyde oxygen of this aldose phosphate would not retain label since it exchanges with the medium and this compound would be readily absorbed into the photosynthetic carbon cycle. The above reaction might proceed either with or without catalysis by an enzyme.

CHAPTER III

ERYTHRONIC ACID - A [^{14}C]CO_2 WILD GOOSE CHASE

Introduction

Erythronic or threonic acid was found in relatively large quantities in the extracts of spinach leaves exposed to 100% oxygen (Chapter II). This four carbon acid will hereafter be referred to as erythronic acid, although it is recognized that it might instead be threonic acid. Since the extraction procedures described in Chapter II involved treatment with alkaline phosphatase it is possible that this acid exists *in vivo* as 4-phosphoerythronic acid.

Little is known of the formation or metabolism of erythronic acid or of its phosphate ester. It would seem unlikely that a compound present in such large quantities could have escaped detection before now if it became labeled during [^{14}C]CO_2 fixation studies. Kornberg and Racker (1955) and Racker *et al.*, (1959) reported that purified triose phosphate dehydrogenase was capable of oxidizing erythrose-4-P to 4-P-erythronate. The rate of this oxidation relative to that of glyceraldehyde-3-P was not reported. Uehara *et al.*, (1963) have

reported the partial purification from beef liver of an enzyme which catalyses the dismutation of D-tetrose into the corresponding polyol and acid.

Erythrose-4-phosphate is a key intermediate in the Calvin cycle. However, its presence in extracts of plants exposed to $[^{14}C]CO_2$ has proven difficult to demonstrate. It is generally agreed that this is due to the small pool size of erythrose-4-phosphate. There exists only one report (Moses and Calvin, 1958) that demonstrates the labeling of erythrose-4-phosphate during short time (3 min) $[^{14}C]CO_2$ photosynthesis of Chlorella. That report describes two adjacent radioactive spots which when eluted and treated with acid phosphatase, behaved in a different chromatographic manner. The first spot subsequently yielded radioactivity which co-chromatographed with erythrose, glycolic, glyceric and erythronic acids. The amount of ^{14}C incorporated from $[^{14}C]CO_2$ into erythrose-4-phosphate was estimated as being not more than 0.1% of the total incorporation. The second spot subsequently yielded material which co-chromatographed with glycolic, glyceric and erythronic acids. However, it should be stressed that the original spots were some distance removed from the spots assigned to phosphoglyceric and phosphoglycolic acid. These spots overlap. Thus Moses and Calvin (1958) concluded that the labeled erythronic acid probably arose by air oxidation of the erythrose on the paper.

Examination of the photosynthesis chromatography maps (Calvin and Bassham, 1962) reveals that erythronic acid has similar chromatographic properties to aspartate in the solvent systems most commonly used. 4-P-erythronate would be expected to chromatograph in the densely populated region of the sugar phosphates. Although remote, it was possible that erythronic acid or its phosphate ester had been overlooked, and it was decided to determine whether or not erythronic acid became labeled, firstly during photosynthesis in $[^{14}C]CO_2$ and/or subsequently during photorespiration in 100% oxygen. This chapter describes the failure to detect any labeled erythronic acid either during photosynthesis or in the subsequent period of photorespiration.

Materials

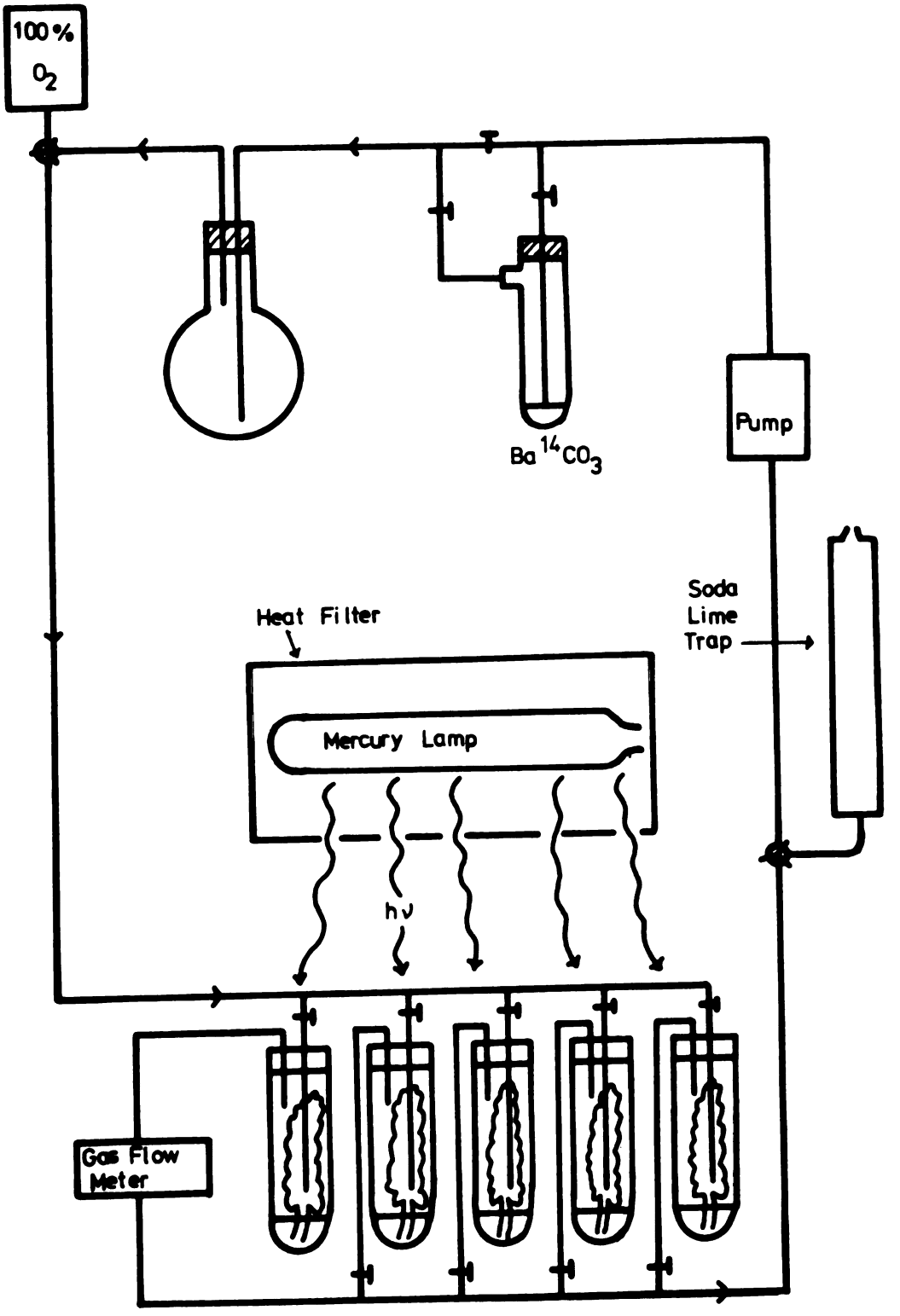
Spinach was grown as previously described (Chapter II). Leaves about 10 cm long and about 2 g fresh weight were used. $[^{14}C]BaCO_3$ (5 μCi/μ mole) was purchased from New England Nuclear Corp.

Methods

The $[^{14}C]CO_2$ Photosynthesis - Photorespiration Pulse Chase Method

A diagramatic representation of the apparatus used is shown in Fig. 17. The volume of the apparatus was about 3 l. Spinach leaves were placed in the tubes

Figure 17. Apparatus for the $[^{14}C]CO_2$ photosynthesis - photorespiration "pulse - chase."


100%
O_2
$Ba^{14}CO_3$
Pump
Soda
Lime
Trap
Heat Filter
Mercury Lamp
hν
Gas Flow
Meter

as shown and equilibrated in a stream of air for about 40 min. The light intensity, measured at the surface of the tubes, was about 3,000 ft. candles. The temperature was 25°. At time zero 100 μmoles $[^{14}C]CO_2$ were released by injection of concentrated lactic acid into the tube containing the $[^{14}C]BaCO_3$. The concentration of CO_2, uncorrected for that originally present, was about 0.07%. Individual leaves were killed by the addition of 30 ml of boiling 90% (v/v) ethanol at times 2.5, 5.0, 6.5, 8.2 and 10.5 min following the release of $[^{14}C]CO_2$. Five and a half min after the release of $[^{14}C]CO_2$, the entire system was flushed with 100% oxygen.

The leaves were extracted as described previously (Chapter II). The extracted leaves were dried to a constant weight in a drying oven. Phase separations were performed on the extracts as previously described (Chapter II). The aqueous fractions thus obtained were used for further fractionation.

Fractionation of the Aqueous Extract

At all stages of the fractionation procedure, aliquots were removed for scintillation counting and in most cases for two dimensional chromatography and autoradiography. The fractionation procedure was essentially the same as that described previously (Chapter II). For purpose of identification, the amino acid

fraction is here defined as that which adheres to Dowex - 50 cation exchange resin and is eluted with 1 M NH_4OH. Similarly, the sugar fraction is defined as that which after treatment with alkaline phosphatase, passes unimpeded through a column of Dowex - 1 anion exchange resin. The organic acid fraction is that which is eluted from the Dowex - 1 column with 4 M acetic acid.

The recovery of radioactivity in each of the fractions obtained by this procedure was essentially quantitative (Table 5).

TABLE 5.--RECOVERY OF RADIOACTIVITY DURING FRACTIONATION

	% of Aqueous Fraction				
Fraction (min)	2.5	5.0	6.5	8.2	10.5
Organic Acids	7	4	2	3	4
Amino Acids	21	25	26	31	32
Sugars	65	68	75	65	63
Total	93	97	103	99	99

Paper Chromatography

An aliquot of each fraction, containing the quantity of radioactivity listed below, was spotted on a 24 x 50 cm sheet of Whatman 3MM Chromatography paper; (total aqueous fraction, 1×10^5 dpm; amino acid fraction, 2×10^4 dpm; sugar fraction, 6×10^4 dpm; organic acid fraction, 5×10^4 dpm). The quantity of

organic acids spotted on the chromatogram was about ten times that present in the aqueous fraction. The chromatograms were developed first in the shorter direction for about 18 hours in phenol (freshly distilled): water (70 : 30 w/w) and then in the longer direction for 16 hours in n-butanol : propionic acid : water (10 : 5 : 7 by vol.). The chromatograms were then dried in preparation for autoradiography.

Autoradiography

Autoradiography was performed for a total of 29 days essentially by the procedures of Benson et al., (1950).

High Voltage Paper Electrophoresis

The procedure of Stevenson (1971) was used. The paper strips were subsequently scanned for radioactivity in a Packard 7201 Radiochromatogram Scanner.

Gas Liquid Chromatography-Mass Spectrometry

The organic acid fractions were evaporated to dryness and silylated as previously described (Chapter II). Two to five μl aliquots of the silylated mixture were analyzed by gas-liquid chromatography-mass spectrometry.

Gas Liquid Chromatography-Radioactivity Measurements

These measurements were performed by analyzing the gaseous eluate from a gas chromatograph equipped with a 2.1m x 0.3cm (i.d.) silanized glass column packed with 1% (w/v) SE-30 on silanized Supelcoport. The temperature programme was as previously described (Chapter II). Radioactivity in the eluate was detected with a Barber-Coleman Radioactivity Monitor, previously optimized with respect to gas flow rate and standardized with $[^{14}C]$ $(Me_3Si)_5$ glucose.

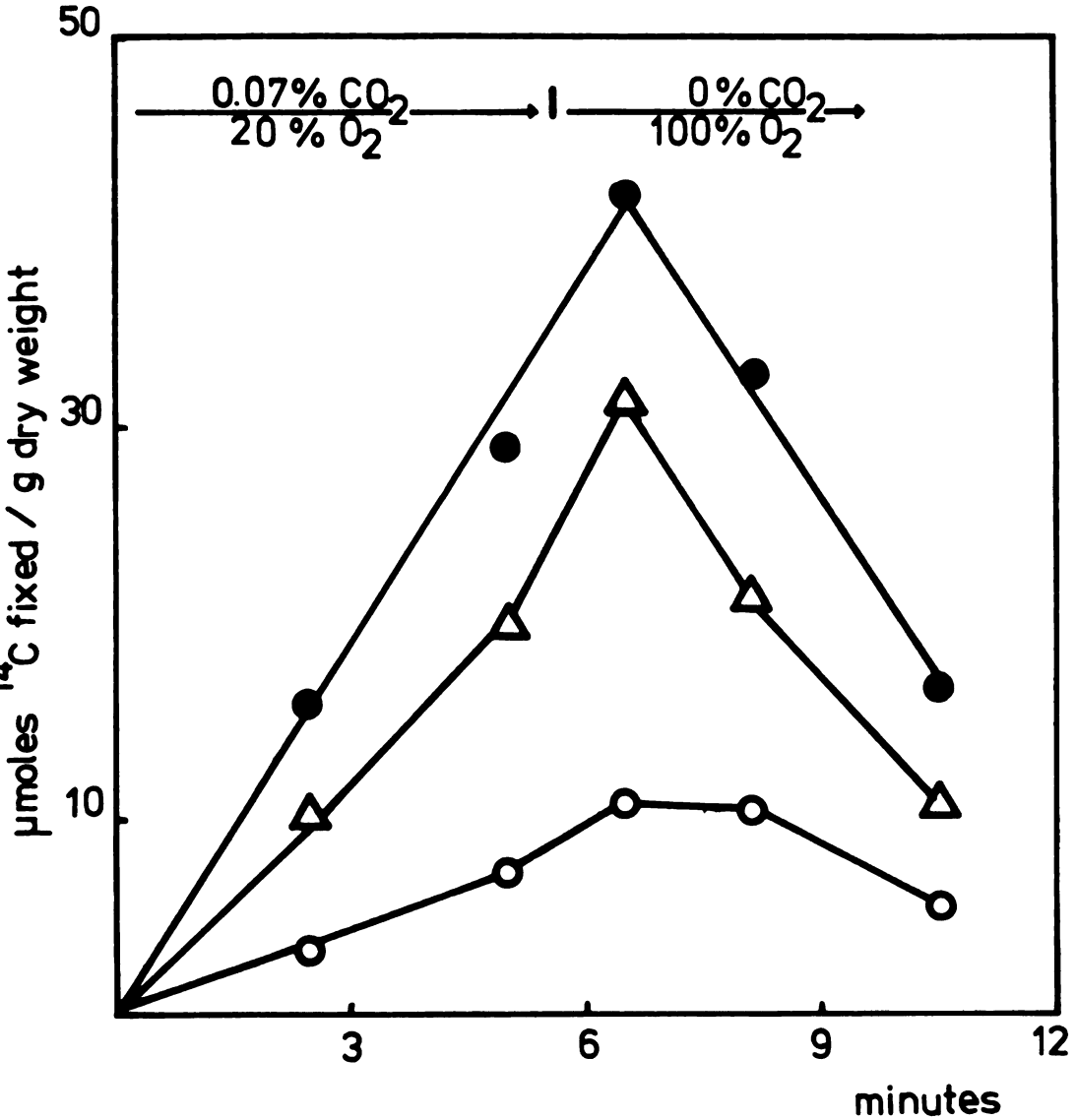
Liquid Scintillation Counting

One to 10 µl aliquots were added to 1.0 ml water followed by 14 ml of scintillation fluid. The scintillation fluid contained 2.0g PPO, 25 mg POPOP, 500 ml toluene and 500 ml Triton X-100. The cross channel ratio method of Herberg (1965) was used to obtain disintegrations per minute.

Results and Discussion

The fixation of $[^{14}C]CO_2$ proceeded linearly at a rate of about 380 µmoles/min/ g dry weight during the period of photosynthesis (Fig. 18). Following the introduction of oxygen, $[^{14}C]CO_2$ was rapidly released at a rate of about 360 µmoles/min/ g dry weight. Thus, under these conditions, the rate of photorespiration is very nearly as great as that of photosynthesis in

Figure 18. The fixation of [^{14}C]CO_2 in photosynthesis and the subsequent photorespiratory loss of ^{14}C. Total fixation ●, fixation into the "sugar fraction" Δ, and fixation into the "amino acid fraction" 0.


50
30
10
0.07% CO2
20 % O2
0 % CO2
100% O2
µmoles 14C fixed / g dry weight
3
6
9
12
minutes

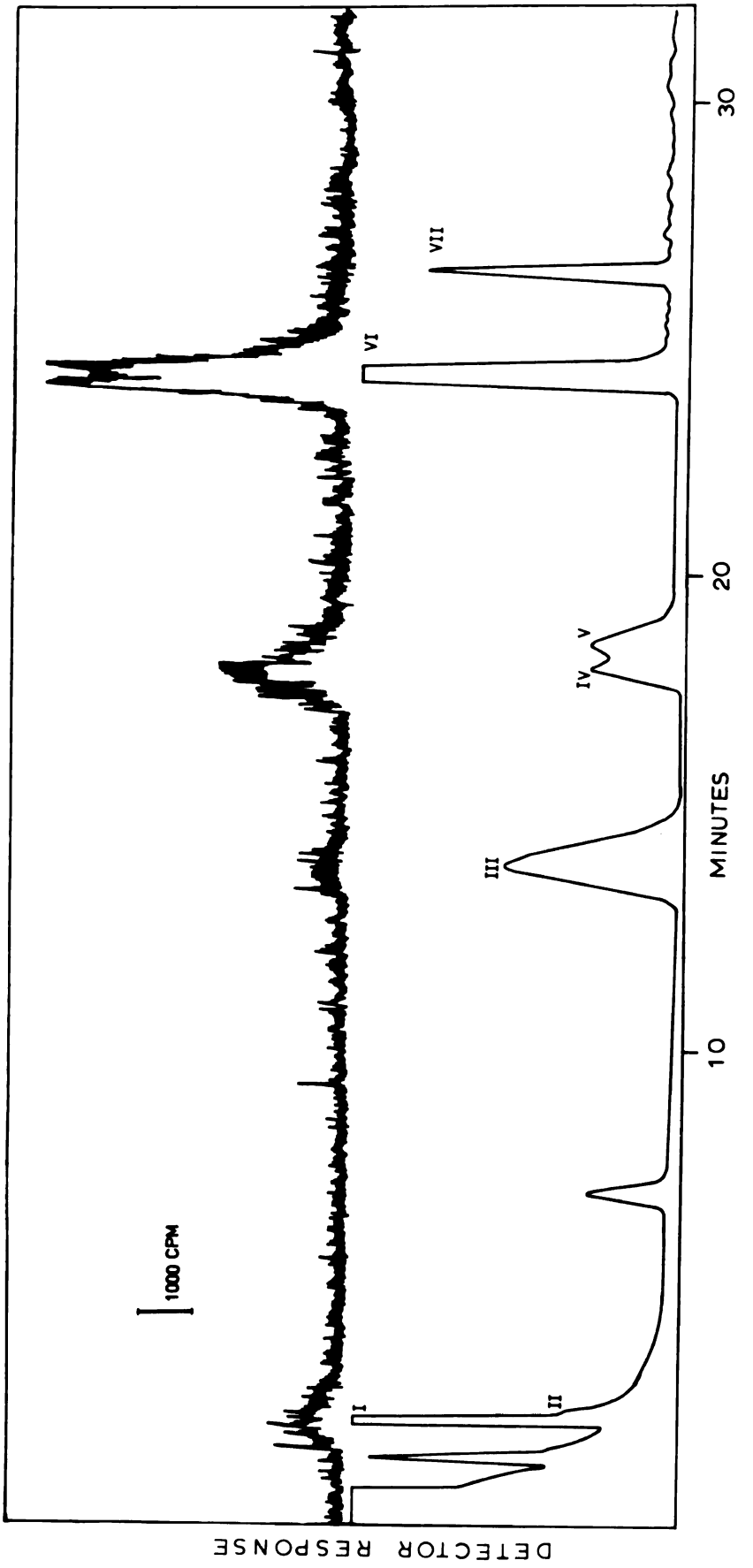
the preceding period. The greatest loss of radioactivity occurred from the sugar fraction.

