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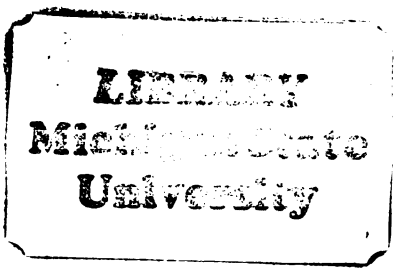

This is to certify that the

dissertation entitled

The Enzymatic Mechanism of Ribulose Bisphosphate

Carboxylase/Oxygenase

presented by

Robert Michael Mulligan

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

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Date June 28, 1983

O-12771

THE ENZYMATIC MECHANISM OF RIBULOSE BISPHOSPHATE CARBOXYLASE/OXYGENASE

By

Robert Michael Mulligan

A DISSERTATION

submitted to
Michigan State University
in partial fulfillment of the requirement
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1983

ABSTRACT

THE ENZYMATIC MECHANISM OF RIBULOSE BISPHOSPHATE CARBOXYLASE/OXYGENASE

By

Robert Michael Mulligan

The mechanism of ribulose bisphosphate carboxylase/oxygenase has been examined. The carboxylated intermediate was liberated from a carboxylase reaction by acidification and stabilized by reduction. The reduction product was identified as carboxyarabinitol bisphosphate and was obtained at a stoichiometry of 0.04 per enzyme active site. An intermediate with a labile phosphate ester was also liberated by acidification of a carboxylase reaction. Phosphate release occurred with a stoichiometry of about 0.04 per enzyme active site, and was probably due to the lability of the carboxylated intermediate. Attempts to demonstrate the presence of other intermediates of the carboxylase and oxygenase reactions failed to verify the existence of these other intermediates.

Glyoxylate is a slowly reversible inhibitor of ribulose bisphosphate carboxylase. Glyoxylate forms an adduct with a catalytic site amino acid residue which maybe involved in catalysis. Glyoxylate did not selectively inhibit either

carboxylase or oxygenase activities. Inhibition of carboxylase activity by glyoxylate exhibited an apparent dissociation constant of 3.3 mM and a maximal psuedo first order rate constant for inhibition of 7×10^{-3} $second^{-1}$. The dissociation of glyoxylate from the enzyme exhibited a first order rate constant of 12×10^{-3} $second^{-1}$. Glyoxylate also elicted a two-fold stimulation in carboxylase activity of the unactivated enzyme. The stimulation of activity by glyoxylate was not due to increased activation of the enzyme due to carbamate formation.

Glyoxylate inhibited photosynthesis by intact chloroplasts, but, carboxylase activity was not substantially inhibited. Other weak acids also inhibited CO_2 fixation, and the inhibition of photosynthesis may be a general effect of weak acids. Glyoxylate was reduced to glycolate by illuminated chloroplasts and glyoxylate reductase may serve to scavenge and detoxify glyoxylate in chloroplasts.

Digestion of activated spinach carboxylase with trypsin rapidly inhibits catalytic activity, but carboxyarabinitol bisphosphate binding is only slightly diminished. Gel electrophoresis of the digested protein demonstrated that limited tryptic digestion of activated carboxylase converted the native large subunit (52,800 daltons) to 2 forms with molecular weights of 51,300 and 49,300 daltons. Thus carboxyarabinitol bisphosphate binding may not be used to determine the catalytically competent active sites if proteolysis is a factor.

ACKNOWLEDGEMENTS

I would like to express my appreciation for financial support and professional guidance provided by Dr. N.E. Tolbert. Gratitude is also extended to Dr. K. Schubert who was involved in securing and maintaining the Jessie Smith Noyes Fellowship which provided financial support for 2 years.

The research presented in this thesis is partially the result of collaborative efforts with other investigators. Dr. Cathy Cook was a co-investigator in the studies presented in Chapter 3. Barbara Wilson was involved in the studies using intact chloroplasts in Chapter 4. Dr. Irwin A. Rose had considerable influence on the experiments presented in Chapters 1 and 2. Dr. Ming Tien provided helpful discussions on oxygen chemistry. Dr. John Pierce contributed to many stimulating discussions. The members of my guidance committee have been very helpful. It has been a pleasure to have ready access to these individuals for scientific discussions.

I would like to thank my parents for their love and patience. Their input into my personal and intellectual development is greatly appreciated. Thanks are also extended to Mr. William Kushner and Dr. Christopher Papasian for stimulating my interest in science. Finally, the love and emotional support of my wife, Maureen Ford Mulligan, has

carried me through the frustrating hours and shared with me the joyous moments.

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LIST OF ABBREVIATIONS

Bicine	N,N'-bis(2-hydroxyethyl)glycine
CABP	2'-C-carboxy-arabinitol-1,5-bisphosphate
chl	chlorophyll
DETAPAC	diethylenetriaminepentaacetic acid
DMPO	5,5'-dimethyl-1-pyrroline-N-oxide
MnSOD	the superoxide dismutase which contains manganese as a cofactor
NBT^{2+}	nitro blue tetrazolium
ribulose-P_2	D-erythro-pentulose-1,5-bisphosphate
xylulose-P	D-threo-pentulose-1,5-bisphosphate

INTRODUCTION

The literature concerning ribulose-P_2 carboxylase/oxygenase is overwhelming in scope but several recent reviews have summarized it effectively (Lorimer, 1981a; Lorimer and Andrews, 1981; Jensen and Bahr, 1977). Major recent developments of the molecular aspects of the synthesis and assembly of the protein have occurred (Bogorad, 1981), and these will be reviewed briefly. In addition, literature on the reaction mechanism of ribulose-P_2 carboxylase/oxygenase will be summarized.

Synthesis and Assembly of Ribulose-P_2 Carboxylase/Oxygenase. Ribulose-P_2 carboxylase from higher plants is a hexadecameric protein of eight identical small subunits of 12,000 to 14,000 daltons and eight identical large subunits of 50,000 to 55,000 daltons (Kawashima and Wildman, 1970). The enzyme is roughly spherical in shape as determined by electron microscopy (Trown, 1965) and ultracentrifugation (Paulsen and Lane, 1966). Crystallized enzyme from tobacco has been studied by X-ray diffraction analysis, and is characterized by a four-fold axis of symmetry and a plane of symmetry parallel to the four fold axis of symmetry (D_4 symmetry group)(Eisenberg et al., 1978).

The gene for the small subunit of ribulose-P_2 carboxylase is located on the nuclear genome (Kawashima and Wildman, 1972). The small subunit is synthesized by free

cytoplasmic ribosomes as a precursor with a molecular weight of 4,000 to 5,000 daltons greater than the processed small subunit (Dobberstein et al., 1977). This precursor polypeptide is taken up, processed and incorporated into ribulose-P_2 carboxylase holoenzyme by intact chloroplasts (Chua and Schmidt, 1978). The precursor of the small subunit has not been detected in intact chloroplasts. Therefore processing probably occurs during or soon after transport. Sequence analysis of the 4,000 dalton peptide, (cleaved by proteolysis from the precursor) demonstrated that it is composed of 44 mostly nonpolar amino acid residues and occupies the N-terminus of the precursor peptide (Chua and Schmidt, 1979). This sequence may be involved in the post-translational transport mechanism, and has been designated as a "transit peptide" to distinguish it from the "signal peptide" of precursors of secretory proteins. Transit peptides may occur on the C or N terminus of precursors of organelle proteins which are synthesized by free cytoplasmic ribosomes. The synthesis of the precursor is not intimately coupled to transport. In comparison, signal peptides occur on the N terminus of secretory proteins which are synthesized by membrane-bound ribosomes and protein synthesis is directly coupled to transport.

The small subunit of the spinach enzyme has been sequenced by amino acid sequencing (Martin, 1979), and the small subunit of the pea enzyme has been sequenced by DNA

sequencing (Bedbrook et al., 1980). The amino acid sequence of the small subunit from these sources is poorly conserved. At present it is not possible to discuss structure-function aspects of the small subunit. This subunit has no known function, except that it dramatically improves the solubility of the large subunit which is essentially insoluble in its absence.

The gene for the large subunit of ribulose-P_2 carboxylase is maternally transmitted (Kawashima and Wildman, 1972) and the location of the gene in the chloroplast genome has been documented by physical techniques (Link and Bogorad, 1980; Bedbrook et al., 1979). The large subunit gene of maize has been cloned and sequenced, and the amino acid sequence is highly conserved when compared to peptides isolated from carboxylases of most photosynthetic organisms (Bogorad, 1981). In fact, the amino acid sequence between residues 170 to 220 is essentially completely conserved in Synechococcus, a blue-green algae, Chlamydomonas, a green algae, and spinach and maize, flowering plants (Lorimer, 1983). The amino acid sequence of the large subunit from the purple sulfur bacterium, Rhodospirullum rubrum, is considerably less homologous when compared to the sequence of the large subunit from other photosynthetic organisms (Hartman et al., 1982). However, considerable homology does exist in several sequences which probably define the active site residues (174-180 and 326-338). The relative lack of

sequence homology between the R. rubrum and other bacterial and plant carboxylases suggests that the enzyme could have evolved independently in the two groups of organisms. This notion could provide new support for the view that ribulose-P_2 oxygenase is an unavoidable consequence of the ribulose-P_2 carboxylase reaction (Lorimer, 1981a).

The Structure of the Catalytic Site. Ribulose-P_2 carboxylase is activated by incubation with CO_2 and Mg^{2+} with commensurate changes in kinetic properties (V_{max} of the carboxylase and oxygenase increase, $K_m(CO_2)$ decreases)(Jensen and Bahr, 1977). The protein reacts with CO_2 in a slow reaction, followed by the rapid association with Mg^{2+}(Eq. 1) (Lorimer et al., 1976).

$$\text{Enz} \overset{\text{slow}}{\longleftrightarrow} \text{Enz-CO}_2 \overset{\text{fast}}{\longleftrightarrow} \text{Enz-CO}_2\text{-Mg}^{2+} \qquad \text{(Eq. 1)}$$

The reaction of CO_2 with a specific lysyl residue of the large subunit (lysine 201) has been verified and localized by methylation of the lysyl-carbamate, isolation of the derivitized peptide and sequence analysis (Lorimer and Miziorko, 1980). Carbamate formation occurs by the reaction of an unprotonated lysyl residue with a CO_2 molecule (Lorimer, 1981b). This form of the enzyme binds Mg^{2+}, and several investigators have speculated that the activating metal ion may directly interact with the carbamate (Miziorko and Sealy, 1980; Pierce et al., 1980), although there is no

data available on this aspect. The Mg^{2+} may pull the equilibrium toward activation, and this may account for the effect of Mg^{2+} on the pH optimum of activation (Sugiyama et al., 1968a). The distance between an activating metal ion, Mn^{2+}, and the substrate CO_2 has been determined as 5.4 $\pm$ 0.1 A by NMR spectroscopy (Miziorko and Mildvan, 1974). CABP, a tight binding inhibitor, renders the activating CO_2 molecule and metal ion nonexchangeable (Miziorko and Sealy, 1980). The stoichiometry of CABP, CO_2 and metal ion incorporated is 1:1:1 per protomer, and ^{14}C labelled CABP or CO_2 may be used to measure the number of sites capable of binding these ligands (Pierce et al., 1982).

The large subunit of ribulose-P_2 carboxylase/oxygenase contains the catalytic site and the activation site. Lysyl residues (175, 334) and cysteinyl residues (172, 459) have been derivitized by treatment of the enzyme from spinach with affinity ligands, and these residues may define the geography of the catalytic site (Schloss et al., 1978b). Of these four amino acid residues, only lysine 175 was derivitized under activating conditions (in the presence of Mg^{2+}). Lysine 175 also forms a Schiff-base with pyridoxal-P (Spellman et al., 1979) or glyoxylate (Cook, Tolbert, and Hartman, unpublished). Lysine 175 exhibits enhanced nucleophilicity toward these active site probes, and the reactivity suggests that this residue could be catalytically functional. It is tempting to speculate that lysine 175 may be

the base which abstracts a proton from C-3 of ribulose-P_2 and catalyzes enolization.