Paper chromatography, followed by autoradiography, of aliquots of each of the fractions failed to yield any spots which could not be rationalized by the known products of photosynthesis. To ensure that the spot assigned to aspartate did not in addition contain erythronate, it was cut out and eluted with water. The eluate was reduced in volume and subjected to high voltage paper electrophoresis at pH 2.0. An aspartate standard was included. All detectable radioactivity co-electrophoresed with the aspartate standard.

The organic acid fractions were silylated and analyzed by gas-liquid chromatography-mass spectrometry as previously described. The resultant chromatograms were similar to one another and to that reported in Fig. 12. All five samples contained erythronate in approximately the same amount.

The eluate from the gas-liquid chromatograph was analyzed for radioactivity. An aliquot of the silylated organic acids containing about 0.2% of the total ^{14}C fixed or about 10% of the ^{14}C in the organic acid fraction was thus analyzed. The radiochromatogram (Fig. 19) indicates that there was no detectable radioactivity associated with the erythronate peak. The sensitivity of this method would permit the detection of about 0.01% of the total ^{14}C fixed.

Figure 19. Gas-liquid radiochromatogram of the silylated organic acids extracted from a spinach leaf exposed to $[^{14}C]CO_2$ for 5.5 min followed by 100% oxygen for 1 min. The various peaks are as follows: I $(Me_3Si)_2$ oxalate, II probably $(Me_3Si)_2$ glycolate, III unknown (probably contains two compounds), IV $(Me_3Si)_3$ glycerate, V $(Me_3Si)_2$ succinate, VI $(Me_3Si)_3$ malate and VII $(Me_3Si)_4$ erythronate. The lower trace is the gas chromatogram, the upper trace a recording from the radioactivity monitor.


DETECTOR RESPONSE
1000 CPM
I
II
III
IV
V
VI
VII
10
20
30
MINUTES

It was therefore concluded that neither erythronate not its phosphate ester became labeled with ^{14}C under these experimental conditions. Even if erythronate were an artifact formed during the extraction and fractionation procedures, and there is no evidence that eliminates this possibility, the failure to detect any ^{14}C in erythronate precludes the possibility that this compound, or its phosphate ester or the compound from which it is artifactually formed, have a direct role in photosynthetic-photorespiratory carbon metabolism. Further investigation of the metabolism of erythronic acid did not therefore seem warranted.

CHAPTER IV

A MODEL SYSTEM FOR THE OXIDATION OF RIBULOSE DIPHOSPHATE TO PHOSPHOGLYCOLATE

Introduction

The results of the *in vivo* [^{18}O] oxygen experiments described in Chapter II indicated that glycolate biosynthesis involved the incorporation of one atom of oxygen, derived directly or indirectly from molecular oxygen. It was not possible to determine which form of oxygen was the reactive species. However, since the oxygen atom of the hydroxyl free radical, $OH\cdot$, exchanges with water (Kasanowsky *et al.*, 1956), the reactive species might be the superoxide radical $HO_2\cdot$ or hydrogen peroxide. None of the oxygen atoms of $HO_2\cdot$ or H_2O_2 exchanges with those of water (Dole *et al.*, 1952; Cahill and Traube, 1952). Therefore, in addition to molecular oxygen *per se*, these species must be considered as potential candidates. The possibility that the reactive species was singlet oxygen could not be excluded either. Indeed the apparent requirement of light for incorporation of ^{18}O could be interpreted as evidence that an "activated" species of oxygen was the reactive species.

The assumption that a 4-electron oxidation of a

ketose sugar by molecular oxygen would proceed by a concerted mechanism with the incorporation of an atom of oxygen into each of carboxyl groups formed, lead to the conclusion that glycolate formation involved a 2-electron oxidation to yield phosphoglycolate and/or glycolate and an aldose sugar phosphate.

In view of the reactivity of hydrogen peroxide and the free radicals derived from it towards carbohydrates (see review by Moody, 1964) it was decided to investigate the reactions of hydrogen peroxide with intermediates of the C-3 photosynthetic carbon reduction cycle.

Initially it was planned first to investigate the non-enzymatic reactions and then proceed with enzymatic studies. However, before this could be completed, the experiments described in the next chapter became imperative. This chapter then describes some limited studies on manganese catalyzed oxidation of ribulose diphosphate by hydrogen peroxide.

Methods

Preparation of [U - ^{14}C] - Ribulose Diphosphate

Uniformly labeled [^{14}C] ribulose diphosphate was synthesized by the method of Wishnick and Lane (1969) with the following modifications. Uniformly labeled [^{14}C] glucose-6-phosphate (35 μCi/μmole) was used rather than glucose thus dispensing with the hexokinase step.

Secondly the pyruvic kinase-ATP regenerating system was omitted. Thirdly, since the preparations were conducted on a much smaller scale, the RuDP was separated from the other reaction components by paper chromatography (Whatman 3MM) in butanol : propionic acid : water (10:5:7). Unlabeled RuDP was run on both sides of the sample streak. Following chromatography, the RuDP markers were treated by the method of Bandurski and Axelrod (1951). A sample strip was scanned for radioactivity. Generally there were two peaks, the major one co-chromatographing with RuDP. The minor peak was probably sugar monophosphates. The major peak was eluted from the paper with water and lyophilized. The residue was redissolved in 1 ml of water and pH adjusted to neutrality by the addition of Tris base.

To ensure radiochemical purity, a sample was subjected to high voltage paper electrophoresis at pH 3.4 as described by Bieleski and Young (1963). Electrophoresis was conducted for 1.5 hours at 1800 Volts, 25 mA. [^{14}C] glucose-6-phosphate and unlabeled RuDP were included as markers. Following electrophoresis, strips were stained for RuDP and scanned for radioactivity. More than 90% of the radioactivity co-electrophoresed with RuDP. The remainder co-electrophoresed with glucose-6-phosphate. Radiochemical purity was further indicated by the chromatographic behaviour of reaction controls in several solvent systems. The specific

activity of the [U-^{14}C] RuDP was assumed to be the same as the [U-^{14}C] glucose-6-phosphate from which it was made.

Manganese Catalyzed Oxidation of Ribulose Diphosphate by Hydrogen Peroxide

All reactions were performed at 25°. The basic reaction consisted of the following components in a total volume of 250 µl: 10 mM bicine-NaOH, pH 8.4, 10 mM $MnCl_2$, 30 mM hydrogen peroxide and 0.2 mM [U-^{14}C] RuDP (2.3 µCi/ µmole). The reaction was generally allowed to proceed for 30 min or 1 hour before termination by the addition of 75 units of catalase. A control omitting hydrogen peroxide was run concurrently.

The reaction products were identified by paper chromatography in several solvent systems. No one solvent was capable of resolving the products and reactants. Other details are given in the figure legends.

After it became apparent that 3-phosphoglyceraldehyde was a reaction product it was possible to follow the reaction spectrophotometrically by coupling the production of 3-phosphoglyceraldehyde to $NADH_2$ oxidation by the use of triose phosphate isomerase and α-glycerophosphate dehydrogenase. The spectrophotometric assay contained the following components in a total volume of 250 µl: 10 mM bicine-NaOH, pH 8.4, 10 mM $MnCl_2$, 30 mM H_2O_2, 0.15 mM $NADH_2$, 0.8 mM RuDP, 10 units triose

phosphate isomerase and 2.7 units of α-glycerophosphate dehydrogenase.

Results

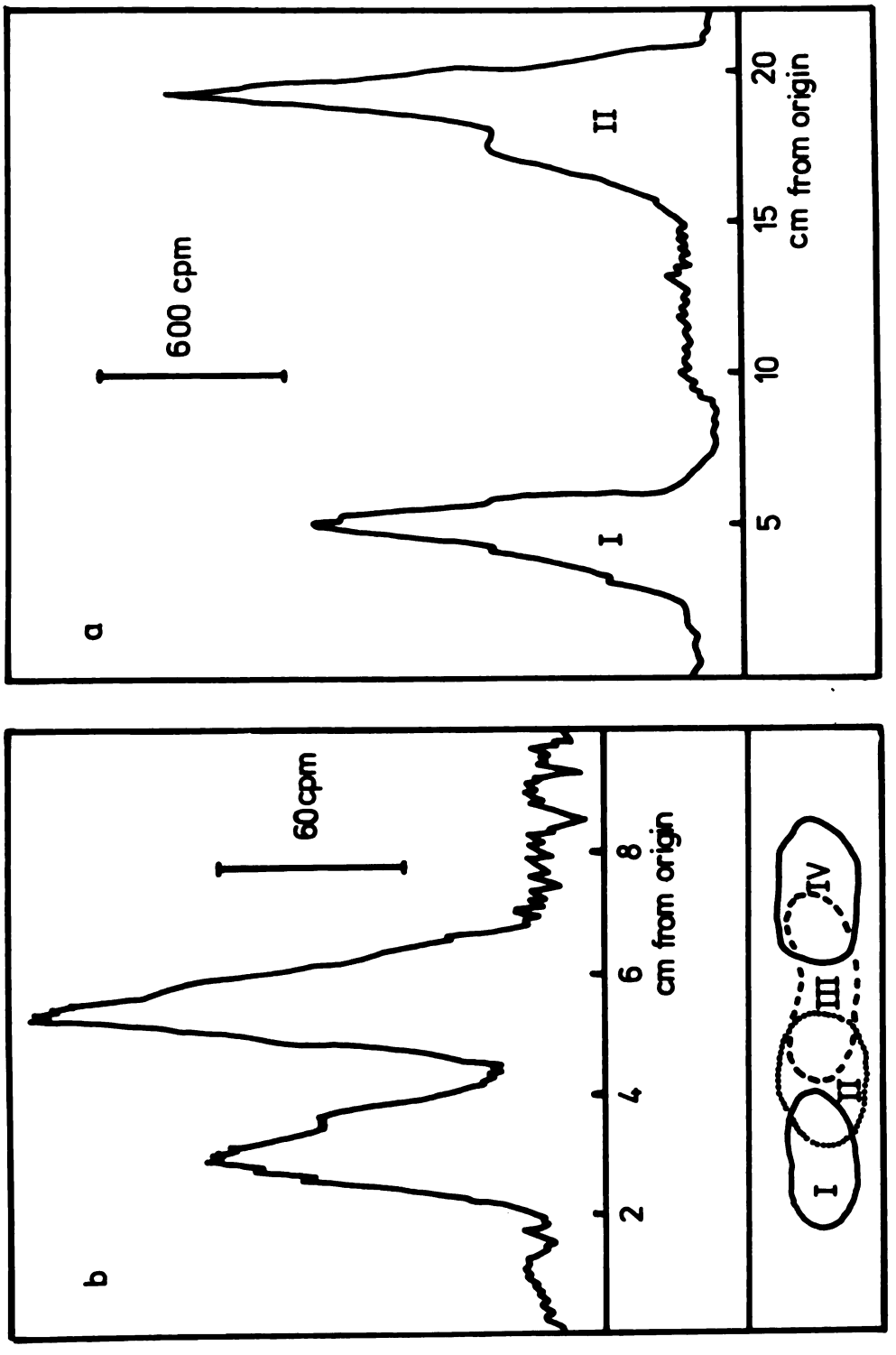
Identification of Reaction Products

A two electron oxidation of RuDP with carbon-carbon cleavage occurring between C-2 and C-3 can conceivably yield 2 sets of reaction products: i.e., 2-phosphoglycolate and 3-phosphoglyceraldehyde and/or 2-phosphoglycolaldehyde and 3-phosphoglycerate. It was not possible to separate all these compounds from one another in any one chromatographic system.

Following the reaction of [U-^{14}C] RuDP with hydrogen peroxide, an aliquot was subjected to paper chromatography in n-butanol : propionic acid : water (10:5:7 by volume) for 24 hours. The 2 and 3 carbon phosphate esters listed above were included as markers. After chromatography the location of the compounds was determined either by use of the phosphate spray reagent of Bandurski and Axelrod (1951) in the case of the markers and by scanning for radioactivity. The resultant chromatogram (Fig. 20a) revealed 2 peaks of radioactivity, the faster moving one having a pronounced shoulder. Peak I corresponded in position to ribulose diphosphate. A decision on the composition of peak II could not be made since the markers all ran in this region. However, the control, [U-^{14}C] RuDP incubated without hydrogen

Figure 20a. Paper chromatography of the reaction products of the Mn/H_2O_2 oxidation of RuDP. The solvent system was butanol:propionic acid:water (10:5:7 by volume). The duration of chromatography was 24 hours. Peak I corresponds to unreacted RuDP, Peak II co-chromatographed with P-glycolate, P-glycolaldehyde, P-glyceraldehyde and P-glycerate.

Figure 20b. Re-chromatography of Peak II of the chromatogram shown in Figure 20a. The solvent system was phenol:water (7:3 by volume). The duration of chromatography was 14 hours. The markers were I P-glycolate, II P-glycerate, III P-glyceraldehyde and IV P-glycolaldehyde.


a
600 cpm
I
II
5
10
15
20
cm from origin
b
60cpm
2
4
6
8
cm from origin
I
II
III
IV

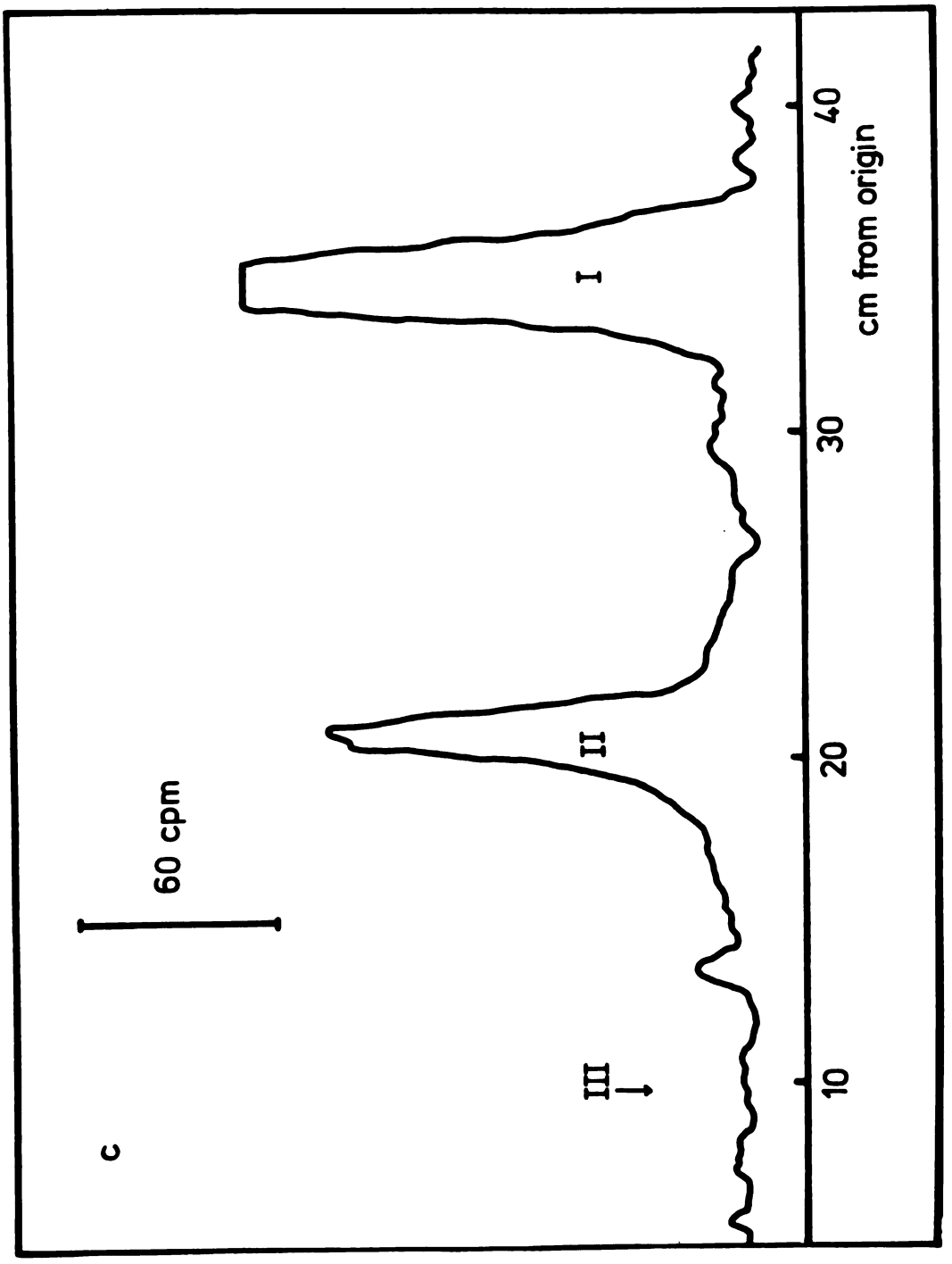
peroxide, showed only one peak, that due to RuDP. Thus, on this alone it can be concluded that at least one of the two sets of reaction products has been formed.

The area of the chromatogram corresponding to peak II was cut out and the radioactivity eluted with water. The solution was reduced in volume and re-chromatographed for 14 hours in phenol : water (7:3 by volume). Markers were included and the resultant chromatogram stained and scanned as before. The chromatogram (Fig. 20b) showed 2 peaks of radioactivity which best corresponded with 2-phosphoglycolate and 3-phosphoglyceraldehyde. Although the other 2 markers overlapped the ratio of radioactivity in the 2 peaks favors assignment to 2-phosphoglycolate and 3-phosphoglyceraldehyde.

In order to confirm that the acid product was 2-phosphoglycolate and not 3-phosphoglycerate, an aliquot of the original reaction solution was treated with alkaline phosphatase and chromatographed for 17 hours in phenol : water as before. [^{14}C] glycolate and glycerate markers were run alongside. Two radioactive peaks were obtained, the faster moving (Peak III, Fig. 20c) corresponding in position to ribulose. This peak probably also contained glyceraldehyde. The slower moving peak (Peak II) co-chromatographed with glycolate. No radioactivity was found to co-chromatograph with glycerate (Fig. 20c).

Further proof that the reaction products were

Figure 20c. Paper chromatography of the reaction products of the Mn/H_2O_2 oxidation of RuDP, after treatment with alkaline phosphatase. The solvent system was phenol:water (7:3 by volume). The duration of chromatograph was 17 hours. Peak I corresponds to unreacted RuDP (and probably contains glyceraldehyde), peak II co-chromatographed with [^{14}C] glycolate. Peak III corresponds to the peak position of [^{14}C] glycerate.


c
60 cpm
III
II
I
10
20
30
40
cm from origin

2-phosphoglycolate and 3-phosphoglyceraldehyde was obtained from the spectrophotometric assay. The requirements for the reactions are given in Table 6. The results indicate that the reaction requires RuDP, $MnCl_2$ and hydrogen peroxide. Equimolar concentrations of magnesium do not substitute for manganese. The oxidant is clearly not oxygen since catalase completely inhibited the reaction. Furthermore the requirement for both coupling enzymes for the oxidation of $NADH_2$ demonstrates that the product of the RuDP oxidation is indeed 3-phosphoglyceraldehyde.