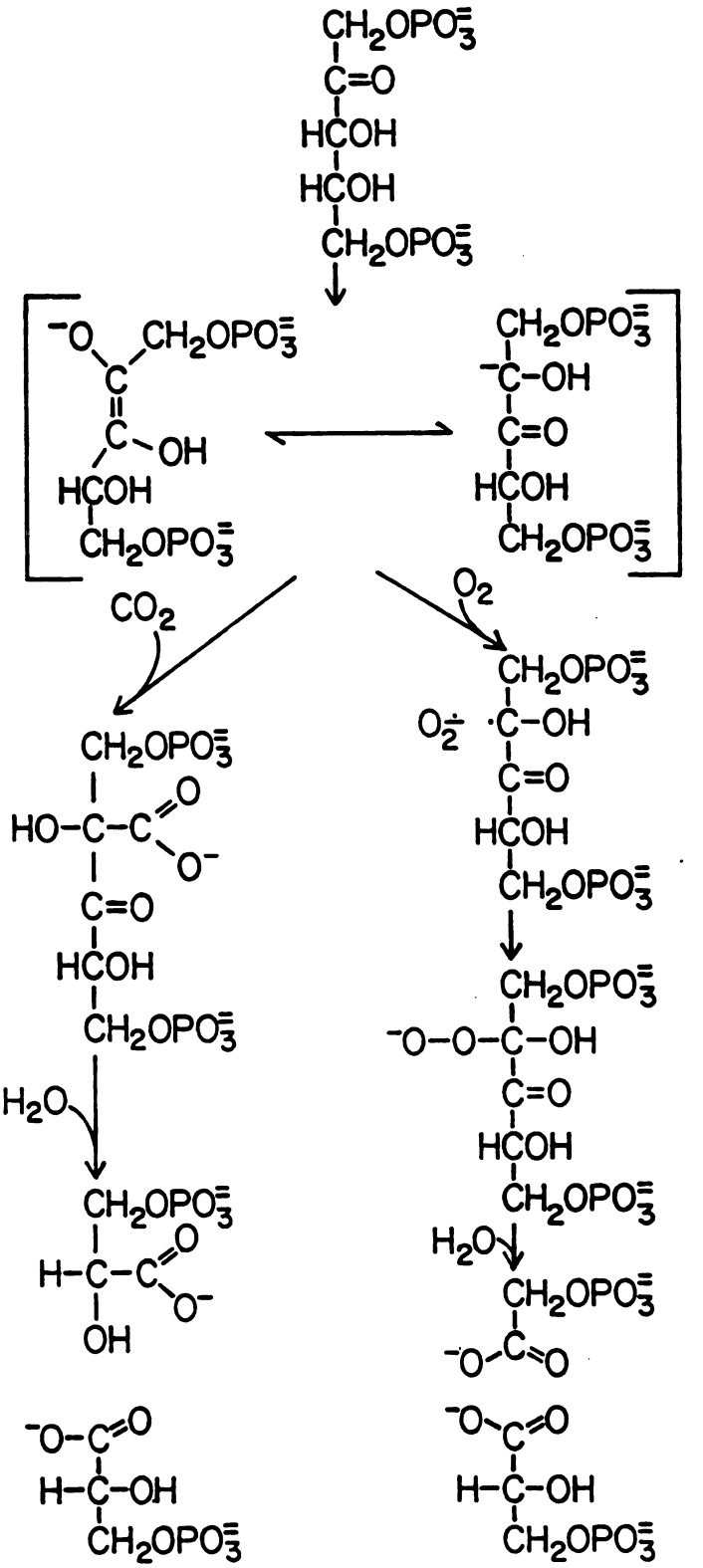
Hartman and colleagues have compared the susceptibility of the R. rubrum enzyme to active site probes. A lysyl residue which corresponds to lysine 175 is derivitized by pyridoxal phosphate. The counterpart of lysine 334 has not been derivitized, although methionine 335 was derivitized by active site directed probes. Thus the assignment of these regions of the polypeptide as part of the catalytic site seems reasonable. The role of cysteinyl residues in the catalytic mechanism of ribulose-P_2 carboxylase has been questioned since these residues (cysteine 84, 99, 172, 192, 221, 247, 427 and 459 of the spinach enzyme) are substituted in the R. rubrum enzyme (Hartman et al., 1982).

Group specific reagents have been utilized by several investigators to establish the involvement of specific amino acid residues in catalysis. Arginine residues have been implicated by reaction with dicarbonyl reagents (Schloss et al., 1978a; Lawlis and McFadden, 1978); tyrosine by reaction with tetranitromethane (Robison and Tabita, 1979); cysteine by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (Rabin and Trown, 1964; Trown and Rabin, 1964); histidine by reaction with diethyl pyrocarbamate (Saluja and McFadden, 1980). Typically the only proof that a catalytic site amino acid residue was involved in the inactivation phenomenon was

based on ribulose-P_2 protection from inhibition. Protection by substrate is not sufficient proof that an active site amino acid is involved in these studies since substrate binding could modify the accessibility or reactivity of an amino acid which is in any part of the protein. Therefore, evidence that enzyme inactivation due to specific reaction with an active site residue has been weak in these studies.

In summary, the amino acid residues which appear to have a specific role in catalysis are lysyl 201, which reacts with CO_2 during activation, and lysyl 175, which exhibits enhanced nucleophilicity and reactivity with catalytic site directed probes under activating conditions. Other amino acids are derivitized by catalytic site probes in the absence of Mg^{2+} (lysyl 334, cysteinyl 173 and 458). This indicates that a comformational change occurs in the enzyme with the addition of Mg^{2+} and that the catalytic site may be defined by amino acid residues from three distinct portions of the polypeptide.

The Reaction Mechanism of Ribulose-P_2 Carboxylase/Oxygenase. Calvin (1954) suggested that the mechanism of ribulose-P_2 carboxylation involved enolization of ribulose-P_2, nucleophilic attack on CO_2 to form a six carbon carboxylated intermediate, followed by hydrolysis to two molecules of glycerate-3-P (Scheme 1, page 8). This mechanism has been essentially verified by recent reports. Other carboxylation mechanisms have been considered, such as those involving



Scheme 1

thiohemiketal (Rabin and Trown, 1964) or ketimine formation (Wishnick and Lane, 1969) between an amino acid residue and ribulose-P_2. Ketimine formation could not be observed by reductive trapping. These mechanisms now seem unlikely because the oxygen atoms at carbon two and three of ribulose-P_2 are retained in the products of the carboxylation reaction (Sue and Knowles, 1978; Lorimer, 1978).

Enolization of ribulose-P_2 by ribulose-P_2 carboxylase/oxygenase from Rhodospirullum rubrum has been extensively studied by Knowles and his associates. Activated ribulose-P_2 carboxylase catalyzes the exchange of solvent protons with the carbon 3 proton of ribulose-P_2 (Saver and Knowles, 1982), and tritium label is lost from carbon 3 of ribulose-P_2 (Sue and Knowles, 1982a). The rates of exchange are slow relative to catalysis. These data were interpreted to indicate that enolization is a slow step in the ribulose-P_2 reaction mechanism, but that enolization is not rate determining. This conclusion was partially confirmed by the primary kinetic isotope effect for 3-deuterio-ribulose-P_2 which is 1.2 (Sue and Knowles, 1982b). This value is too small to be an isotope effect for the rate-limiting removal of the carbon 3 proton.

Siegel and Lane (1973) reported that the epimeric mixture of 2-carboxy-arabinitol-P_2 and -ribitol-P_2 was inhibitory to ribulose-P_2 carboxylase, and suggested that a six carbon carboxylated intermediate was formed during the car-

boxylase reaction. Pierce et al. (1980) resolved the mixture of epimeric carboxy-pentitol-P_2 and demonstrated that 2-carboxy-arabinitol-P_2 (CABP) was the species which the enzyme bound exceedingly tightly (K_D= 10 pM). Since 2-carboxy-arabinitol-P_2, not the ribitol epimer, had potent inhibitory properties, CABP was proposed to be a transition state analogue of the carboxylation reaction.

Recently, Schloss and Lorimer (1982) isolated the reduction product of the carboxylated intermediate and confirmed the structure of the enzyme-bound intermediate as 2-carboxy-3-keto-D-arabinitol-1,5-bisphosphate. This intermediate could undergo carbon bond breakage by attack of hydroxyl ion on the carbonyl (carbon 3) with formation of two molecules of D(-)glycerate-3-P.

An aspect of ribulose-P_2 oxygenase which is poorly understood is the mechanism of the reaction with dioxygen. Dioxygen exists in the ground state as a triplet; it has two unpaired electrons and the direction of spin for each electron is the same. The products of the oxygenase reaction, glycerate-3-P and glycolate-2-P, are singlet molecules, i.e. all electrons are paired in orbitals with opposite spin directions. The lowest singlet state of dioxygen is 22 Kcal per mol above ground state and this species is probably not involved in the oxygenase reaction. Angular momentum must be conserved in a chemical reaction, just as in all physical interactions. Therefore, over the course of the oxygenase

reaction the angular momentum of the two spinning electrons of dioxygen must ultimately cancel each other in the products. The electron spins may become opposite through spin inversion, a process where the direction of electron spin switches orientation, or if spatial separation occurs, the electron spins may become uncoupled and lose their original orientation. The time required for spin inversion varies between 1 to 10^{-9} s depending on the environment (Hamilton, 1974), and is much slower than most chemical reactions. As a consequence of the conservation of the angular momentum of the spinning electrons and the slow rate of spin inversion, the direct reaction of a singlet and triplet to give singlet products is impossible.

Oxygenases typically utilize either a transition metal or an organic cofacter to catalyze a reaction with O_2. The d-orbitals of transition metals may overlap with the p-orbital of dioxygen. The total angular momentum of the electrons in the metal-oxygen complex is involved, and oxygen can react by a nonradical (ionic) mechanism (Hamilton, 1969). In this case, the net angular momentum of the electrons is the same after the substrates have reacted. Organic cofactors readily react with O_2 by virtue of their ability to form resonance stabilized radical intermediates. For example consider the reaction of reduced flavin with O_2 (Eq. 2) (Massey et al., 1969):

$$\text{Flavin-}H_2 + O_2 \text{ ---> Flavin-}H^{\cdot} + O_2^{-} + H^{+} \text{ ---> Flavin} + H_2O_2 \quad \text{(Eq. 2)}$$

In this reaction, a singlet (reduced flavin) and a triplet (dioxygen) react with a one electron transfer to form two doublets (two free radicals, flavin semiquinone and superoxide). Note that the net angular momentum has not changed, but the electron spins become uncoupled when they reside on different molecules. Subsequent reaction may occur between the two radicals, yielding oxidized flavin and hydrogen peroxide. Therefore, it is a spin allowed process for a triplet and singlet to react and give two doublet intermediates, which may recombine to singlet products (Hamilton, 1974).

Ribulose-P_2 oxygenase seems to be an anomoly among enzymes which catalyze oxygenase reactions, since it contains no transition metal or organic cofactor (Chollet et al., 1975; McCurry, 1979; this thesis, Chapter 2). The enzyme is an internal monooxygenase, in which one atom of dioxygen is incorporated into product (the carboxy of glycolate-2-P) and one atom into H_2O (Lorimer et al., 1973). An oxygen atom from dioxygen is not incorporated into the carboxy of glycerate-3-P; thus formation of a dioxetane ring with carbons two and three during oxygenation seems unlikely. The enzyme has been reported to contain copper and iron, although these metals may be removed to a fraction of the enzyme active site concentration without commensurate loss in catalytic activity (McCurry, 1979). The enzyme also contains a trace

of flavin (Chapter 2), although this was present at far less than 1:1 stoichiometry. In summary, there is very good evidence that ribulose-P_2 lacks transition metals or organic cofactors at stoichiometric levels.