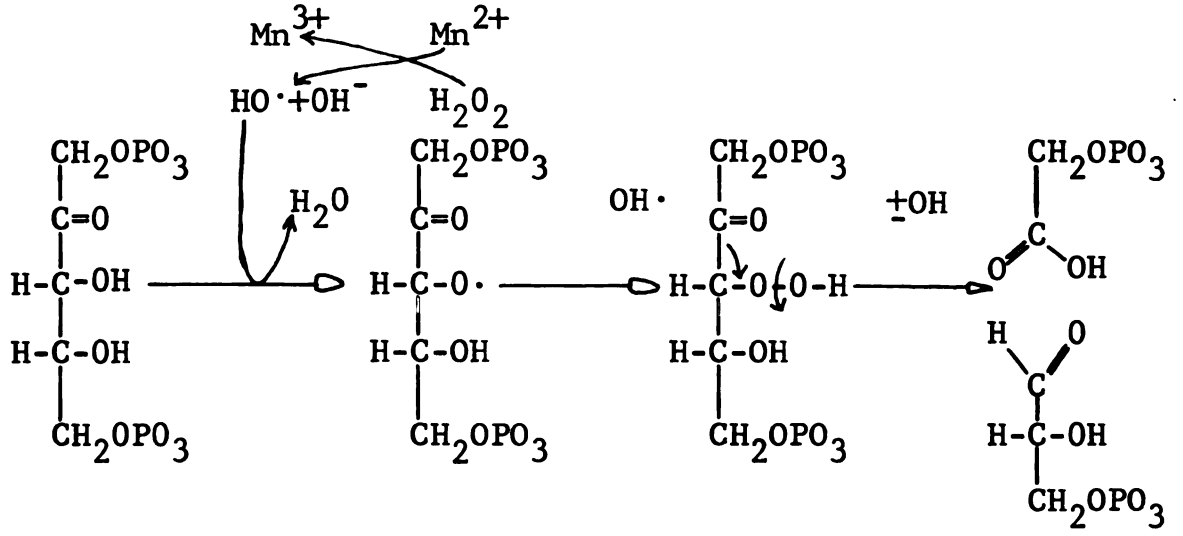
TABLE 6.--REACTION REQUIREMENTS FOR THE MANGANESE CATALYSED OXIDATION OF RIBULOSE DIPHOSPHATE BY HYDROGEN PEROXIDE

Reaction	P-glyceraldehyde Assay n moles $NADH_2$ oxidised / min.
Complete	1.15
- RuDP	0.12
- $MnCl_2$	0.32
- $MnCl_2$ + 0.01 M $MgCl_2$	0.32
- TPI, or - α GPD	0
Complete + 152 units catalase	0

The complete reaction is described in the Methods section.

The requirement for manganese ions in the hydrogen peroxide dependent oxidation of RuDP strongly suggests that the oxidation proceeds by a free radical mechanism.

A plausible mechanism is shown below. However, apart from the nature of the reaction products, there is no experimental evidence to support this mechanism or, for that matter, to exclude other mechanisms.



CHAPTER V

THE ENZYMATIC OXIDATION OF RIBULOSE DIPHOSPHATE BY MOLECULAR OXYGEN

Introduction

The results of the *in vivo* $^{18}O_2$ experiments described in Chapter II indicated that glycolate biosynthesis involved the incorporation into the carboxyl group of one atom of oxygen, derived directly or indirectly from molecular oxygen. In a brief communication Bowes *et al.*, (1971), reported that a purified preparation of soybean ribulose diphosphate carboxylase catalysed the oxidation of ribulose diphosphate by molecular oxygen to yield P-glycolate and, they assumed, P-glycerate. This chapter confirms and considerably extends that initial observation with a purified preparation of spinach ribulose diphosphate carboxylase. The reaction to be considered is:

Ribulose diphosphate + O_2 → P-glycolate + P-glycerate

The reaction was catalysed by purified preparations of RuDP carboxylase, but the oxidative reaction is hereafter referred to as RuDP oxygenase.

The experiments described in this chapter were

part of a cooperative project with Dr. T. John Andrews.

Materials

Spinach was grown as previously described in Chapter I. DEAE-cellulose (0.66 meq.g^{-1}), hydroxylapatite (Bio-Gel HT) and Dowex - A950W -XZ (200-400 mesh) were obtained from Bio-Rad Laboratories. Sephadex G-25, medium, was a product of Pharmacia. All commercial preparations of enzymes were obtained from Sigma Chemical Co. The barium salt of 3-PGA was obtained from Boehringer-Mannheim Corp. and converted to the Tris salt by treatment with Dowex 50^{+} form and adjustment to neutrality with Tris base. 2-P-glycolate was from General Biochemicals. Silylating reagents were obtained from Regis Chemical Co. All other chemicals were from Sigma Chemical Co.

[^{14}C] $NaHCO_3$ was obtained from Amersham Searle Corp., [^{14}C] fructose diphosphate from New England Nuclear Corp., and [^{14}C] glucose-6-phosphate and [^{14}C]-fructose-6-phosphate from Calatomic. [^{14}C]-ribulose-diphosphate was prepared (Chapter IV). [^{18}0] oxygen (93.5 atoms %) and [^{18}0] water (20.6 atoms %) were obtained from Miles Laboratories.

Methods

Exploratory Assays

In developing an assay, it was decided to concentrate

upon methods which would reflect oxygenation and which could not possibly be confused with carboxylation. Methods based on the disappearance of RuDP or appearance of P-glycerate were bypassed, since carboxylation would clearly interfere with either. Attempts were made to develop assays for glycolate which could be formed from P-glycolate by the action of alkaline phosphatase. They were discarded due to lack of sensitivity, interference by ribulose and/or glycerate or non-stoichiometric conversion. For example, ribulose interfered with the colorometric Calkin's test for glycolate and with the phenylhydrazone assay of glyoxylate. Attempts were then made to assay oxygen consumption. For economic reasons (RuDP costs \$1.50 mg^{-1}) reaction volumes had to be kept to a minimum. When a one ml solution was equilibrated with 100% oxygen and oxygen consumption measured with a standard Clark oxygen electrode, back diffusion of oxygen into the electrode chamber resulted in an unacceptably high blank rate.

RuDP Oxygenase Assay

A manometric method was used in most of this work, even though this method has several disadvantages. Single side arm Warburg flasks with a total volume of about 3 ml were used. The Gibson Constant Pressure Respirometer was run at 160 shakes min^{-1}. During initial investigations the temperature was 30^{o}. Subsequently it

was found that by operating at 25°, a temperature nearer to ambient, the large pressure fluctuations associated with tipping in the contents of the side arm could be minimized. The apparatus was equipped with Tygon tubing which was permeable to oxygen and thus it was not ideally suited for use with 100% oxygen. However, by flushing the apparatus with 100% oxygen for two to three hours prior to use, the blank rate due to leakage of oxygen was substantially reduced. The reason for this effect is not known.

The standard reaction mixture contained the following components in a total volume of 1.0 ml: in the flasks, 100 μmoles ammediol-Cl, pH 9.3, 10 μmoles $MgCl_2$, 1 μmole EDTA, 0.4 μmoles dithiothreitol and enzyme (commonly 1 to 2 mg protein) to give a rate of oxygen consumption of between 2 and 8 μl per min, and in the side arm 2 μmoles RuDP. With the side arm vent open, the system was flushed with humidified 100% oxygen for 3 minutes. The oxygen supply was turned off, the vent closed and the system allowed to equilibrate for a further 9 min. The reaction was then initiated by tipping in the RuDP. Readings were taken at 90 sec intervals. A water blank or a reaction blank (less enzyme or with boiled enzyme) was run concurrently. The values reported are corrected to standard temperature and pressure.

RuDP Carboxylase Assays

(a) ^{14}C Assay: The reaction contained the following components in a total volume of 250 µl: 25 µmoles Tris-Cl, pH 7.80, 2.5 µmoles $MgCl_2$, 0.015 µmoles EDTA, 1.25 µmoles dithiothreitol (freshly prepared), 12.5 µmoles [^{14}C] $NaHCO_3$ (0.72 µc/µmole), 0.125 µmoles RuDP and sufficient enzyme to give rates less than about 16,000 dpm per min of enzyme reaction. The enzyme was preincubated at the reaction temperature of 30° for 10 min before initiating the reaction with RuDP. The reaction was terminated after 1 min by the addition of 0.5 ml 2N HCl. The solution was then quantitatively transferred to a glass scintillation vial and evaporated to dryness in an oven at 95°. To ensure complete removal of the unreacted $^{14}CO_2$, an additional 0.5 ml 2N HCl was then added and the samples redried. After cooling, 1.0 ml water was added followed by 14 ml of the scintillation cocktail (Chapter III). The cross channel ratio method of counting was used. The rate of reaction was proportional to enzyme concentration. However, the progress of the reaction was not consistently linear but appeared to depend upon factors related to the state of purity of the preparation and the previous treatment of the enzyme. This may be related to the activation phenomena reported by Pon et al., (1963). In general, activity declined after a short time. Since this was not the primary purpose of this study, it was not extensively

investigated. In order to avoid this non-linearity, the reaction was terminated after 1 min.

(b) Spectrophotometric Assay: The reaction contained the following components in a volume of 250 µl: 25 µmoles Tris-Cl, pH 7.80, 2.5 µmoles $MgCl_2$, 0.015 µmoles EDTA, 1.25 µmoles dithiothreitol (freshly prepared), 12.5 µmoles $NaHCO_3$, 0.125 µmoles NADH, 2.5 µmoles ATP, 10 µl of coupling enzyme containing 1.07 units yeast P-glycerate phosphokinase, 1.17 units rabbit muscle P-glyceraldehyde dehydrogenase, 1.00 unit yeast triose phosphate isomerase and 0.90 unit rabbit muscle α-glycerophosphate dehydrogenase, and sufficient carboxylase to give a reaction rate not exceeding 2.0 nmoles/min. The temperature was 25°. The enzyme was preincubated for 10 min before initiating the reaction by addition of RuDP. A reaction blank less carboxylase was routinely included. The rate of reaction was proportional to enzyme concentration. A rapid non-linear rate lasting about 2 minutes was followed by a rate which was linear for the succeeding 10 min. The rapid non-linear rate was also observed in the reaction blank. The rate was determined from the linear portion of the reaction.

A mixture of the coupling enzymes for the carboxylase assay was prepared in the following manner. Fifty µl yeast P-glycerate phosphokinase containing 160 units, 125 µl rabbit muscle P-glyceraldehyde dehydrogenase containing 175 units, 15 µl yeast triose-P-isomerase

containing 150 units, and 100 µl rabbit muscle α-glycerophosphate dehydrogenase containing 134 units were added to 710 µl 0.05 M Tris-Cl, pH 7.7, 0.002 M EDTA and 0.005 M DTT. $(NH_4)_2SO_4$, which inhibits the carboxylase reaction, was removed by desalting on a 0.7 x 15 cm column of Sephadex G-25 equilibrated with 0.05 M Tris-Cl, pH 7.7, 0.002 M EDTA and 0.005 M DTT. The enzymes were recovered in 1.5 ml and stored under N_2 at 4^o. Under these conditions the coupling system was active for at least 2 weeks.

Standardization of [^{14}C] $NaHCO_3$

A 0.35 ml aliquot of [^{14}C] Na_2CO_3 (ostensibly 58 µCi/µmole) was added to 1.65 ml 0.303 M $NaHCO_3$. To a scintillation vial was added 0.1 ml 1 M Hymamine hydroxide in methanol and 1 µl of the diluted [^{14}C] $NaHCO_3$. This was then followed by 0.9 ml water and 14 ml of scintillation cocktail (Chapter III). Quadruplicate samples were prepared. A dummy vial containing no added ^{14}C was prepared since phosphorescence generated by the scintillant in alkaline solution caused spurious counts. The sample vials were counted after the activity in the dummy vial had declined to normal background levels. They were recounted 2 or 3 hours later to ensure that they too had reached a constant count rate.

Standardization of RuDP

Stock solutions of RuDP were standardized by the ^{14}C carboxylase assay and also by the spectrophotometric carboxylase assay. Both methods involved the use of excess amounts of carboxylase. The RuDP from Sigma was found to be 85% pure by weight. The RuDP synthesised by Paulsen and Lane (1966) was 63% pure by weight. Spectrophotometric analysis of the RuDP revealed some UV absorbing material, with a peak at 260 nm. This was most probably adenine nucleotides, which are used in the enzymatic synthesis of RuDP. On a molar basis it amounted to no more than 1% contamination.

Protein Determination

Protein was determined spectrophotometrically at A_{280} and A_{260} against appropriate buffer blanks. The formula suggested by Layne (1957) was used.

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

Polyacrylamide Gel Electrophoresis

Fifty to 100 µl aliquots of the purified preparation were subjected to polyacrylamide gel electrophoresis by the method of Davis (1964) on 5.5% or 6% gel as indicated. The samples were applied as a solution containing 10% glycerol. Electrophoresis was performed at 5 ma/tube for a period of time corresponding to twice the time taken for the tracker dye to run through. The gels were stained for a minimum of 3 hours

in 0.5% (v/v) Amido Schwartz in 7.5% (v/v) acetic acid and destained in 7.5% (v/v) acetic acid.

Analytical Ultracentrifugation

Ten ml of the uniformly suspended $(NH_4)_2SO_4$ precipitated enzyme was centrifuged at 27,000 g for 15 minutes. The precipitate was dissolved with 1.0 ml of 0.025 M glycylglycine, pH 7.7, 0.10 M KCl and 0.001 M DTT, and the resultant solution desalted on a 0.7 x 15 cm column of Sephadex G-25 equilibrated with the same buffer. Analytical ultracentrifugation was conducted in a Spinco Model E Analytical Ultracentrifuge at 3.9° and a protein concentration of 11.6 mg/ml. After a speed of 50,740 rpm was obtained, photographs were taken at 4 min intervals. The phase angle was 70°.

Identification of Reaction Products by Mass Spectrometry

Following the reaction, the solution was transferred to a test tube and frozen until use. After thawing the tube plus contents were held in a boiling water bath for about 40 sec to precipitate the protein. (It was not necessary to remove the coagulated protein since most of it adhered to the test tube and what little was carried over was trapped at the top of the ion exchange column.) After cooling, the solution was applied to a 0.5 x 4 cm column of Dowex 50 H^+ form, in order to remove the buffer and Mg^{2+}. The column was washed with 1.5 ml water and the eluate evaporated to dryness. To

ensure anhydrous conditions 0.1 ml of absolute ethanol was added and re-evaporated to dryness. Forty μl BSTFA containing 1% (v/v) trimethylchlorosilane were added to the dried residue and heated at 110° for 10 min to facilitate the silylation reaction. Standards containing P-glycolate and P-glycerate (2.5 μmoles/ml) were similarly prepared.

Aliquots (0.5 to 1 μl) of the above silylated sample were analyzed using an LKB-9000 combined gas chromatograph - mass spectrometer, equipped with a 1.4 m x 3 mm (i.d.) silanized glass column packed with 3% (w/v) SE-30 on silanized Supelcoport (100-200 mesh, Supelco Inc., Bellefonte, Pa.). The column temperature was 150° and the flash heater temperature was 170°. The flow rate of the helium carrier gas was 30 cc/min. The temperature of the ion source was 290° and the ionizing voltage 70 eV.

Results

The report of Bowes et al., (1971) that a purified preparation of soybean RuDP carboxylase catalysed the formation of P-glycolate from RuDP was confirmed. Details of these experiments are in manuscript (Andrews, Lorimer and Tolbert). A sample of soybean RuDP carboxylase, purified by the method of Paulsen and Lane (1966), was kindly furnished by Dr. W. L. Ogren, USDA Soybean Laboratory, Urbana, Ill. [^{14}C]- RuDP prepared

as described in Chapter IV was incubated with this enzyme in the presence of 100% oxygen. Treatment of the products with alkaline phosphatase was followed by paper chromatography with n-pentanol saturated with 5 M formic acid. Besides unreacted [^{14}C]- ribulose, 2 additional radioactive products were formed which co-chromatographed with marker [^{14}C] glycolate and [^{14}C] glycerate. No glycolate formation occurred under 100% N_2. In addition the reaction appeared to occur much more rapidly at pH 9.0 than at pH 7.8.

Purification of RuDP Carboxylase from Spinach Leaves

All operations were performed at 0 to 4°C. At each stage of the purification a 1.0 ml aliquot was removed and applied to a 0.7 x 15 cm column of Sephadex G-25 (medium) equilibrated with 0.025 M Tris-Cl, pH 8.0 and 0.001 M DTT, in order to remove phenolic material and $(NH_4)_2SO_4$ which interfere with the protein and carboxylase assays respectively. Prior to use, the pH of the saturated $(NH_4)_2SO_4$ was adjusted with NH_4OH so that a 1:5 dilution gave a pH of 7.3.

Initial Enzyme Extraction: About 100 g (fresh weight) washed, deribbed spinach leaves were homogenized for 45 sec in a Waring Blender with 250 ml 0.1 M Tris-Cl, pH 8.0, 0.002 M EDTA and 0.05 M mercaptoethanol. The brei was filtered through 2 layers of cheesecloth and one layer of "Mirah cloth" and the filtrate centrifuged

at 18,000 g for 30 min. The clear yellow supernatant solution constituted the initial extract.

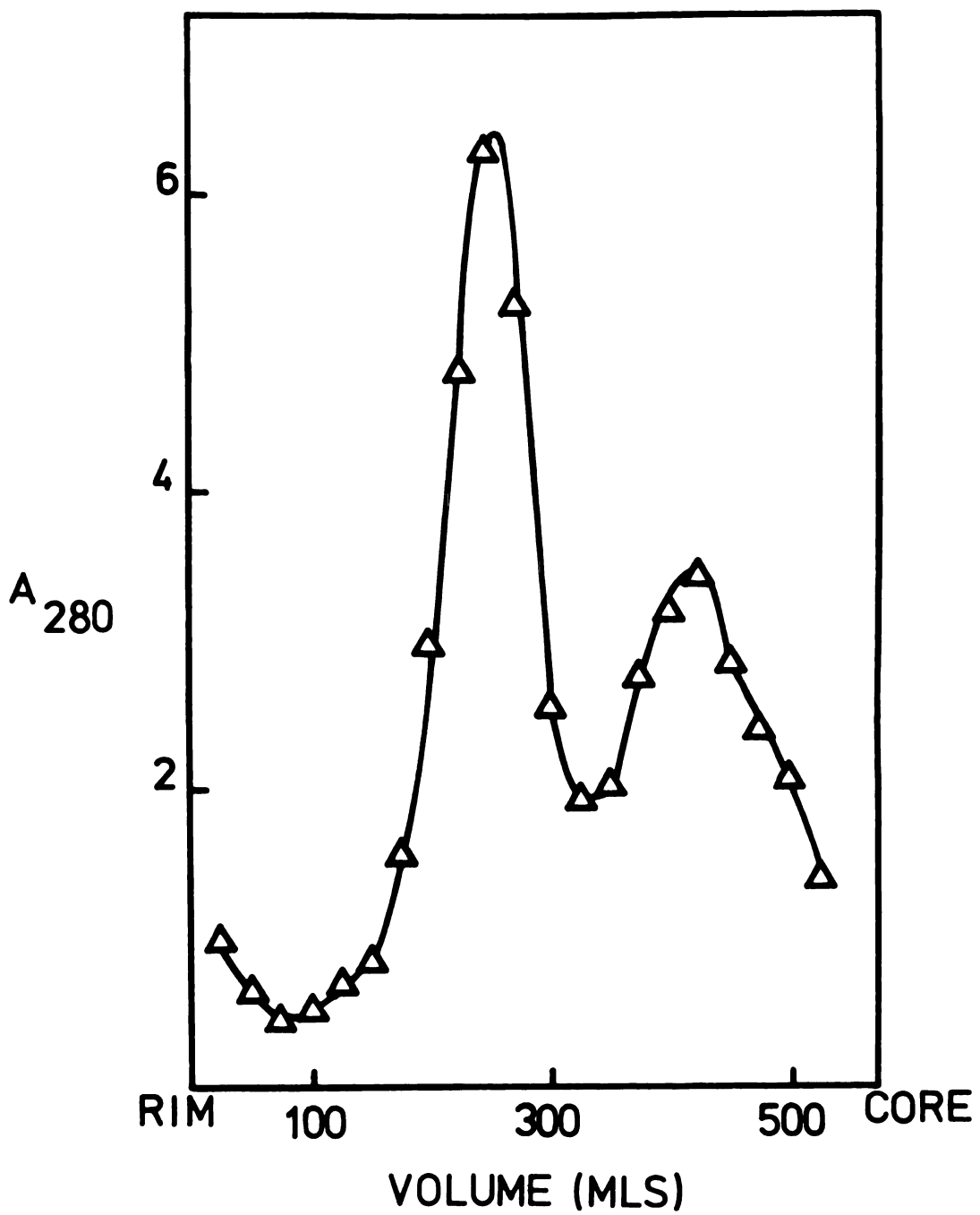
$(NH_4)_2SO_4$ Fractionation: Sufficient saturated $(NH_4)_2SO_4$ was added to the initial extract to give 30% saturation. After standing for 30 min the suspension was centrifuged at 18,000 g for 10 min and the precipitate discarded. The supernatant solution was brought to 50% saturation by the addition of saturated $(NH_4)_2SO_4$. After standing for 30 min the suspension was centrifuged at 18,000 g for 30 min. The enzyme was stored as the precipitate overnight, and redissolved with about 15 ml 0.1 M Tris-Cl, pH 8.0, 0.002 M EDTA and 0.05 M mercaptoethanol. Insoluble material was removed by centrifugation at 18,000 g for 20 min. This supernatant solution constituted the $(NH_4)_2SO_4$ fraction.