Two mechanisms for ribulose-P_2 oxygenase have been proposed (Lorimer, 1981a; Lorimer and Andrews, 1981). Scheme 2 (page 57) shows a possible mechanism for ribulose-P_2 oxygenase. The enediol(ate) may tautomerize to the carbanion and this species may reduce dioxygen by one electron, indicated by the single headed arrow. The intermediates of this reaction would be a carbon based radical and superoxide radical, and these two radicals may recombine to form 2-peroxy-3-keto-pentitol-P_2. This putative intermediate of the oxygenase reaction may decompose by addition and elimination of hydroxyl ion. This mechanism involves the reaction of a triplet and a singlet to form two doublets, followed by a reaction to form two singlets, essentially as discussed with the reduction of dioxygen by reduced flavin. Thus ribulose-P_2 is proposed to react in a manner analogous to flavin. Further discussion of the mechanism of ribulose-P_2 oxygenase is deferred to Chapter 2.

The Role of Ribulose-P_2 Carboxylase/Oxygenase in Photosynthetic Carbon Metabolism. Ribulose-P_2 carboxylase/oxygenase mediates the flux of carbon between two cycles, the reductive photosynthetic carbon cycle and the oxidative photosynthetic carbon cycle. If the kinetic parameters for

ribulose-P_2 carboxylase and oxygenase could be modified, perhaps photosynthesis and crop productivity could be improved. Therefore many investigators have attempted to modify the ratio of carboxylase to oxygenase activities. Hydroxylamine has been reported to selectively inhibit ribulose-P_2 oxygenase (Bhagwat et al., 1978a; Okabe et al., 1979), although this could not be repeated in other laboratories (Brown et al., 1980; Mulligan, unpublished). Superoxide dismutase and NBT^{2+} have been reported to inhibit ribulose-P_2 oxygenase (Bhagwat and Sane, 1978b), and this too could not be repeated in our laboratory (McCurry, 1979; this thesis, Chapter 2). Aliphatic aldehydes have been reported to selectively inhibit ribulose-P_2 oxygenase (Martin and Tabita, 1982), but selective inhibition was not observed with either glyoxylate or trans-2-hexenal in our laboratory (this thesis, Chapters 3 and 7). The reasons for the frequent discrepancies in results from different laboratories are unclear, but may relate to the procedures used to assay the enzyme. For example, an artifact could be generated by preincubation of ribulose-P_2 with hydroxylamine to form the corresponding hydroxamate. Different periods of preincubation for the carboxylase and oxygenase assays might result in different concentrations of substrate, and a differential effect might be suggested due to this artifact. Similarly, time-dependent inactivation by aldehydes might cause a similar artifact if aldehyde is preincubated with enzyme for

different periods before assaying for carboxylase or oxygenase activities.

The ratio of ribulose-P_2 carboxylase to oxygenase is dependent on the relative concentrations of CO_2 and O_2, since these gases are linearly competitive inhibitors with respect to each other (Laing et al., 1974). C_4 plants and some algae utilize this phenomenon by concentrating CO_2 at the site of carboxylation and consequently suppress the oxygenase activity and photorespiration. The activation energies of the carboxylase and oxygenase activities differ, and therefore the ratio of carboxylase to oxygenase varies with temperature (Badger and Collatz, 1977). The metal ion included in activation and assay modifies the kinetic parameters of the enzyme (Christeller, 1981). Enzyme activated with Mn^{2+} exhibits a greatly decreased $K_m(O_2)$ and modified ratio of carboxylase to oxygenase. No compound which has been tested has selectively inhibited the oxygenase, and the ratio of carboxylase to oxygenase activity from any given source is constant within the constraints indicated above. An important point is that the ratio of carboxylation to oxygenation does vary when the carboxylase from various sources is surveyed (Jordan and Ogren, 1981). These authors claim that the kinetic properties of the enzyme are mutable and therefore may be manipulated genetically. Although the ratio of carboxylase to oxygenase varied widely from prokaryotes to higher plants, little difference existed in

higher plant carboxylases. The contention that the kinetic properties of the enzyme vary and are therefore mutable seems sound. Whether genetic manipulation may further improve the specificity of the enzyme for carboxylation must await proof.

The role that ribulose-P_2 carboxylase/oxygenase plays in plant metabolism has been firmly established. It is responsible for photosynthetic carbon fixation and for initiating photorespiration. The enzyme shows changes (1-4 fold) in activity with dark to light transitions (Bahr and Jensen, 1978), but these changes are small relative to the 20 to 40 fold activation of ribulose-5-P kinase, fructose-bisphosphatase and sedoheptulosebisphosphatase (Laing et al., 1981). Therefore, the physiological significance of CO_2 and Mg^{2+} activation has not been clearly demonstrated and remains controversial.

Ribulose-P_2 oxygenase catalyzes a reaction which initiates glycolate synthesis. The source of glycolate has been very controversial. Oxidation of the dihydroxyethyl derivative of thiamine pyrophosphate (an intermediate of the transketolase reaction) by H_2O_2 has been fostered by some as the source of glycolate (Shain and Gibbs, 1971). This reaction is exceedingly slow and is unable to account for physiological rates of glycolate formation (Christen and Gasser, 1980). In addition, a mutant of Arabidopsis which lacks glycolate-2-P phosphatase will grow under low O_2 ten-

sions, but the lesion is lethal under photorespiratory conditions (Somerville and Ogren, 1979). This mutant accumulates glycolate-2-P, even when glycolate oxidase is inhibited by a suicide inactivator. At present, it is generally agreed that ribulose-P_2 oxygenase initiates glycolate synthesis and is principally responsible for photorespiration.

Ribulose-P_2 oxygenase is not solely responsible for light-dependent O_2 Consumption. Photosystem I carries out the reduction of O_2 and this phenomenon is termed psuedo-cyclic electron transport or the Mehler reaction (Marsho et al., 1979). Under ambient CO_2 and O_2 tensions, it has been estimated that 65% of the dioxygen consumption is due to photorespiratory uptake, presumably via ribulose-P_2 oxygenase and that the remaining O_2 consumption is due to O_2 reduction (Behrens et al., 1982).

The outstanding question in the field of photorespiratory carbon metabolism is: Why does this phenomenon exist? The process was proposed to be wasteful, since several oxidative reactions are involved which are not coupled to pyridine nucleotide reduction or phosphorylation (Andrews and Lorimer, 1978). A related viewpoint proposed that ribulose-P_2 oxygenase is an unavoidable consequence of the reaction mechanism of ribulose-P_2 carboxylase (Lorimer and Andrews, 1973b). This may be true whether photorespiration has a useful role or is a wasteful process. A third view-

point suggests that photorespiration is a means for the orderly dissipation of excess reducing equivalents (Powles et al., 1979; Heber and Krause, 1980). Leaves exposed to high irradiant flux (2000 μEinsteins$\cdot m^{-2} \cdot s^{-1}$) in the absence of CO_2 and reduced O_2 tension showed decreased rates of photosynthesis (Powles et al., 1979). Leaves exposed to the same light intensity with CO_2 at the compensation point or atmospheric O_2 tension suffered essentially no photoinhibition. Thus a plant appears to suffer irreversible inhibition of the photosynthetic apparatus when exposed to light in the absence of electron acceptors. Photosystem II is sensitive to photoinhibition (Powles et al., 1979) and addition of CO_2 provides a sink to accept reducing equivalents and phosphorylation energy from the light reactions. Similarly O_2 stimulates the oxidative photosynthetic carbon cycle which effectively consumes reducing equivalents and phosphorylation energy. Thus, either CO_2 or O_2 may provide a sink for electrons from the light reactions and protect the plant from photoinhibition. Although this rationale for the existence of photorespiration is not yet well documented, the model predicts an important physiological role for photorespiration, which is amenable to experimentation and is substantiated by the limited literature on this topic.

CHAPTER 1

THE LABILITY OF AN INTERMEDIATE OF THE RIBULOSE-P_2 CARBOXYLASE REACTION

The mechanism of ribulose-P_2 carboxylase was probed by liberation of an enzyme-bound intermediate through interruption of the catalytic cycle by acid denaturation. This technique was used by Iyengar and Rose (1981a,b) to study the ene-diol intermediate of the reaction catalyzed by triose-P isomerase. In their study, acid treatment of the enzyme-substrate complex liberated an intermediate with a labile phosphate ester. Schloss and Lorimer (1982) utilized this technique for the reductive trapping of the carboxylated intermediate of the ribulose-P_2 carboxylase reaction. In this study, acid denaturation of ribulose-P_2 carboxylase/oxygenase also liberated an intermediate with a labile phosphate ester. The intermediate is probably the carboxylated intermediate rather than the ene-diol(ate) intermediate. A manuscript with results from this chapter has been accepted for publication in Archives of Biochemistry and Biophysics.

Materials and Methods

Ribulose-P_2 was prepared from ribose-5-P and ATP as described by Horecker et al. (1956) and purified by chromatography on Dowex 1-Cl (X8) with a linear 0-0.4 M LiCl gradient prepared in 5 mM HCl. The fractions containing ribulose-P_2 were determined by analysis for alkaline labile P_i or by enzyme assay with ribulose-P_2 carboxylase. These fractions were pooled and the ribulose-P_2 was precipitated as the barium salt in the presence of 50% ethanol. The precipitate was washed by resuspension in 95% ethanol and lyophilized. For the synthesis of [1-^{32}P]ribulose-P_2, [γ-^{32}P]ATP was prepared from $^{32}P_i$ and unlabeled ATP by the exchange reaction catalyzed by glycerate-3-P kinase and glyceraldehyde-3-P dehydrogenase (Glynn and Chappel, 1964). Inorganic phosphate contamination of the ribulose-P_2 preparations was 3-5 % of the ribulose-P_2 concentration. The barium salt of ribulose-P_2 was dissolved by treatment with Dowex 50-H^+. Ribulose-P_2 solutions were converted to the Na^+ salt by treatment with Dowex 50-Na^+, by titration with 0.1N NaOH with rapid stirring, or converted to the triethanolamine salt by titration with 1 M triethanolamine. The pH of ribulose-P_2 solutions was maintained at less than 6.5 for stability, and ribulose-P_2 was added to buffered reaction mixtures (pH 8.2) immediately before use.

The dimethyl ketal of DHAP was obtained from Sigma and converted to the keto form according to the instructions provided. Xylulose-P_2 was prepared as previously described (McCurry and Tolbert, 1977). Other chemicals were of reagent quality and used without further purification.

Ribulose-P_2 carboxylase/oxygenase was purified from Spinacia oleracea L. leaves, which were obtained from a local market. The

purification procedure involved ammonium sulfate fractionation, molecular sieve chromatography and ion exchange chromatography as described elsewhere (McCurry et al., 1982). The enzyme was precipitated with 50% saturated ammonium sulfate and frozen into pellets by dropwise addition to liquid N_2 and stored in a -80°C freezer. Aldolase (rabbit muscle) and other enzymes were obtained from Sigma and were dialyzed exhaustively before use.

Protein concentration was determined by the absorbance at 280 nm (absorbance x 0.61 = concentration in mg/ml) (McCurry et al., 1982). Enzyme active site concentrations were calculated by dividing the protein concentration (mg/ml) by the molecular weight of a carboxylase protomer (68,750 daltons).