Zonal Centrifugation: The density of the $(NH_4)_2SO_4$ fraction was adjusted by dilution with buffer so that it was just less than that of 12.5% (w/w) sucrose. The B-30 zonal rotor (International Equipment Co.) was used. With the rotor spinning at about 2,500 rpm, 60 ml of buffer were pumped into the rotor via the rim line at a rate of about 10 ml/min. The enzyme solution was then pumped into the rotor, followed by a 520 ml linear (by volume) gradient from 12.5% to 30% (w/w) sucrose in 0.05 M potassium phosphate pH 7.60, 0.1 mM EDTA, 0.001 M DTT. This displaced the enzyme solution inwards towards the core. The gradient was then developed at 50,000 rpm for

5 hours. After decelerating to about 2,500 rpm the gradient was displaced out of the rotor via the rim line by pumping distilled water into the core. 25 ml fractions were collected. Alternative methods of loading and unloading the rotor have also been attempted without significantly decreasing the half-band width of the carboxylase. The results of the zonal centrifugation step are shown in Fig. 21. RuDP carboxylase constitutes such a large proportion of the soluble protein of the leaf, that it is not always necessary to assay for carboxylase activity. Experience showed that the largest peak of protein invariably contained the carboxylase. Thus, the fractions indicated in Fig. 21 were pooled solely on the basis of absorbance. The enzyme was precipitated by the addition of 2 volumes of saturated $(NH_4)_2SO_4$ to bring the solution to 66% saturation. The suspension was centrifuged at 18,000 g for 20 min and the precipitate dissolved with 15 ml 0.005 M potassium phosphate, pH 7.60, containing 1 mM DTT. The resultant solution was desalted on a 2.3 x 2.5 cm column of Biogel P-6 equilibrated with the same buffer. The desalted enzyme was designated the zonal centrifuge fraction.

Hydroxylapatite Column Chromatography: The zonal centrifuge fraction was applied to a 1.6 x 11 cm column of hydroxylapatite, equilibrated with 0.005 M potassium phosphate, pH 7.60 containing 1 mM DTT. The column was then washed with 40 ml of this buffer followed

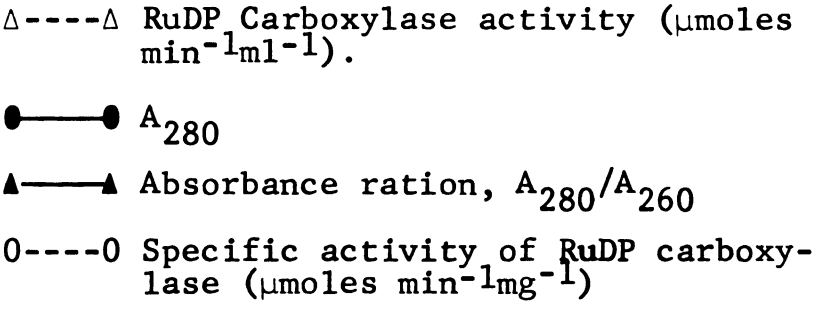
Figure 21. Zonal Centrifugation of RuDP Carboxylase after $(NH_4)_2SO_4$ Fractionation. The fractions indicated were pooled on the basis of the absorbance at 280 nm. Previous experiments indicated that this peak contained the carboxylase activity.


6
4
2
A 280
RIM
100
300
500
CORE
VOLUME (MLS)

by a 120 ml linear potassium phosphate gradient from 0.005 M to 0.050 M. The flow rate was about 15 ml/hr and 10 ml fractions were collected. The results are shown in Fig. 22. Fractions containing more than 5 μmoles/min of carboxylase activity were pooled. An aliquot was removed for assay and the remainder precipitated by the addition of 2 volumes of saturated $(NH_4)_2SO_4$. EDTA and mercaptoethanol were then added to produce final concentrations of 0.1 and 5.0 mM respectively. The suspension was stored at 0 to 4^o. Before use, an aliquot was withdrawn and centrifuged at 10,000 g for 10 min. The precipitate was dissolved with 1.0 ml 0.25 M glycylglycine at pH 8.6, 0.01 M EDTA and 0.01 M DTT and desalted on a 0.7 x 15 cm column of Sephadex G-25 equilibrated with the same buffer. A summary of two such purifications is shown in Table 7.

The specific activity of the carboxylase was marginally less than the value of 1.4 μmoles $min^{-1}mg^{-1}$ reported by Lane's group (Paulsen and Lane, 1966 and Wishnick and Lane, 1971). Note that in order for a comparison of the specific activities to be made, the values reported in Table 7 should be multiplied by 1.92 to correct for differences in protein determination. The corrected specific activities were 1.25 μmoles min^{-1} mg^{-1} for preparation A and 1.39 μmoles $min^{-1}mg^{-1}$ for preparation B. The specific activity of the carboxylase was constant (within experimental error) across the

Figure 22. Hydroxylapatite Column Chromatography of RuDP Carboxylase.



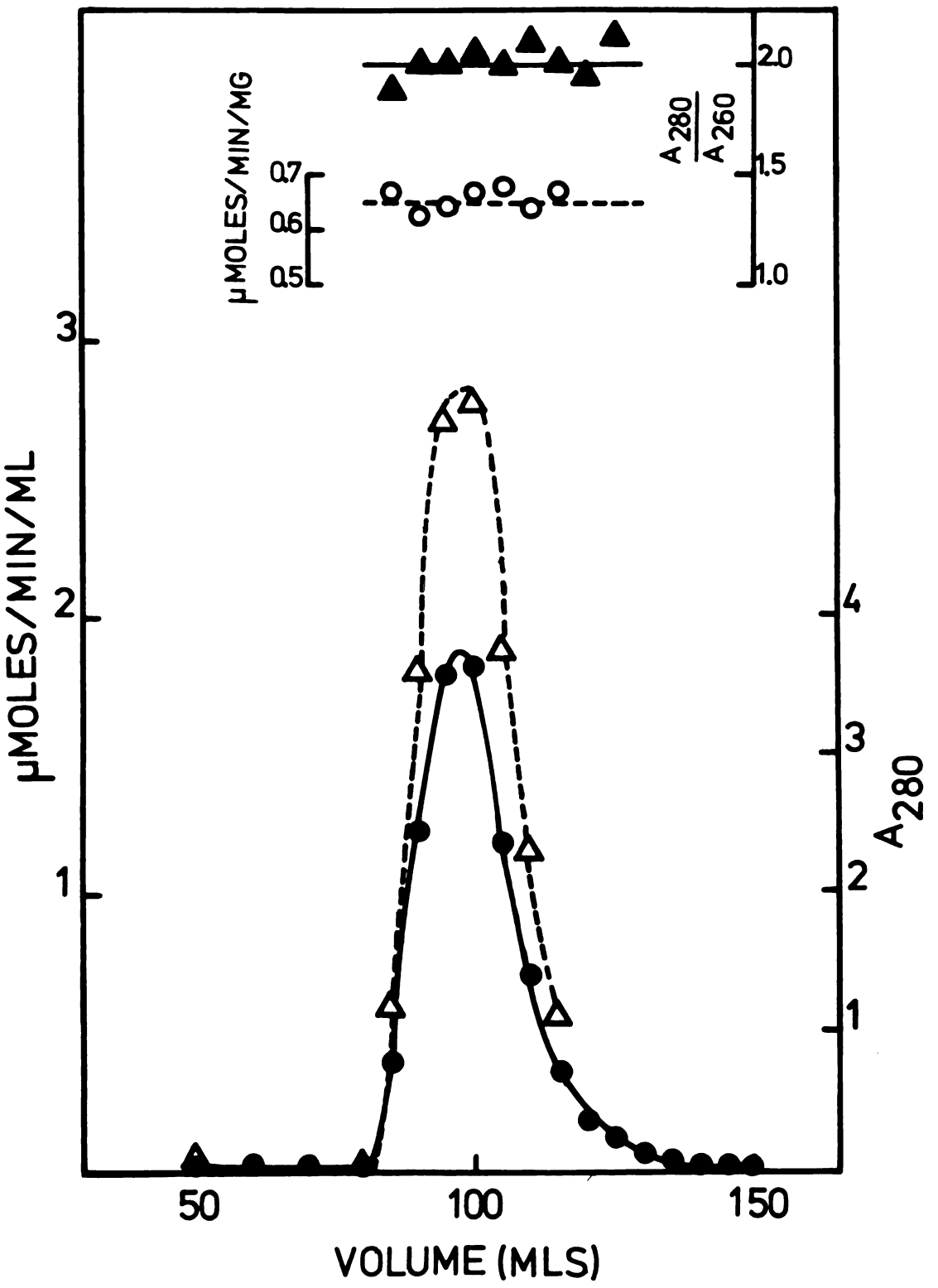

μMOLES/MIN/MG
0.7
0.6
0.5
A280/A260
2.0
1.5
1.0
μMOLES/MIN/ML
3
2
1
A280
4
3
2
1
50
100
150
VOLUME (MLS)

TABLE 7.--PURIFICATION OF RuDP CARBOXYLASE FROM SPINACH LEAVES

	Protein mg	Ratio 280/260	μmoles min^{-1}	μmoles $min^{-1}mg^{-1}$ Protein	Yield %
Preparation A					
Initial Extract	1715	0.9	343	0.20	100
30-50% $(NH_4)_2SO_4$ Fractionation	790	0.8	330	0.42	96
Zonal Centrifugation	286	1.8	169	0.58	49
Hydroxylapatite*	178	2.0	115	0.65	34
Preparation B					
Initial Extract	1280	0.9	565	0.44	100
35-50% $(NH_4)_2SO_4$ Fractionation	662	0.9	360	0.55	64
Zonal Centrifugation	396	1.1	275	0.69	49
Hydroxylapatite**	242	2.0	174	0.72	31

*Column developed by a linear phosphate gradient.

**Column developed by a step gradient with 0.005 M potassium phosphate at pH 7.6 followed by 0.025 M phosphate at pH 7.6.

peak obtained from the hydroxylapatite column. The absorbance ratio, A_{280}/A_{260}, was also constant. An absorbance ratio of 1.95 to 2.00 is a characteristic, but not unique, feature of purified RuDP carboxylase. Additionally, polyacrylamide disc gel electrophoresis of the purified enzyme revealed only one band of protein (Fig. 25a).

Preparations of the carboxylase purified by this method catalysed the oxidation of RuDP to P-glycolate and P-glycerate. However, at this stage in the research programme the oxygenase was assayed by first determining the blank rate on each flask prior to tipping in the RuDP. It was subsequently learned that the enzyme inactivates during the 30 minute period under 100% oxygen when the blank rate was being determined. The measured rate of oxygen uptake at pH 9.3 under 100% oxygen when the blank rate was being determined. The measured rate of oxygen uptake at pH 9.3 under 100% oxygen at 30^{o} was 15 to 20% that of carboxylation at pH 7.8. A correction, estimated from subsequent work, placed the oxygenase rate at between 30 and 40% of the carboxylase rate.

Alternative Procedure for the Purification of RuDP Carboxylase

This procedure was used primarily to investigate the co-purification of RuDP carboxylase and RuDP oxygenase.

Initial Extraction: 260 g (fresh weight) of washed, deribbed spinach leaves were homogenized in a Waring Blender at top speed for 35 sec in 500 ml 0.025 M glycylglycine, pH 7.65, 1 mM EDTA and 0.05 M 2-mercaptoethanol. The brei was filtered through 4 layers of cheesecloth and 1 layer of Mirah cloth, and centrifuged at 40,000 g for 30 min. The clear, yellow supernatant solution constituted the initial extract.

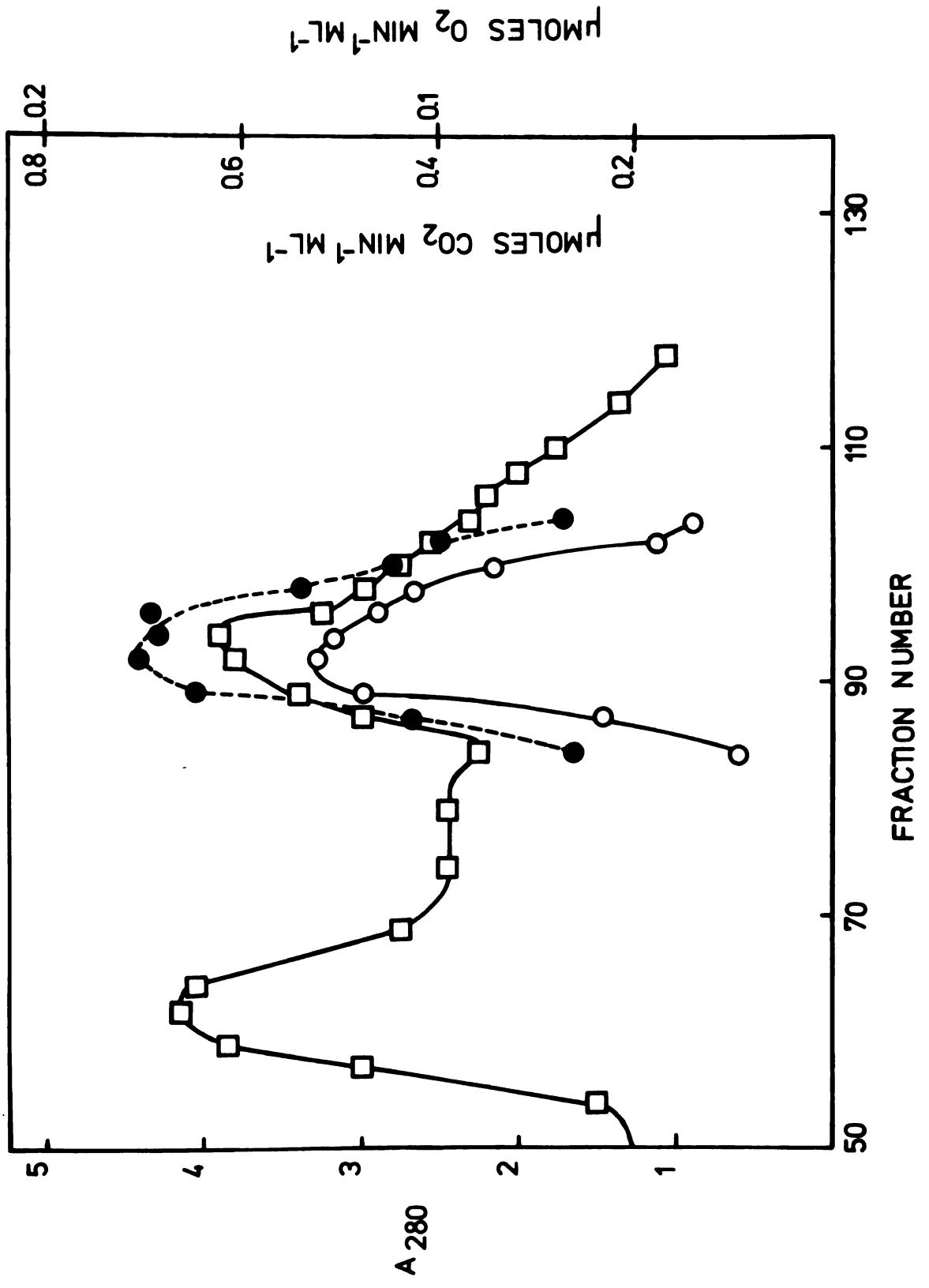
DEAE Cellulose Chromatography: The initial extract was applied directly to a 6 x 24 cm column of DEAE-cellulose equilibrated with 0.025 M glycylglycine pH 7.6, 0.001 M DTT. The column was then washed with 600 ml of the same buffer followed by a 2 l linear gradient from 0 to 1 M NaCl in 0.025 M glycylglycine pH 7.6, 0.001 M DTT. The flow rate was about 2 ml/min. Fractions containing 22 ml were collected. The results are shown in Fig. 23. Fractions 84 to 104 were pooled, the protein precipitated by the addition of 1.5 volume of saturated $(NH_4)_2SO_4$ and the suspension stored overnight. The precipitate was collected by centrifugation at 15,000 g for 20 min and dissolved in about 30 ml 0.025 M glycylglycine pH 7.6, 0.001 M EDTA and 0.001 M DTT. Insoluble material was removed by centrifugation at 40,000 g for 30 min. The supernatant solution constituted the DEAE-cellulose fraction.

Zonal Centrifugation: The density of the DEAE-cellulose fraction was checked to ensure that it was less

Figure 23. Co-purification of RuDP carboxylase and RuDP oxygenase by DEAE cellulose column chromatography.

□——□ A_{280}, 0——0 RuDP carboxylase,

●----● RuDP oxygenase.


μMOLES O2 MIN-1 ML-1
0.2
0.1
μMOLES CO2 MIN-1 ML-1
0.8
0.6
0.4
0.2
A280
5
4
3
2
1
50
70
90
110
130
FRACTION NUMBER

than that of 12.5% (w/w) sucrose. Buffer was added, when necessary to reduce the density of the protein solution. With the B-30 zonal rotor spinning at about 2,500 rpm, a 500 ml linear (by volume) sucrose density gradient from 12.5% to 30% (w/w) sucrose in 0.025 M glycylglycine pH 7.6, 0.001 M EDTA, 0.001 M DTT, was pumped into the rotor via the rim line at a rate of about 10 ml/min. Sufficient 35% (w/w) sucrose was then pumped into the rotor until the 12.5% (w/w) sucrose emerged from the core. The protein solution (about 30 ml) was carefully layered over the 12.5% sucrose, a further 50 ml overlay of buffer layered over the protein solution, and these were then pumped into the rotor. The gradient was centrifuged for 4.5 to 5 hours at 50,000 rpm. After decelerating to about 2,500 rpm the gradient was displaced out of the rotor via the core line by pumping 35% (w/w) sucrose into the rotor at the rim. 25 ml fractions were collected. The results are shown in Fig. 24. Fractions 8 through 14 were pooled and 1.5 volumes saturated $(NH_4)_2SO_4$ were added. The resultant suspension was stored at 4^o. Before use, the enzyme was desalted as previously described. A summary of the co-purification of RuDP carboxylase and RuDP oxygenase is given in Table 8.

RuDP carboxylase and RuDP oxygenase purified together as if they were the same protein. However, the ratio of carboxylase activity to oxygenase activity did

Figure 24. Co-purification of RuDP carboxylase and RuDP oxygenase by zonal centrifugation.

□——□ A_{280}, ●----● RuDP oxygenase, 0····0 RuDP carboxylase.

μMOLES O2 MIN-1 ML-1
1.0
0.5
2.0
1.0
μMOLES CO2 MIN-1 ML-1
8
6
4
2
A280
"CORE"
100
200
300
400
500 "RIM"
VOLUME (MLS)

TABLE 8.--CO-PURIFICATION OF RuDP CARBOXYLASE AND RuDP OXYGENASE

	Protein (mg)	$\frac{A_{280}}{A_{260}}$	RuDP Carboxylase		RuDP Oxygenase		$\frac{\text{Carboxylase}}{\text{Oxygenase}}$
			µmoles/min	Yield %	µmoles/min	Yield %	
Initial Extract	2,460	0.90	510	100	125	100	0.42
DEAE Cellulose	900	1.93	194	38	79	63	0.25
Zonal Centrifugation	705	2.00	208	41	88	70	0.24

not remain constant throughout the purification (Table 8). From a value of 4.2 in the initial extract, the ratio dropped to 2.5 in the fractions obtained from the DEAE-cellulose column. This change in the ratios of activity was reproducible and also occurred when the first step was an $(NH_4)_2SO_4$ fractionation. Analyses of the DEAE-cellulose column fractions (Fig. 23) showed that both activities peak in the same fraction and had nearly identical profiles. Similarly during zonal centrifugation in a sucrose density gradient both activities sedimented together (Fig. 24). The carboxylase/oxygenase activity ratio increased slightly towards the rim or bottom of the gradient. This suggested that the oxygenase might be fractionally heavier than the carboxylase and that two proteins were present. However, on centrifuging for a longer period of time the reverse pattern for the activity ratio was found, rather than a reinforcement of the first pattern. The differences in the activity ratio are most probably due to the inherent insensitivity and inaccuracies associated with the oxygenase assay.

Assessment of Purity and Homogeneity

Polyacrylamide gel electrophoresis performed immediately following purification of the enzyme by either method revealed only one protein band (Fig. 25 a and b). Eleven days after storing the enzyme as a precipitate in

Figure 25. Polyacrylamide gel electrophoresis of purified RuDP carboxylase.

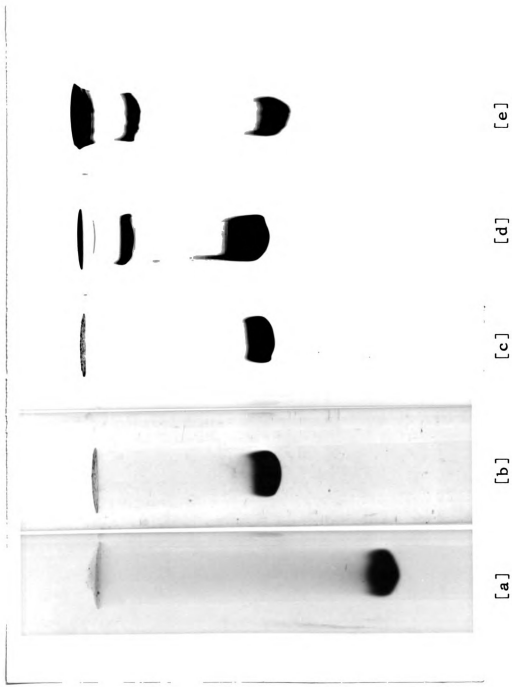
[a] Enzyme (60 μg protein) was prepared by method I and electrophoresed immediately after purification on a 5.5% gel for 90 min.

[b] Enzyme (62 μg protein) was prepared by method II and electrophoresed immediately after purification on a 6.0% gel for 100 min.

[c] Enzyme (65 μg protein of the same preparation as [b]) was prepared by method II and stored as a precipitate in 66% $(NH_4)_2SO_4$ for 12 days before electrophoresis as in [b].

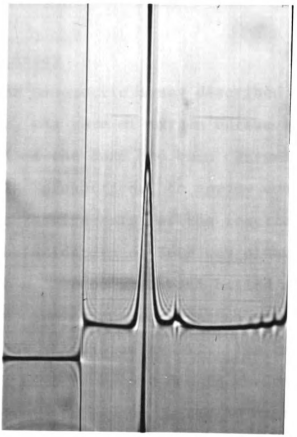
[d] Same as in [b] and [c] except enzyme was stored for 26 days.

[e] Same as in [b], [c] and [d] except enzyme was stored for 49 days.

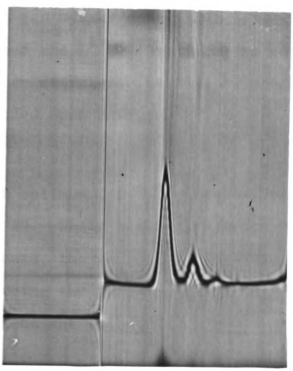

[e]
[d]
[c]
[b]
[a]

60% saturated $(NH_4)_2SO_4$, the preparation was run in the Model E Analytical Ultracentrifuge. A minor contaminant was revealed (Fig. 26a). Electrophoresis of the same enzyme preparation on the following day also revealed the presence of additional bands which had not been present immediately after preparation (Fig. 25c). These results were taken as evidence that the enzyme was polymerizing. Such behaviour had been observed before by Kleinkopf et al., (1970), for the purified barley enzyme, and by Ridley et al., (1967) for the purified spinach enzyme. To confirm that polymerization was indeed continuing, gel electrophoresis and analytical ultracentrifugation were again performed on the same enzyme preparation after 25 days storage in $(NH_4)_2SO_4$. Electrophoresis revealed additional bands which were much more intense than previously (Fig. 25d). Analytical ultracentrifugation performed under exactly the same conditions as before showed that the contaminant had increased in concentration and that in addition an even faster sedimenting peak was now apparent (Fig. 26b). The calculated sedimentation coefficients (S_{20}, buffer) for the three species are 18.6, 26.2 and 34.1. Polyacrylamide gel electrophoresis of a different preparation of enzyme, stored in the same manner for 49 days, revealed evidence of further polymerization (Fig. 25e). Thus, apart from this known aggregation phenomenon, the preparation appeared to be homogeneous by these commonly

Figure 26. Schlieren patterns of purified RuDP carboxylase obtained in the Spinco Model E ultracentrifuge. Both photographs were taken 24 min after reaching a speed of 50,740 rpm. The temperature was 3.9° and the phase angle was 70°. The protein concentration was 11.6 mg ml^{-1} in both cases, and the buffer was 0.025 M glycylglycine, pH 7.7, 0.1M KCl and 0.001M DTT. The upper figure was obtained after storing the enzyme as a precipitate in $(NH_4)_2SO_4$ for 11 days and the lower figure was obtained after 25 days of storage in $(NH_4)_2SO_4$.



a



b

used criteria.

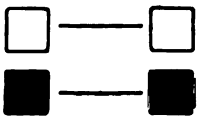
RuDP Oxygenase Assay

With the manometric assay described in the Methods section, the rate of oxygen uptake was linear until about 50% of the RuDP had been consumed (Fig. 27). The rate was also proportional to enzyme concentration (Fig. 28). The requirements for the reaction are listed in Table 9. The oxidation of RuDP was clearly enzymatic since no oxygen uptake occured with boiled enzyme. Like the carboxylase, the oxygenase reaction was dependent upon Mg^{2+} and was stimulated by DTT. The oxygen uptake in the presence of DTT was not due to the oxidation of DTT, since the rate of oxygen uptake by controls without RuDP or enzyme was the same whether or not they contained DTT. The reaction also depended upon oxygen, there being no gas uptake under 100% nitrogen. This result confirmed the [^{14}C] data in which no P-glycolate was formed under nitrogen. Lastly, of course, the reaction depended upon RuDP.

pH Optimum

The pH optimum for the oxygenase reaction is shown in Fig. 29. While these measurements are complicated by buffer effects, the pH optimum appears to be about 9.3. Details concerning the pH optimum are in manuscript (Andrews, Lorimer and Tolbert).

Figure 27. Oxygen consumption by RuDP oxygenase as a function of time. The reaction was initiated by the addition of RuDP.



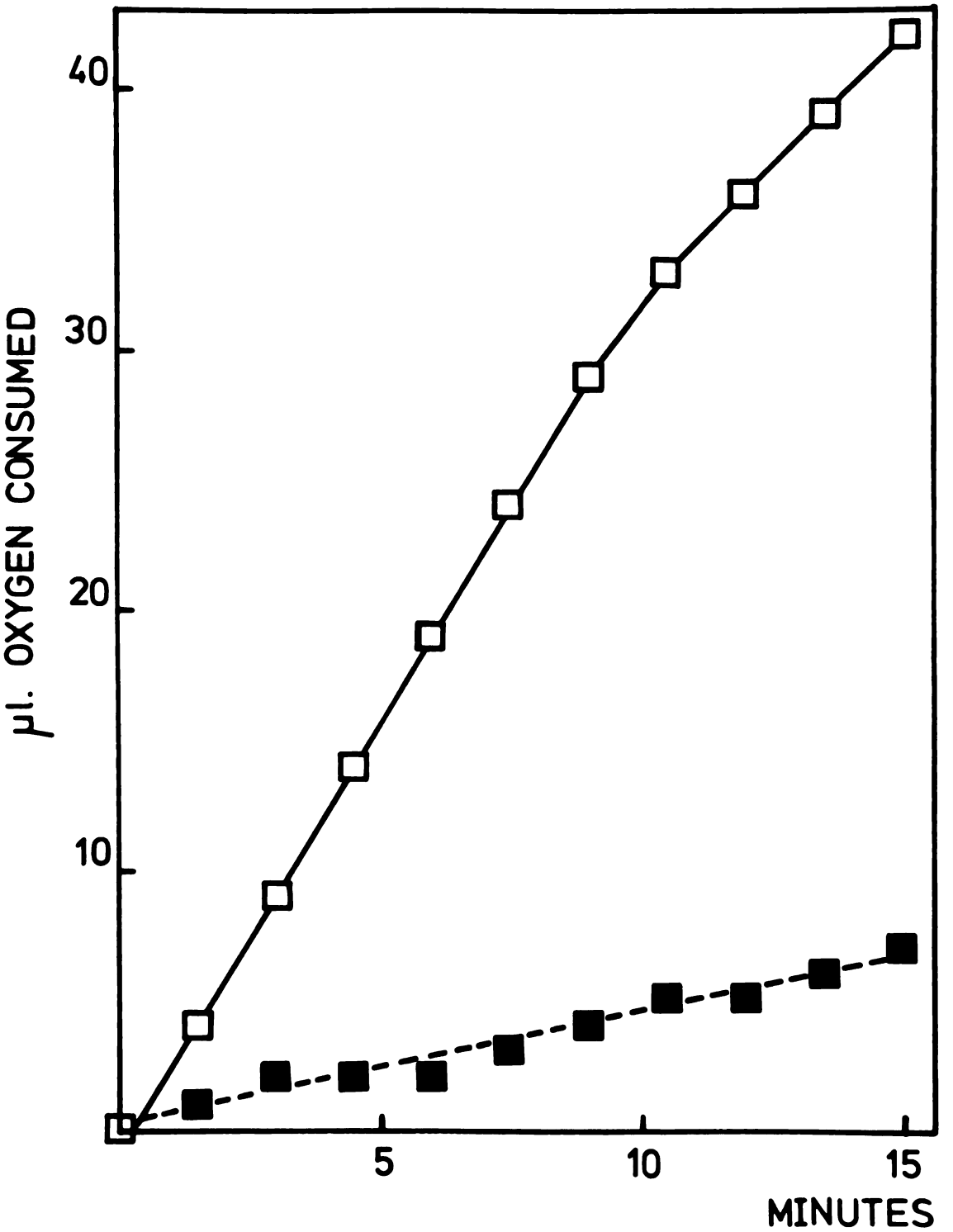

40
30
20
10
µl. OXYGEN CONSUMED
5
10
15
MINUTES

Figure 28. Oxygen consumption by RuDP oxygenase as a function of enzyme concentration.

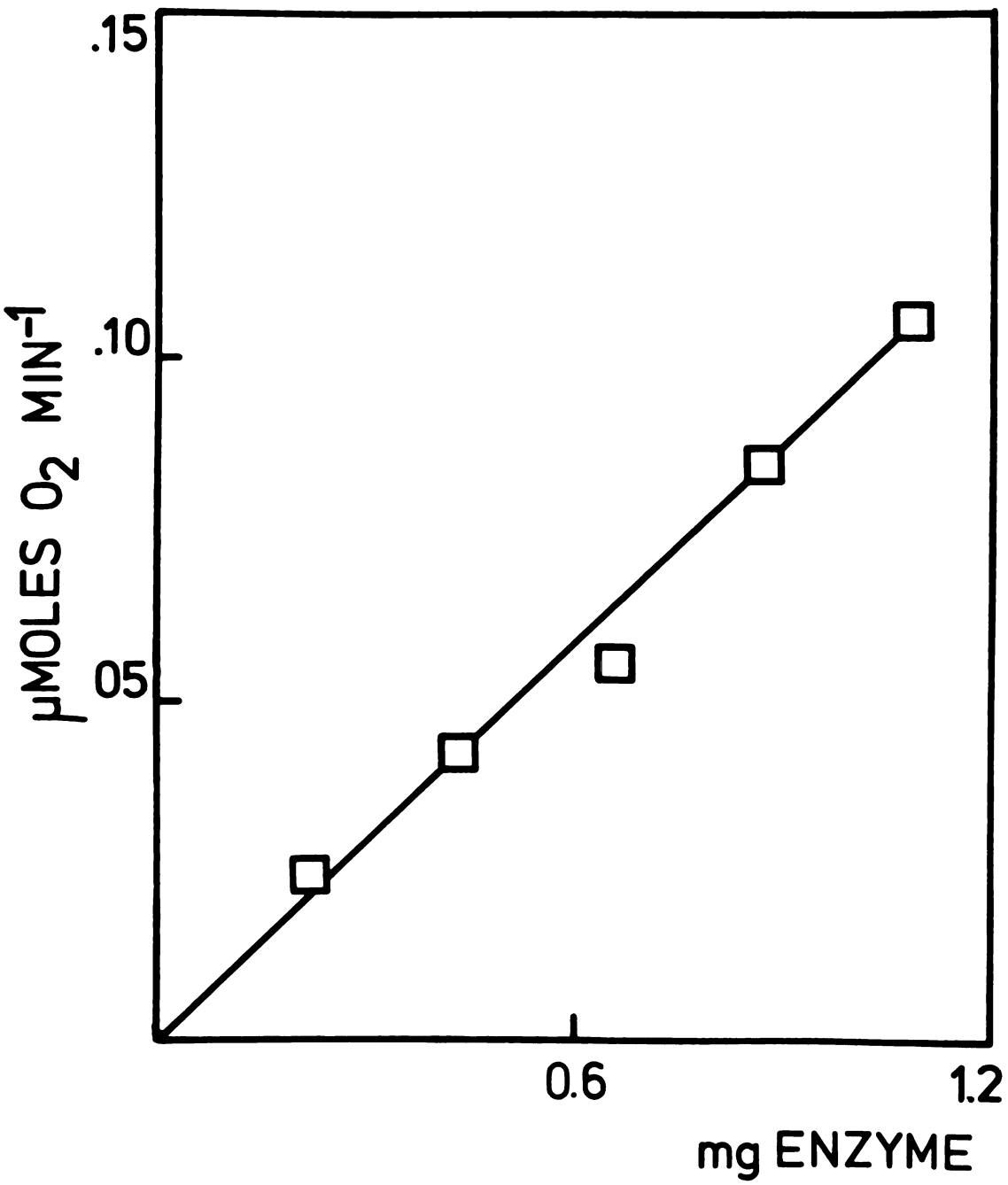

.15
.10
.05
μMOLES O_2 MIN^{-1}
0.6
1.2
mg ENZYME

Figure 29. The pH Optimum for RuDP Oxygenase

□——□ in 0.1 M ammediol-Cl buffer.

■——■ in 0.1 M glycine-KOH buffer.

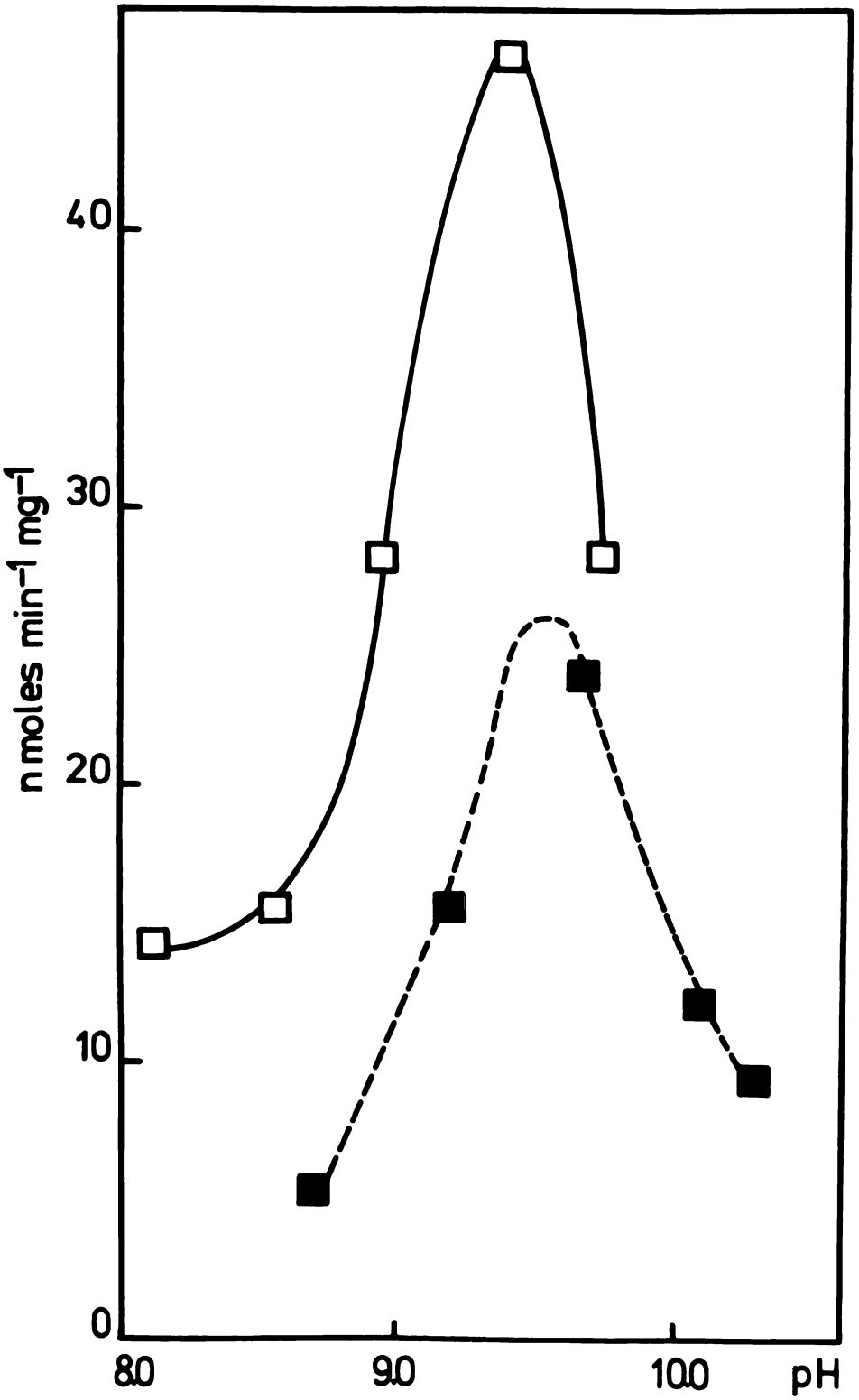

40
30
20
10
0
n moles min⁻¹ mg⁻¹
8.0
9.0
10.0
pH

TABLE 9.--OXYGEN UPTAKE BY RuDP OXYGENASE. THE REACTION MIXTURE WAS AS DESCRIBED IN THE METHOD SECTION LESS THE COMPONENTS INDICATED.