Ribulose-P_2 carboxylase/oxygenase assays were performed as described elsewhere (Pierce et al., 1982). The enzyme was activated with 10 mM $NaHCO_3$, 20 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM EDTA and 100 mM Bicine-Na^+ (pH 8.2) for at least 30 min at 30°C. The carboxylase activity was assayed by the addition of enzyme (4-100 µg) to a 0.25 or 0.50 ml assay mixture containing 0.5 mM ribulose-P_2, 10 mM $NaH^{14}CO_3$ ($\geq$0.14 Ci/mol), 20 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM EDTA, and 100 mM Bicine-Na^+ (pH 8.2). Assays were terminated by the addition of 0.1 or 0.2 ml of 2 N HCl, reduced to dryness at 95°C, and analyzed for ^{14}C by liquid scintillation counting. Oxygenase assays were initiated by the addition of 160 µg of activated enzyme to a 1 ml assay mixture containing 0.5 mM ribulose-P_2, 100 mM Bicine-Na^+ (pH 8.2), 20 mM $MgCl_2$ and 0.2 mM EDTA. The initial rate of O_2 consumption was followed polarigraphically with a Rank Brothers oxygen electrode.

Experiments which interrupted the enzyme catalyzed reaction at approximately one turnover were performed using one of two procedures. In one technique, activated ribulose-P_2 carboxylase/oxygenase (0.25 ml) was added to a rapidly stirred solution of ribulose-P_2 (0.25 ml) in 100 mM Bicine-Na^+ (pH 8.2). The ribulose-P_2 solution contained twice the number of moles of ribulose-P_2 as the number of moles of enzyme active sites which would be added. Catalysis was terminated after approximately 0.5 s by the addition of 0.1 ml of 1.2 N trichloroacetic acid. In control reactions, the acid was added to the enzyme before mixing with the ribulose-P_2 solution. Denatured protein was removed by centrifugation and an aliquot was withdrawn for P_i determination. Phosphate was determined spectrophotometrically by an ultrasensitive procedure which was based on the ability of the phosphomolybdate to complex with malachite green (Tashima and Yoshimura, 1975). The colorimetric reagent was prepared by mixing one volume of 3% (w/v) ammonium molybdate with one volume of 0.06% (w/v) of malachite green·HCl in 6 N HCl. Occasionally it was necessary to filter this solution before use. The sample was diluted to 1 ml, and 0.5 ml of the colorimetric reagent was added. The solutions were mixed and the absorbance at 650 nm was determined after 15 min at room temperature. The absorbance was linear up to 10 nmol of P_i with a molar extinction coefficient of 9×10^4.

Since the procedure described above was subject to considerable variability of the reaction time, a simple, rapid mix and quench device was constructed based on the principles outlined by Froehlich et al. (1976). An aluminum block with two grooves (0.5 cm deep) held two 1 ml disposable tuberculin syringes. A plexiglass top with 4 wingnuts

clamped the syringes in place. A hand operated drive plate provided simultaneous and constant flow to the two syringes. Each 1 ml syringe was fitted with an 18 gauge needle which was connected to 1/32" inside diameter (I.D.) silicone tubing (Dow Corning). The solution from each syringe was divided into two with the use of a tee connector and each side was joined to a second tee connector with 1/32" tubing. Each second connector mixed one half of the flow of one syringe with one half of the flow of the other syringe. The solutions then flowed into 5/32" I.D. tubing to facilitate turbulent mixing. The two solutions, which were mixed at the second tee connector, were mixed together and combined in a final tee connector, also followed by 5/32" tubing. The mixed solution then passed into 1/32" tubing which could be varied in length for different reaction times. The reaction was terminated as the mixed solutions passed into a vial containing 0.28 ml or 0.5 ml of 2 N trichloroacetic acid. The acid solution was rapidly stirred with a magnetic stirbar. The apparent reaction time was calibrated by the base catalyzed hydrolysis of 2,4-dinitrophenyl acetate as described by Froehlich et al. (1976). An apparent reaction duration of 150 ms was used in these experiments.

In a rapid mix and quench experiment, [1-^{32}P]ribulose-P_2 was loaded in one syringe in 0.70 ml of solution containing 70 μM ribulose-P_2, 100 mM Bicine-Na^+ (pH 8.2), 20 mM $MgCl_2$, and 10 mM $NaHCO_3$. Ribulose-P_2 carboxylase/oxygenase in activation solution was loaded in the other syringe in 0.70 ml of solution containing 35 μM active sites. In control experiments, enzyme was omitted from the activation solution. The reaction was terminated by passage of the reaction mixture into trichloroacetic acid as described above. An

aliquot was taken from the terminated reaction mixture and analyzed for $^{32}P_i$ by extraction of the phosphomolybdate complex into isobutanol as described by Berenblum and Chain (1938). Alkaline labile phosphate, representing unreacted ribulose-P_2, was determined by addition of NaOH to a final concentration of 0.5 N and incubation at 37°C for 15 min. This sample was then acidified and extracted for P_i.

Results

Phosphate Lability of an Enzyme Liberated Intermediate. The alkaline lability of phosphate esters which are β to a carbonyl carbon is well established. Ribulose-P_2 is known to eliminate P_i under alkaline conditions and rearrange to a compound which is probably 1-deoxy-2,3-diketo,pentitol-5-P (Paech et al., 1978). A similar reaction is catalyzed by triose-P isomerase. Iyengar and Rose (1981a,b) reported that triose-P isomerase catalyzes the formation of the ene-diol form of dihydroxyacetone-P, an enzyme-bound intermediate, which may subsequently rearrange to methylglyoxal by P_i elimination. Iyengar and Rose extended this observation by liberation of the ene-diol from the active site through acid treatment of the enzyme, and approximately 5% of the enzyme-bound phosphate ester was labile.

Since the reaction mechanism of ribulose-P_2 carboxylase/oxygenase is proposed to proceed via an ene-diol(ate) intermediate, the ability of this enzyme to catalyze the formation of an intermediate with a labile phosphate ester was examined. Activated ribulose-P_2 carboxylase/oxygenase (9 to 105 nmol of active sites) was added to a

rapidly stirred solution of ribulose-P_2. The reaction was quenched with 0.1 ml of 1.2 N trichloroacetic acid after approximately 0.5 s under these conditions. This protocol should quench the enzyme catalyzed reaction during steady state catalysis since the turnover time is approximately 0.5 s under these conditions. More P_i was present in the reaction mixture when enzyme reacted with substrate than in the control, in which acid was added to the enzyme before the substrate (Fig. 1). Linear regression of the P_i released as a function of the amount of enzyme present indicates that the P_i released was equivalent to about 5% of the enzyme active sites. With the hand quench technique, ribulose-P_2 carboxylase/oxygenase activated with 1 mM $MnCl_2$ also liberated P_i. When xylulose-1,5-P_2 was mixed with ribulose-P_2 carboxylase, no P_i was liberated. Xylulose-1,5-P_2 is a competitive inhibitor with respect to ribulose-P_2 in the ribulose-P_2 carboxylase/oxygenase reaction (McCurry and Tolbert, 1977).