Contents	O_2 uptake nmoles/min/mg protein
Complete	70
- DTT	38
- $MgCl_2$	0
- RuDP	0
- enzyme	0
boiled enzyme (2 min., 100°)	0
- O_2, + N_2	0

The Stoichiometry of the Reaction

When an accurately determined quantity (2 μmoles) of RuDP, standardized as described in the Methods section, was incubated with the enzyme under 100% oxygen, and the reaction allowed to run to completion, a total of 2 μmoles of oxygen was consumed indicating that oxygen and RuDP were consumed with a stoichiometry of 1 : 1 (Figure 30). One may conclude, additionally, that under these conditions there is little or no carboxylation of RuDP.

Proof of Reaction Products

The reaction products were identified by combined gas liquid chromatography - mass spectrometry. Two major compounds (peak I and II in Fig. 31) were present which co-chromatographed with authentic standards of the trimethylsilyl derivatives of P-glycolate (peak III) and P-glycerate (peak IV). Mass spectra (Fig. 32 and 33) confirmed that the products were indeed P-glycolate and P-glycerate, i.e. the mass spectra obtained from peak I and II were identical to those obtained from peaks III and IV respectively. The RuDP used for these studies did not contain any detectable P-glycolate or P-glycerate. On one occasion, the small peak preceding peak I (Fig. 31a) was sufficiently large to merit identification. Its mass spectrum was consistent with it being the trimethylsilyl derivative of

Figure 30. The stoichiometric consumption of oxygen by RuDP oxygenase. The reaction was initiated by the addition of 2 μmoles RuDP.

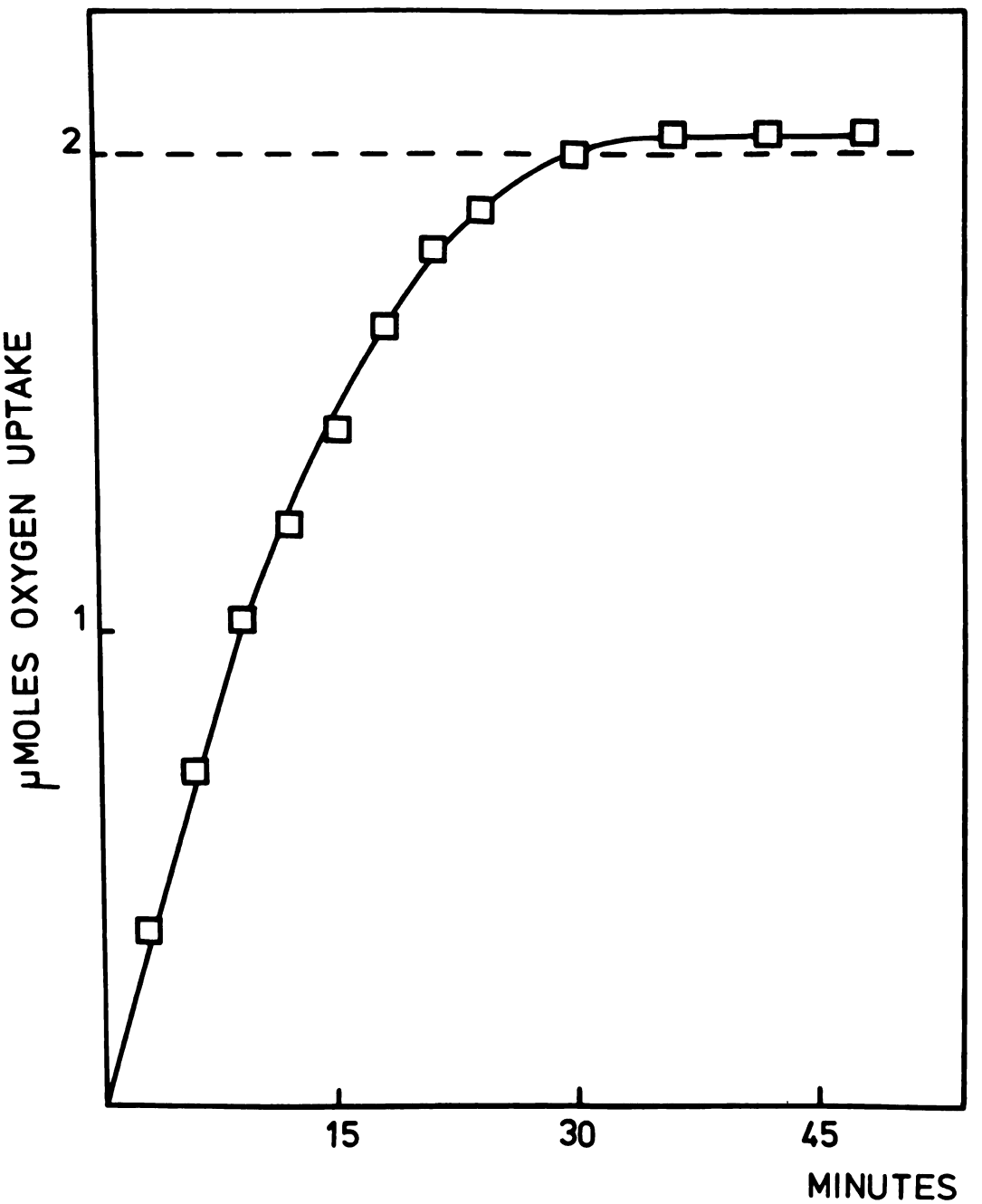

2
1
μMOLES OXYGEN UPTAKE
15
30
45
MINUTES

Figure 31. (A) Gas liquid chromatographic separation of the silylated reaction products from the RuDP oxygenase reaction. (B) The trimethylsilyl derivatives of authentic P-glycolate (III) and P-glycerate (IV). Mass spectrometry confirmed that peaks I and III were $(Me_3Si)_3$ - P-glycolate (Figure 32) and peaks II and IV were $(Me_3Si)_4$ P-glycerate (Figure 33).

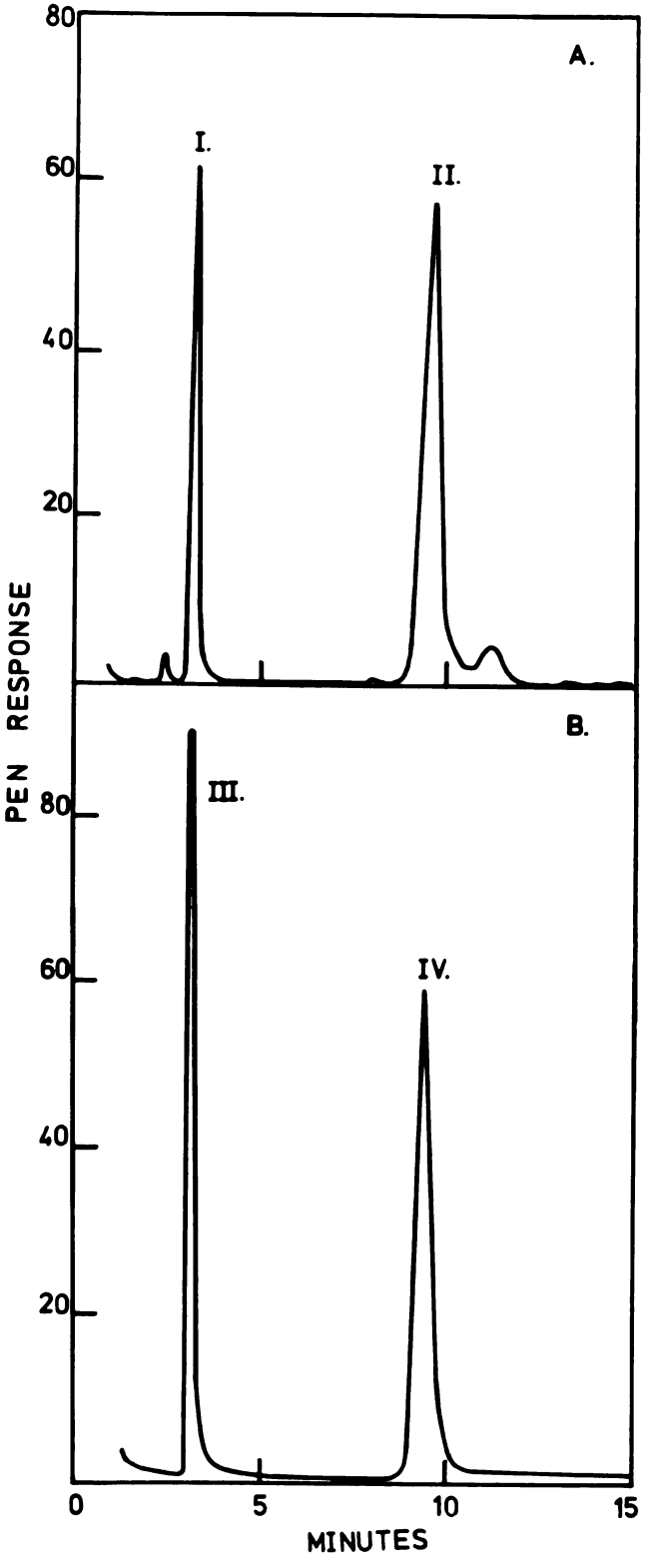

A.
I.
II.
B.
III.
IV.
80
60
40
20
PEN RESPONSE
0
5
10
15
MINUTES

Figure 32. Mass spectrum of $(Me_3Si)_3$-P-glycolate (70 eV.)

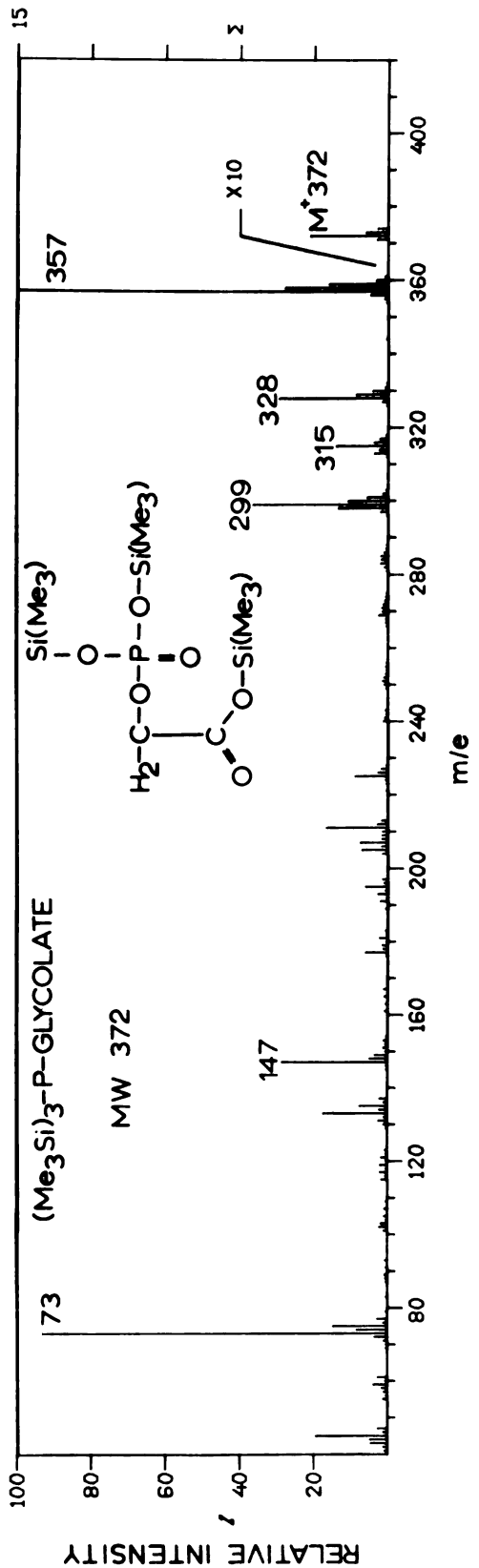
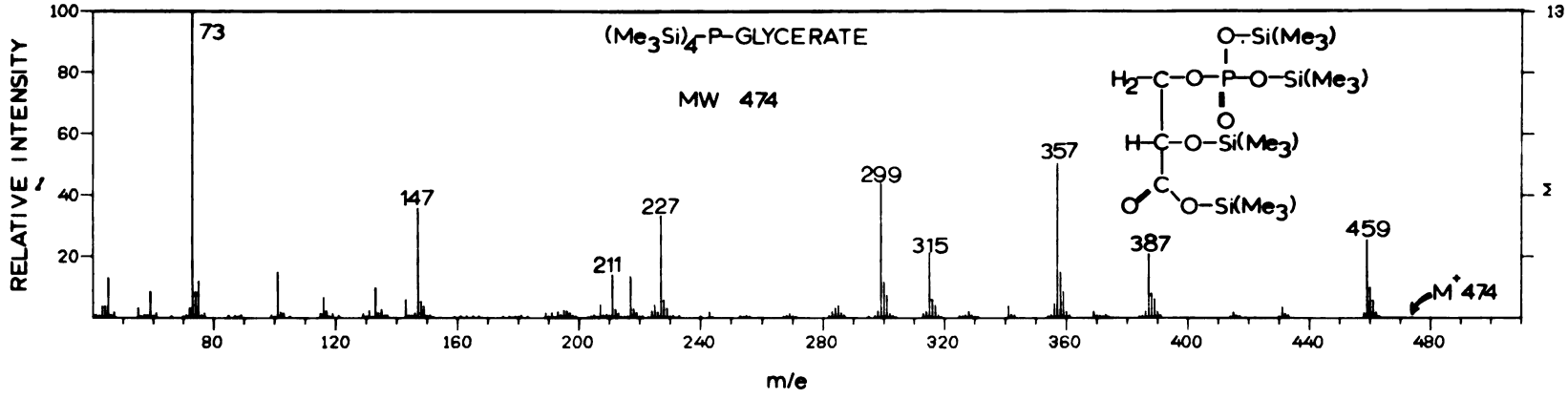

(Me3Si)3-P-GLYCOLATE
MW 372
Si(Me3)
O
O-P-O-Si(Me3)
O
H2-C-O
C
O
O-Si(Me3)
73
147
299
315
328
357
M+372
X10
RELATIVE INTENSITY
m/e

Figure 33. Mass spectrum of $(Me_3Si)_4$-P-glycerate (70 eV.)


RELATIVE INTENSITY
100
80
60
40
20
73
147
211
227
299
315
357
387
459
M+ 474
(Me3Si)4-P-GLYCERATE
MW 474
O-Si(Me3)
H2-C-O-P-O-Si(Me3)
O
H-C-O-Si(Me3)
C
O
O-Si(Me3)
80
120
160
200
240
280
320
360
400
440
480
m/e

the ethyl ester of P-glycolate. This ester was presumably formed during the drying of the sample with absolute ethanol. At this stage, following ion exchange chromatography on Dowex 50 H^+, the solution was acidic, which would catalyse esterification. This peak was absent from the standards which were not treated with Dowex 50 H^+ form or ethanol.

Analysis of the Mass Spectra of $(Me_3Si)_3$ P-glycolate

The mass spectrum of $(Me_3Si)_3$ P-glycolate contains the molecular ion at m/e 372 (Fig. 32). The most intense ion occurs at m/e 357 (M-15), an ion which arises by the loss of a methyl group from the molecular ion. The ion at m/e 328 (M-44) most probably arises by a rearrangement involving the loss of the carboxyl group to give an ion with the structure, $CH_2 = {}^{+}O - P - (OMe_3Si)_3$. The assignment of structure was confirmed by the absence of ^{18}O in this ion in samples of $(Me_3Si)_3$-P-glycolate containing ^{18}O. The ions at m/e 315 and m/e 299 are common to the trimethylsilyl derivatives of phosphate esters (Zinbo and Sherman, 1970) and are related to one another by the metastable ion at m/e 284. The ion at m/e 315 most probably arises by a rearrangement of the molecular ion to give the ion with the structure, $(Me_3SiO)_2 - P(OH) = {}^{+}OMe_3Si$, and a metastable ion at m/e 268 supports this contention. It should be noted that the ability of trimethylsilyl

groups to undergo migration in the mass spectrometer is well documented (DeJongh et al., 1969 and McCloskey et al., 1968). The ion at m/e 299, which has the structure $(Me_3SiO)_2$ - P(O) - O^+ = $SiMe_2$, clearly arises from two sources. Firstly it may arise by rearrangement from the ion at m/e 357, and a metastable at m/e 250 supports this transition. Secondly, it may arise from the ion at m/e 315 by the loss of $-CH_3$ plus H and a metastable at 284 supports this transition. Interestingly, in samples containing ^{18}O in the carboxyl group, the ions at m/e 315 and 299 were also found to contain ^{18}O. Thus, the rearrangement leading to the formation of the ions at m/e 315 and 299 involves a transfer of $-OMe_3Si$ to the phosphorus atom rather than simply the transfer of $-Me_3Si$ to one of the available oxygen atoms of the phosphate group. Other phosphate ions are located at m/e 227 and 211 with a metastable ion at m/e 196 relating these ions.

Analysis of the Mass Spectra of $(Me_3Si)_4$ P-glycerate

The $(Me_3Si)_4$-P-glycerate spectrum contained no molecular ion (Fig. 33). The ion at m/e 459 is due to the loss of a methyl group from the molecular ion. The prominent ion at m/e 357 most probably corresponds to the structure $(Me_3SiO)_2$-P-(O)-OCH_2-CH = ^+O-Me_3Si, i.e. the loss of the silylated carboxyl group from the molecular ion, and the metastable ion at m/e 269 supports

this interpretation. The structure of the ion at m/e 387 is most perplexing. This ion is common in the mass spectra of the Me_3Si derivatives of sugar phosphates (Zinbo and Sherman, 1970). On the basis of deuterium labeling, they assigned the structure $Me_3Si\text{-}^{+}O = P\text{-}(OMe_3Si)_3$ to this ion. In the case of the sugar phosphates this ion is thought to be derived from that m/e 459 and a metastable at m/e 326 supports this contention. The spectrum of $(Me_3Si)_4$ P-glycerate also contains an ion at m/e 459 and a metastable ion at m/e 326. However, in this case the ion at m/e 459 contains a total of 11 methyl groups, while the structure proposed by Zinbo and Sherman (1970) for the ion at m/e 387 contains a total of 12 methyl groups, an obvious discrepancy. In samples containing ^{18}O in the carboxyl group the ion at m/e 387 was found not to be labeled. A possible structure consistent with this observation is $[Me_3SiO\text{-}CH_2\text{-}O\text{-}P(OMe_3Si)(OSiMe_2)_2]^{+}$. The spectrum also contains the familiar phosphate ions at m/e 315 and 299 (and the related metastable at m/e 284) and at m/e 227 and 211 (and the related metastable at m/e 196).

Substrate Specifity

A number of substrates, other than RuDP, were tested for activity by the standard oxygenase assay (Table 10). Fructose diphosphate and fructose-6-phosphate gave a very marginal rate above that of the

TABLE 10.--SUBSTRATE SPECIFITY OF RuDP OXYGENASE

Substrate	Conc. (M)	Relative Rate	
		O_2 Uptake	[^{14}C]P-glycolate or ^{14}C [glycolate] formed
Ribulose diphosphate	$2x10^{-3}$	100	100*
Ribulose 5-phosphate	$2x10^{-3}$	1	-
Ribulose	$2x10^{-3}$	-	0*
3-Phosphoglycerate	$3x10^{-3}$	1	-
Fructose diphosphate	$3x10^{-3}$	2	0
Fructose 6-phosphate	$3x10^{-3}$	5	0

*Determined by reaction with the preparation of soybean RuDP carboxylase.

blank. In view of the previous identification of erythronic acid, it was conceivable that the enzyme catalysed the oxidation of fructose-6-phosphate and fructose diphosphate to 4-P-erythronate and glycolate or P-glycolate. However, this possibility was carefully evaluated by the use of [^{14}C] fructose-6-phosphate and [^{14}C] fructose diphosphate. Paper chromatography of the reaction products revealed no trace of P-glycolate, glycolate, P-erythronate or erythronate. The enzyme did not catalyse the oxidation of P-glycerate, thereby eliminating the possibility that RuDP was first carboxylated to P-glycerate by a trace of CO_2 followed by the oxidation of P-glycerate. Ribulose-5-phosphate and ribulose were inactive as substrates. Thus the oxygenase appeared to be quite specific for RuDP.

Mechanism of RuDP Oxygenase Studied with [^{18}O] Oxygen and [^{18}O] Water

(a) [^{18}O] oxygen:

Experiments with $^{18}O_2$ have been performed on 3 occasions, with modifications (improvements) of the technique in each case. On all 3 occasions the same standard 1 ml RuDP oxygenase assay was run for 60 min at 25^{o} with the addition of isotopic oxygen.

In experiment I, the reaction was run in a Warburg flask equipped with a serum rubber cap with the components in the centre and RuDP in the side arm. The

total volume of the flask was about 4 ml. The flask was first flushed with 100% nitrogen and then 6 ml of $^{18}O_2$ (ostensibly 93.5 atoms %) was injected through the serum cap. The reaction was initiated by tipping in the RuDP. The reaction was terminated after 60 min by freezing. After thawing the solution was transferred to a test-tube and the procedure used for the identification of the reaction products followed. Isotope incorporation was determined as described in Chapter II. The results are based upon analyses of the ions at m/e 357 and m/e 359 in the case of P-glycolate and at m/e 459 and m/e 461 in the case of P-glycerate. The results of experiment 1 are shown in Table 11. Qualitatively it was evident that the reaction proceeded with the incorporation of a single atom of oxygen-18 into the carboxyl group of P-glycolate. No incorporation of oxygen-18 into P-glycerate occurred. However, it was evident that in experiment 1, the incorporation of oxygen-18 into P-glycolate was substantially less than that expected. Before considering that the reaction did not proceed in the expected manner, three hypotheses were tested to explain the low level of ^{18}O incorporation. (a) There may have been some P-glycolate present in the RuDP before the reaction with $^{18}O_2$. However, a control experiment without the enzyme revealed no P-glycolate, eliminating the possibility of isotope dilution due to the presence of P-glycolate in the RuDP used. (b) The

TABLE 11.--THE MECHANISM OF RuDP OXYGENASE.

Substrate	Label	^{18}O atoms % in label	^{18}moles %	
			P-Glycolate	P-Glycerate
RuDP (1)	$^{18}O_2$	-	56.3	0
(2)	$^{18}O_2$	84.3	40.0(47.5)	0
(3)	$^{18}O_2$	82.0	74.1(90.4)	0
RuDP	$H_2{}^{18}O$	10.3	10.3(100)	9.8(95.1)
P-glycolate + P-glycerate	$H_2{}^{18}O$	10.3	0	0.5

^{18}O content of the gas phase could well have been substantially less than 93.5 atoms %. The second and third experiments were therefore designed to include a determination of the atoms % ^{18}O in the gas phase. (c) Some loss of ^{18}O by exchange from the carboxyl group of P-glycolate might occur during the "work up" procedure prior to mass spectrometry.

In experiment 2, the reaction was performed in a similar manner. However, after the reaction had proceeded for 60 min it was rapidly frozen in an acetone-dry ice bath and the oxygen-18 content of the gas phase measured. This was done by analyses with the Varian MAT Mass Spectrometer GD 150 equipped with the HTE-DE Inlet System. The instrument was operated at a pressure of 1.5×10^{-6} tor. After subtraction of the background, the atoms % ^{18}O was determined by the formula

$$\frac{M_{36} + M_{34/2}}{M_{32}+M_{34} + M_{36}} \times 100\%$$

where M_{32}, M_{34} and M_{36} are the peak height of masses 32, 34, and 36 respectively. The contribution of ^{17}O-^{17}O to M_{34} was ignored, since there was a negligible amount of ^{16}O-^{17}O (M_{33}) or ^{17}O-^{18}O (M_{35}). The isotope incorporation into the products was determined by the procedure previously described. The ^{18}O incorporation into P-glycolate was still substantially less than the ^{18}O content of the gas phase (Table 11). Additionally,

a third peak was apparent in the gas chromatogram. This was identified as the trimethylsilyl derivative of the ethyl ester of P-glycolate. It was labeled with ^{18}O. To ensure anhydrous conditions for the silylation reaction it was the practice to redry the samples with absolute ethanol. Evidently in the acidic conditions following chromatography on Dowex 50 H^+ form, there had been some esterification. Since esterification is reversible it was possible that the loss of label occurred at this stage. Experiment 3 was performed to eliminate this possibility.

Experiment 3 was run in a 4 x 1 cm vial equipped with a serum rubber cap. Before introduction of the isotopic oxygen the vial was evacuated. Six ml of ^{18}O were then injected giving a slight positive pressure within the vial. The reaction was initiated by injection of RuDP. After 60 min the reaction was again frozen and the ^{18}O content of the gas phase determined as previously described. However, the "work up" procedure was modified so that the eluate from the Dowex 50 H^+ form column was immediately neutralized with KOH and the addition of absolute ethanol eliminated from the dehydration process. Isotope analysis of the silylated products showed the incorporation of ^{18}O into P-glycolate was 90.4% of that expected (Table 11). Evidently there was still a small loss of label. In Fig. 34 and 35, the relevant regions of the mass spectra of the (Me_3Si)

Figure 34. Comparison of part of the mass spectrum of authentic $(Me_3Si)_3$-P-glycolate (a) with that of the same compound isolated from an incubation of RuDP with the enzyme in 82% [^{18}O] oxygen (b). The incubation and subsequent preparation of the $(Me_3Si)_3$-P-glycolate were carried out as described in the Methods section.

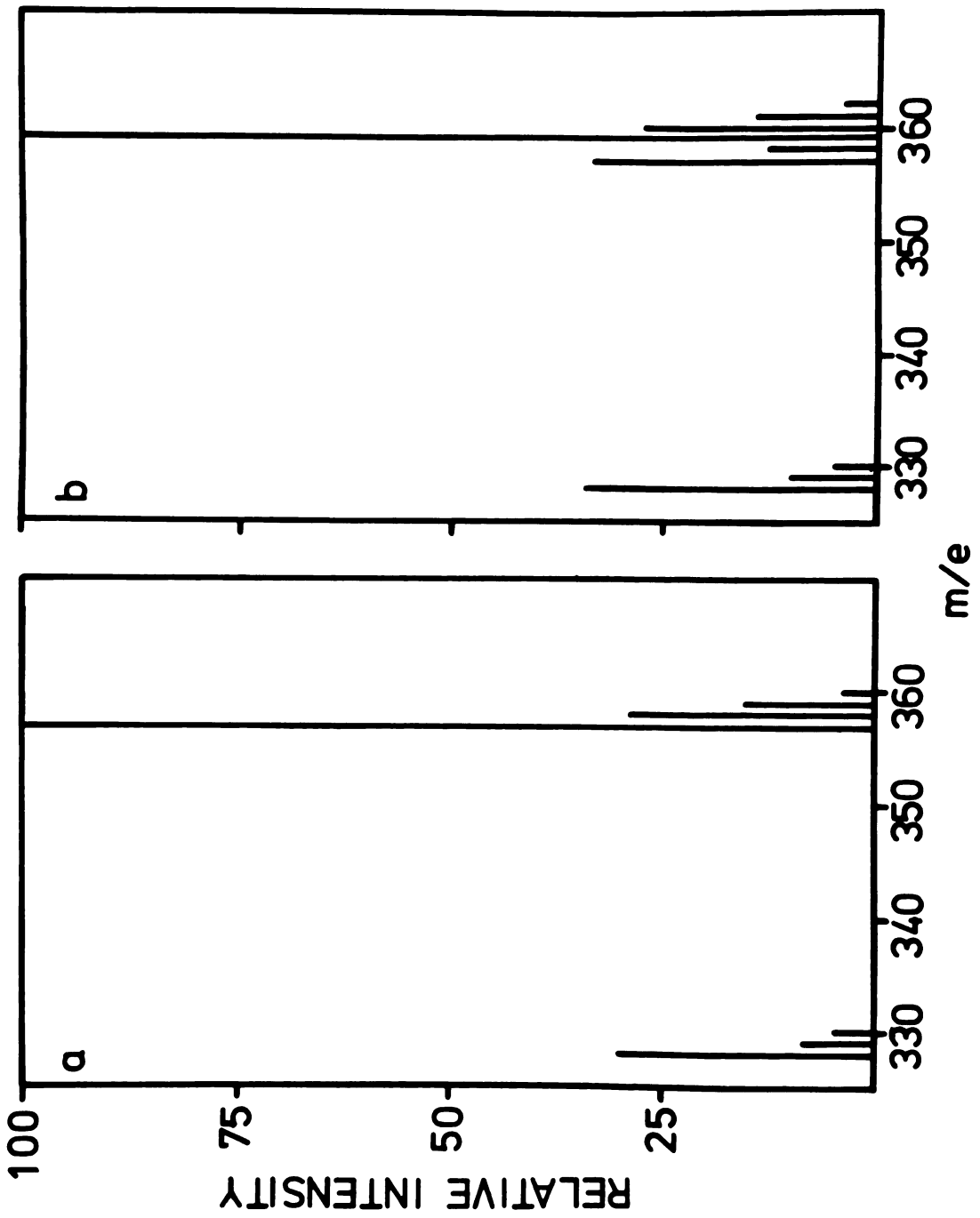
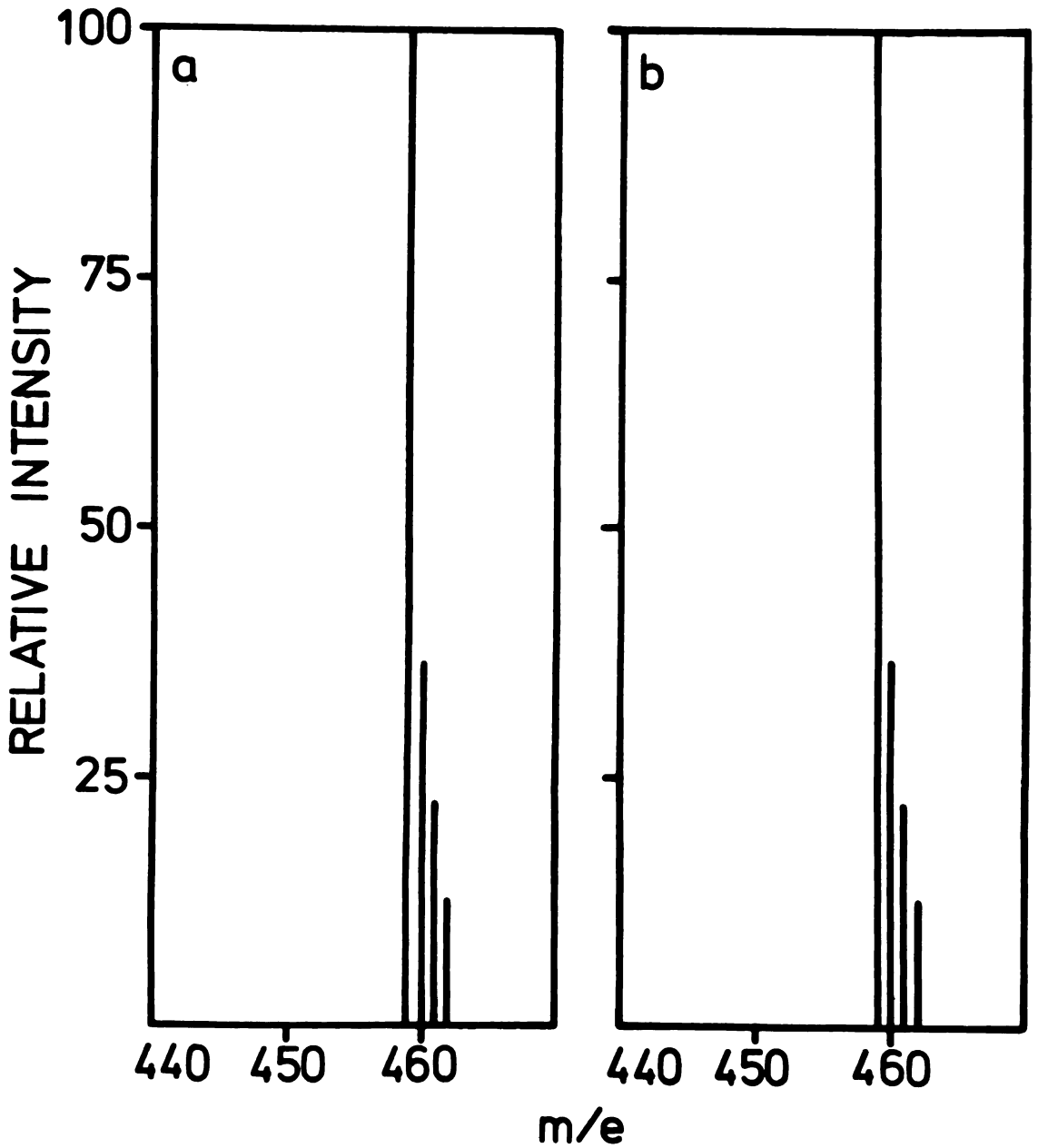

RELATIVE INTENSITY
100
75
50
25
a
b
330
340
350
360
m/e

Figure 35. Comparison of part of the mass spectrum of $(Me_3Si)_4$-P-glycerate (a) with that of the same compound isolated from an incubation of RuDP with the enzyme in 82% [^{18}O] oxygen (b). The incubation and subsequent preparation of the $(Me_3Si)_4$-P-glycerate was carried out as described in the text.

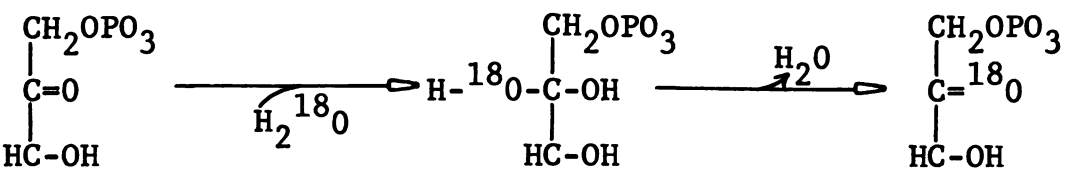

100
75
50
25
RELATIVE INTENSITY
a
b
440
450
460
440
450
460
m/e

derivatives of authentic samples of P-glycolate and P-glycerate are compared with similar spectra for P-glycolate and P-glycerate obtained after incubation of RuDP with the oxygenase under an atmosphere containing 82% $^{18}O_2$. In the case of the P-glycolate spectra, a comparison of the relative intensities of the ions at m/e 357, 359 and 361 clearly shows that one, and only one, atom of ^{18}O has been incorporated. The apparent increase in the relative insensity of the ion at m/e 361 is due to the combination of one atom of oxygen-18 with the naturally occurring isotopes of silicon, ^{29}Si (natural abundance 4.7%) and ^{30}Si (natural abundance 3.1%). It is not due to the incorporation of two atoms of oxygen-18. Additionally, a comparison of the ions at m/e 328 and 330, ions lacking the carboxyl group, shows the absence of ^{18}O. These results therefore prove that one atom of ^{18}O was incorporated into the carboxyl group of P-glycolate. For the P-glycerate spectra, a comparison of the relative intensities of the ions at m/e 459 and 461 clearly shows that no oxygen, derived from molecular oxygen, has been incorporated. By default then, the carboxyl oxygen of P-glycerate must be derived from water. In order to confirm this, the oxygenase assay was run in the presence of $H_2{}^{18}O$.

(b) [^{18}O] water:

In order to perform these experiments, the

reaction was reduced in volume and run in 4 x 1 cm vials equipped with serum rubber caps. The components were: 20 µl 0.25 M Ammediol-Cl pH 9.3, 1 µl 1.0 M $MgCl_2$, 2 µl 0.01 M DTT, 2 µl 0.05 M EDTA, 20 µl enzyme (20.7 mg/ml) and 60 µl $[^{18}O]H_2O$ (20.63 atoms %). The vial was flushed with 100% oxygen and the reaction initiated by the injection of 15 µl 0.025 M RuDP. The final $[^{18}O]H_2O$ content was thus 10.3 atoms %. The reaction was allowed to proceed at 25° for 60 min with shaking, and terminated by freezing. The Me_3Si-derivatives of P-glycolate and P-glycerate were prepared as in experiment 2 above. The results (Table 11) confirmed that the carboxyl oxygen of P-glycerate was derived from water. However, oxygen-18 was also incorporated into the carboxyl group of P-glycolate. The labeling of the P-glycolate carboxyl group is to be expected since the keto oxygen of RuDP most probably exchanges with the medium via the hydrated form:



The absence of incorporation of ^{18}O, from molecular oxygen, into P-glycerate during the RuDP oxygenase reaction might have been due to an enzyme catalysed exchange of the carboxyl oxygens with the medium. To ensure that the enzyme did not catalyse any exchange of the carboxyl oxygens of the products with those of the

medium, the enzyme was incubated with P-glycolate and P-glycerate in the presence of $[^{18}O]H_2O$ under 100% oxygen. The reaction was performed in 4 x 1 cm vials equipped with serum rubber caps, and consisted of the following components: 20 µl 0.25 M Ammediol-Cl pH 9.3, 1 µl 1.0 M $MgCl_2$, 2 µl 0.01 M DTT, 2 µl 0.05 M EDTA, 10 µl 0.05 M P-glycerate, 10 µl 0.05 M P-glycolate and 65 µl $[^{18}O]H_2O$ (20.63 atoms %). The vial was flushed with 100% oxygen and the reaction initiated by the injection of 20 µl enzyme (20.7 mg/ml). The final $[^{18}O]$ H_2O content was thus 10.3 atoms %. The reaction was allowed to proceed at 25° for 60 min with shaking and terminated by freezing. The Me_3Si-derivatives were prepared as in experiment 2 above. The results (Table 11) indicate that the enzyme did not catalyse the exchange of the carboxyl oxygens of either phosphate ester with the medium.

Summary of Other Properties of RuDP Oxygenase

The experiments described in this section were performed by Dr. John Andrews. Like all experiments described in this chapter they were the outcome of joint experimental planning. They are included here for the sake of completeness.

Activity as a Function of Oxygen Concentration

At both 25° and at 30°, oxygenase activity was a linear function of the oxygen concentration in the gas phase from 0 to 100% oxygen. This indicates that

the enzyme was not saturated with 100% oxygen in the gas phase. The physiological significance of this result will be discussed later.

Activity as a Function of RuDP Concentration

Owing to the insensitivity of the oxygenase assay, attempts to determine the K_M for RuDP have proven difficult to perform. However, a Lineweaver-Burk plot of the best data indicates a K_M for RuDP under 100% oxygen of about 0.2 mM. This must be regarded as an operational K_M value, since the other substrate, oxygen, was present in sub-saturating concentrations.

Stability

Inactivation experiments have not yielded consistently reproducible results. Upon storage in $(NH_4)_2SO_4$, one preparation showed a constant level of both oxygenase and carboxylase activity for about 3 weeks. Then both activities declined precipitously, suggesting that they were one and the same protein. However, in other preparations there has generally been a slow loss of carboxylase activity which has not been paralleled by equivalent losses in oxygenase activity. The oxygenase activity in general seems to be more stable. This result suggests that the two activities are associated with different proteins.

Cyanide Inhibition

The oxygenase activity was inhibited about 50% by 1 x 10^{-4}M cyanide. This inhibition appeared to be dependent upon the presence of RuDP. When the enzyme was incubated with different concentrations of cyanide between 0.5 and 2.0 x 10^{-4}M and the reaction initiated by the addition of RuDP, there was a lag before inhibition was observed. This behaviour was strikingly similar to the lag in the cyanide inhibition of the carboxylase as reported by Wishnick and Lane (1969). When the enzyme was incubated for 1 hour at 4^{o} with 5 x 10^{-3}M KCN and then the cyanide removed by Sephadex, the oxygenase activity was fully restored. This has also been reported to be true for the carboxylase activity.

Copper Content of RuDP Carboxylase

Wishnick *et al.*, (1969) have reported the presence of 1 g atom of copper per mole of enzyme. However, Atomic Absorption analyses of two purified enzyme preparations failed to confirm this report, but revealed the presence of only 0.1 g atom of copper per mole of enzyme. This discrepancy cannot be attributed to the small differences in the specific activity of the various preparations. Furthermore addition of copper sulphate to a concentration 20 times that of the carboxylase did not stimulate the rate of oxygen uptake.

Reaction Intermediates derived from Oxygen

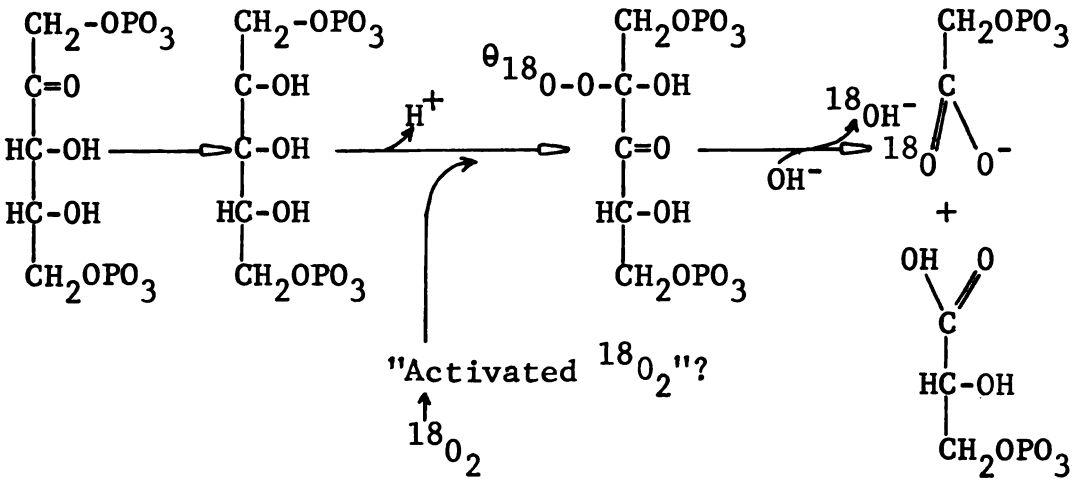
Addition of catalase or erythrocuprein (superoxide dismutase) did not effect the RuDP oxygenase reaction. These results suggest that neither hydrogen peroxide nor the superoxide radical are involved in the oxygenase reaction, although negative results do not unequivocally eliminate the possibility of their involvement.

Discussion

The term RuDP oxygenase is new. This activity is best described by the rules in Enzyme Nomenclature by the International Union of Biochemistry as ribulose-1,5-diphosphate:oxygen oxidoreductase (E.C. 1.13.1.-). Enzymes in the group 1.13 catalyse reactions "acting on single donors with incorporation of oxygen (oxygenases)". In this group there are no enzymes acting upon sugar phosphates. With the exception of myo-inositol oxygenase and lipoxygenase. Most other enzymes in this group act upon aromatic substrates.

Mechanism of RuDP Oxygenase

The results of the ^{18}O experiments (Table 11) are consistent with the following mechanism for RuDP oxygenase activity.



The oxidative cleavage is depicted as occurring between C-2 and C-3 of the enediol structure of RuDP. Although the results do not eliminate the possibility that the cleavage occurs between C-3 and C-4, this is considered less likely. Specifically labeled [^{14}C]-RuDP would be needed to clarify this point. The reaction is depicted as proceeding via a peroxide intermediate formed by attack of oxygen on the C-2 of RuDP. The results do not indicate how this intermediate peroxide is formed. Recently a great deal of attention has been devoted to determining the reactive species of oxygen in oxygenase reactions and Hirata and Hayaishi, (1971) and Strobel and Coon, (1971) have reported evidence for the involvement of the superoxide radical in the reactions of tryptophas dioxygenase and the microsomal cytochrome P-450 hydroxylation system respectively. Preliminary evidence indicated that RuDP oxygenase was not inhibited by superoxide dismutase, but this negative result does

not unequivocally eliminate the possible involvement of the superoxide radical any more than a positive result would unequivocally establish that the reactive species of oxygen was the superoxide radical. Preliminary evidence also indicated that the purified carboxylase did not contain the expected 1 g atom of copper per mole of enzyme reported by Wishnick et al., (1969). This was disappointing since the association of ferrous or copper ions with oxygenases is widespread. There are no reports of other metal ions in purified RuDP carboxylase. Since the above points remain to be clarified, the mechanism is for the meantime depicted as involving some form of activated oxygen.

The mechanism of decomposition of the peroxide intermediate clearly involves the attack and release of hydroxyl ion rather than an intramolecular concerted mechanism, since the latter would result in the incorporation of molecular oxygen into P-glycerate, which was not observed. The mechanism of decomposition of the peroxide thus appears to be similar to the mechanism of decomposition of the peroxide intermediate formed during the reaction of hydrogen peroxide with α-diketones studied by Bunton (1961) and discussed in the Literature Review.

Enzyme Purity

An intriguing question raised by these results is whether RuDP oxygenase and RuDP carboxylase are the same or different proteins. The simplest conceptual model would assign the carboxylative and oxygenative activities to different proteins. The consistent differences in percentage yield can be interpreted to support this model. So, too, does the fact that upon storage in $(NH_4)_2SO_4$ the carboxylase activity declines more rapidly than does the oxygenase activity. Different pH optima may be interpreted as evidence for two independent proteins. Although evidence for homogeneity was obtained by gel electrophoresis and analytical ultracentrifugation, these results must be interpreted with caution. The carboxylase has an extremely low catalytic activity. Taking the value of 1300 moles RuDP carboxylated per hr per mole of enzyme at 30^{o} reported by Paulsen and Lane (1966) and assuming 8 catalytic sites, calculations reveal that each catalytic site operates at a rate of only about 3 carboxylations per second. Given such low activity and the fact that the oxygenase assay required 1 to 2 mg of protein, it is quite conceivable that a contaminant, undetectable by the techniques used, could well account for the observed oxygenase activity.

The second model would assign the carboxylative and oxygenative activities to the same protein and there

are several variants of this model. This model is supported by an ostensibly pur enzyme and an apparently similar substrate specificity. One such variant of this model would be the existence of two independent catalytic sites, one for carboxylation, the other for oxygenation. Another variant would be the existence of a common catalytic site for both carboxylation and oxygenation and that this site is capable of undergoing subtle transformations such that in one state it is capable of catalysing carboxylation while in the other it is capable of catalysing oxygenation. For example, carboxylation might require that the sulphydryl groups, known to be involved at the active site, be in the reduced state, while oxygenation might require that the same sulphydryl groups be in the oxidised disulphide state. The differences in the pH optima for carboxylation and oxygenation can be rationalized in such a manner. For example, carboxylation might require an amino group to be protonated while oxygenation may require the same group to be unprotonated. The failure to separate the two activities is consistent with this one protein hypothesis. A consistent decrease in the carboxylase to oxygenase activity ratio that is observed during the purification might simply be due to the removal of an activator of the carboxylase and/or an inhibitor of the oxygenase. If this is the case then these effectors must be of high molecular weight since the initial extract was

always subjected to gel filtration on Sephadex G-25 before assay. There is precedent for such an effector of the carboxylase. Wildner and Criddle (1969) have described what is termed the "light activating factor" which they isolated from mutant tomato leaves. This factor has a molecular weight of about 6000 and stimulated purified tomato leaf carboxylase activity by about 2.7 fold. Alternatively, the decrease in the carboxylase to oxygenase activity ratio might be due to transformation of the catalytic site from a form suited to carboxylation to one suited to oxygenation. The differences in the rates of loss of activity observed upon storage in $(NH_4)_2SO_4$ can be rationalized in a similar manner. The observation by Ogren and Bowes (1970) that the carboxylase is inhibited by oxygen and that the inhibition is competitive with respect to CO_2, suggests that the carboxylase and the oxygenase are one and the same protein. Another item of circumstantial evidence concerns the nature of the cyanide inhibition. The pronounced lag in the inhibition of the oxygenase reaction is strikingly similar to that observed for the carboxylation reaction by Wishnick and Lane (1969). These authors demonstrated the formation of an inactive ternary complex of enzyme, RuDP and cyanide. While a more detailed study of the cyanide inhibition of the oxygenase reaction has not been conducted, owing to the insensitive nature of the assay, the general features of

the inhibition are in agreement with those reported for the carboxylase.

One final piece of evidence favoring one protein for both carboxylase and oxygenase activity is of a physiological nature. It is the remarkable constancy of the CO_2 compensation point within a species of plants. The CO_2 compensation point is the atmospheric concentration of CO_2 at which there is no net gain or loss of CO_2 during photosynthesis. This is clearly a reflection of the relative carboxylase and oxygenase activities. A low carboxylase to oxygenase ratio would result in a higher CO_2 compensation point and vice versa. If, on one hand, the carboxylase and the oxygenase activities were associated with different proteins, one might expect to find varietal differences in the CO_2 compensation point, depending on the relative amounts of each protein. If, on the other hand, the two activities are associated with one and the same protein, then the relative activity of each is tightly coupled or fixed, and one would not expect to find varietal differences in the CO_2 compensation point. Moss et al., (1969) have measured the CO_2 compensation point at 25° in 100 varieties of wheat. The value they found was 52 ± 2 ppm CO_2. Similar measurements on 44 genotypes of soybean by Cannell et al., (1969) gave a constant value of 73 ± 0.9 ppm CO_2. While such evidence may be quite fortuitous, it is consistent with the concept that the

carboxylase and the oxygenase are one and the same protein.

The question whether there are one or two proteins does not invalidate the results obtained here which clearly demonstrate the enzymatic oxidation of RuDP by molecular oxygen, an activity described here as RuDP oxygenase.

The Relationship of RuDP Oxygenase to Photorespiration

Both the *in vivo* and *in vitro* [^{18}O] oxygen results are consistent with the view that the oxygenation of RuDP is the primary photorespiratory event in precisely the same manner that the *in vivo* and *in vitro* [^{14}C]CO_2 results are consistent with the view that the carboxylation of RuDP is the primary photosynthetic event. The Warburg effect, photorespiration and glycolate synthesis are most evident under conditions of limiting CO_2 concentration and high oxygen concentration. Furthermore, these processes may, in large part, be overcome by increasing the concentration of CO_2. It is clear that *in vivo* the concentrations of CO_2 and oxygen must govern the relative activities of the carboxylase and the oxygenase. It is proposed that the properties of RuDP oxygenase are consistent with its function as the primary oxidation reaction in photorespiration. It was previously established (Chapter I) that the other oxygen consuming reaction of

photorespiration, glycolate oxidase, is effectively saturated under an atmosphere of about 60% oxygen. Yet photorespiration is known not to be saturated even under atmospheres of 100% oxygen. It was therefore particularly interesting that the oxygenase activity was a linear function of the oxygen concentration and that it was not saturated under atmospheres of 100% oxygen.

The high pH optimum of the oxygenase reaction is also of physiological interest, since $[^{14}C]CO_2$ fixation studies *in vivo* with algae (Orth *et al.*, 1966) and with cell free chloroplast preparations (Dodd and Bidwell, 1971) have demonstrated that the percentage of the total carbon fixed into the intermediates of the photorespiratory pathway, glycolate, glycine and serine, increases dramatically at pH values in excess of 9.0. Dodd and Bidwell (1971) eliminated the possibility that this effect might be due to limiting CO_2 concentrations at the higher pH values. This response to pH has lacked any enzymatic explanation. This explanation, based on the response of the oxygenase to pH, is attractive.

Photorespiration, the Warburg effect and glycolate biosynthesis are known to be stimulated by high light intensity, limiting CO_2 concentrations and high oxygen concentrations. These conditions would be expected to lead to RuDP accumulation were it not for the activity of RuDP oxygenase. The rather high pH optimum of the oxygenase is consistent with the phenomenon Jagendorf

and Neumann (1965) and others have reported that, when unbuffered chloroplasts are illuminated, there is a rapid rise in the pH of the medium. The broken chloroplasts used in such studies are not intact nor are they capable of fixing CO_2. A rise in the pH of the medium is interpreted as being due to the transport of protons into the space enclosed by the lamellar membrane. Thus, in vivo, illumination will result in the stroma becoming more alkaline. This pH shift, of itself, would be expected to lead to conditions favoring oxygenation of RuDP with a concomitant decrease in the carboxylation of RuDP. But additionally a shift to more alkaline pH's will effectively shift the equilibrium between CO_2 and bicarbonate towards bicarbonate. Since CO_2 is the substrate for the carboxylase, this pH shift will further limit the extent of carboxylation. In addition, illumination will clearly result in an increase in the oxygen concentration in the chloroplast. Therefore, the selective requirement of high light intensity for photorespiration and glycolate synthesis can be explained firstly by a light dependent pH shift from conditions favoring carboxylation of RuDP to conditions favoring oxygenation of RuDP. This effect is reinforced by the light dependent increase in oxygen concentration in the chloroplast. Whether or not the pH ever rises as high as 9.3 in vivo is unknown, but any shift in this direction will favor oxygenation. Indeed the control

of photosynthesis and photorespiration might ultimately centre around subtle shifts in the pH of the chloroplast.

While the above discussion appears to make teleological sense if nothing else, there is a major discrepancy in the fact that the pH optimum of P-glycolate phosphatase, also a chloroplast enzyme, is about 6.3 (Richardson and Tolbert, 1961) with very little activity above pH 8.0. Although evidence is lacking, the suggestion that P-glycolate phosphatase is a membrane bound enzyme involved in the transport of glycolate out of the chloroplast, is attractive. If this were the case, the pH optimum of the solubilized enzyme may be artifactual.

Although the activity of RuDP carboxylase in crude extracts is insufficient to account for the *in vivo* rates of photosynthetic CO_2 fixation, few would challenge the pivotal role of the carboxylase. Whether the activity of RuDP oxygenase in crude extracts is sufficient to account for the observed rates of glycolate synthesis and photorespiration *in vivo* is difficult to answer meaningfully. The optimum oxygenase conditions of 100% oxygen and pH 9.3 probably do not exist *in vivo*. The activity of RuDP oxygenase in crude extracts is about 25% that of RuDP carboxylase when both are assayed under optimum conditions. However, if the activities were determined at an intermediary pH, say 8.6, under natural atmospheric conditions of 0.03% CO_2 and 21%

oxygen, oxygenation would exceed carboxylation. This is perhaps an unfair comparison since the K_M for CO_2 for the purified carboxylase is known to be anomolously high (see Literature Review for a discussion of this feature of the carboxylase). The results in Fig. 18 indicate that under 100% oxygen a spinach leaf is capable of photorespiring at rates approaching those of photosynthesis. Under no circumstances, however, has the rate of oxygenation exceeded that of carboxylation when the assays are performed under their respectively optimal conditions.

CONCLUDING DISCUSSION

The inhibition of net photosynthetic CO_2 fixation by molecular oxygen, the Warburg effect, can now be explained in terms of the action of RuDP oxygenase which results in the formation of P-glycolate and P-glycerate. Not only does this reaction reduce the pool of RuDP available for carboxylation and thus the rate of CO_2 fixation but it also leads to the formation of P-glycolate, the subsequent metabolism of which results in the loss of CO_2. Thus, the oxygenation of RuDP both inhibits CO_2 fixation and stimulates CO_2 release.

As the glycolate pathway is now conceived, photorespiration involves the uptake of oxygen at two sites, firstly in the synthesis of P-glycolate and secondly in the oxidation of glycolate by glycolate oxidase. The release of CO_2 occurs at another site, namely glycine decarboxylase. Since the conversion of 2 moles of RuDP to 2 glycolates and then to one serine involves the consumption of 3 moles of oxygen and the release of 1 mole of CO_2, the photorespiratory quotient is theoretically 3 : 1. This contrasts with the respiratory quotient for dark respiration of 1 : 1.

In order to release one mole of CO_2, two moles of RuDP must first be oxygenated. Therefore at the CO_2 compensation point one can predict that the rate of RuDP oxygenation is twice the rate of RuDP carboxylation. Furthermore one would also predict that at the CO_2 compensation point the rate of oxygen uptake is three times the rate of CO_2 uptake. Bulley and Tregunna (1970) have attempted to measure the rates of CO_2 exchange in soybean leaves at the CO_2 compensation point. In 21% oxygen, the CO_2 exchange rates were determined to be 61 and 87 μmoles $dm^{-2}hr^{-1}$ at light intensities of 6.5×10^4 and 28×10^4 ergs $cm^{-2}sec^{-1}$. At comparable light intensities, the rate of oxygen exchange of soybean leaves at the CO_2 compensation point in 19% oxygen has been measured by Mulchi *et al.*, (1971). They reported values ranging from 300 to 350 μmoles $dm^{-2}hr^{-1}$. While these 2 sets of values are not strictly comparable, it is interesting to note that the rates of oxygen exchange exceed those of CO_2 exchange by 4 to 5 fold. The values for the rates of CO_2 exchange are underestimates owing to the internal recycling of photorespiratory CO_2.

The requirement of light for photorespiration and for the incorporation of molecular oxygen into the carboxyl groups of glycine and serine is indirect and dependent upon the formation of RuDP, in the same sense that the CO_2 fixation reactions of photosynthesis are

truely dark reactions. Thus light is required solely to regenerate the reducing equivalents and ATP that is consumed in the regeneration of RuDP from P-glycerate.

The ultimate question as to the function of photorespiration remains problematic. There is a tacit assumption throughout the literature that photorespiration is a deleterious process. After all, the synthesis of glycine and serine by the glycolate pathway could just as easily be achieved by synthesis from P-glycerate. It would be remarkable if natural selection had not eliminated such a deleterious process. The fact that photorespiration has not been removed by selection suggests that its occurrence confers an advantage on the plant, albeit at some expense.

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