When 70 μM [1-^{32}P]ribulose-P_2 was mixed with 35 μM active sites of ribulose-P_2 carboxylase and quenched with acid after approximately 150 ms, additional $^{32}P_i$ was extractable which was equivalent to 4.1 ± 0.7% of the enzyme active site concentration (mean ± S.E., 6 determinations). Analysis of the unreacted ribulose-P_2 by extraction of P_i after alkaline treatment indicated that each active site turned over an average of 0.13 times. In an experiment in which the enzyme catalysis was allowed to go to completion, no P_i was liberated in the normal course of catalysis. Thus P_i liberation is totally dependent on the interruption of the catalytic cycle, and was not a phenomenon associated with normal catalysis.

Figure 1. Phosphate Liberation as a Function of the Amount of Ribulose-P_2 Carboxylase Active Sites. Activated enzyme (9 to 105 nmol of active sites in 0.25 ml) was added to a rapidly stirred solution (0.25 ml) of 0.1 M Bicine-Na^+ (pH 8.2) containing twice the number of mol of ribulose-P_2 as active sites added. Trichloroacetic acid (0.2 ml of 1.2 N) was added after about 0.5 s. Phosphate was determined by the malachite green procedure as described in materials and methods. The line calculated by linear regression has a slope of 0.048 ± 0.009 (mean ± S.E.).

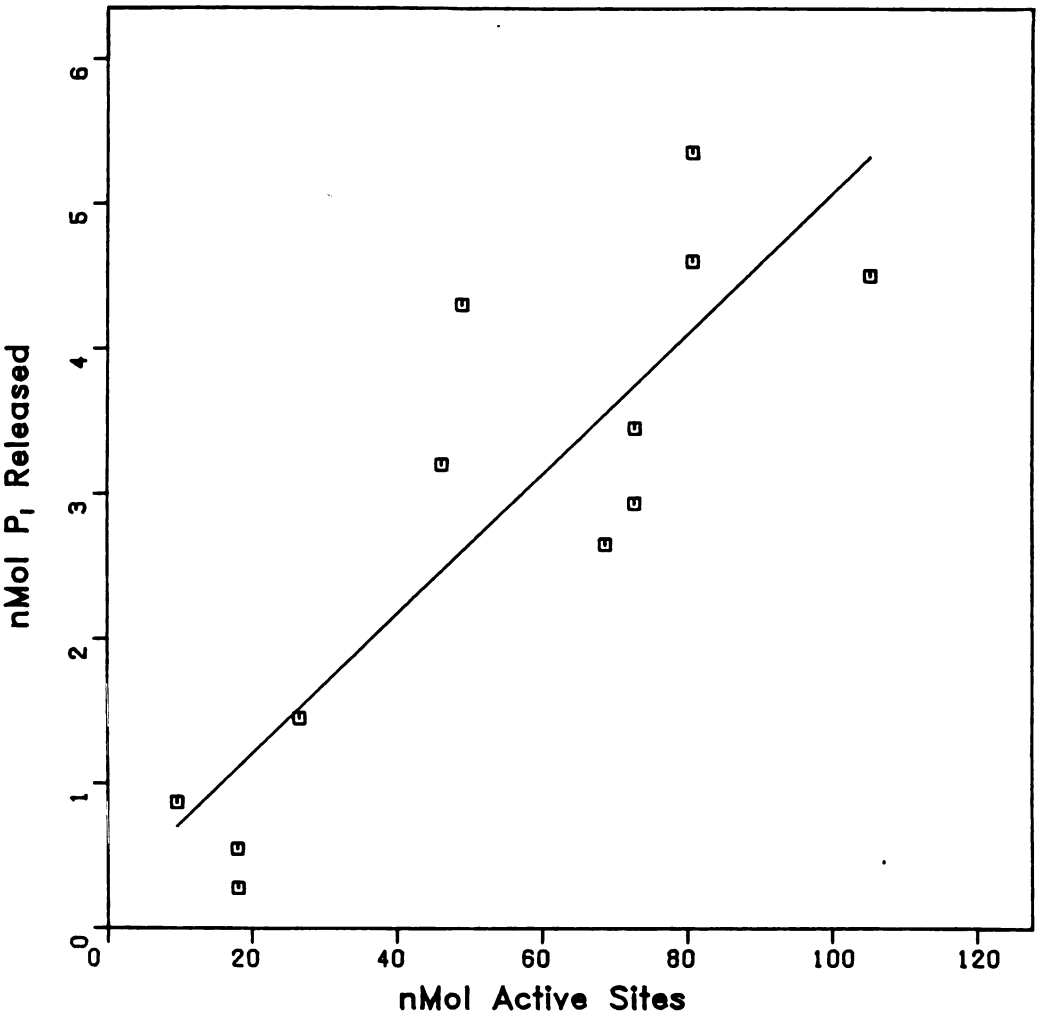

nMol P_i Released
0
1
2
3
4
5
6
0
20
40
60
80
100
120
nMol Active Sites

Figure 1.

The intermediate with the labile phosphate ester is probably either the ene-diol(ate) or the carboxylated intermediate. The ene-diol intermediate liberated from triose-P isomerase by acid denaturation is rapidly labile, with a half life for phosphate elimination between 12 and 17 ms (Iyengar and Rose, 1981b). Therefore the stability of the liberated intermediate from the carboxylase reaction may provide some information regarding its identity. Schloss and Lorimer (1982) stabilized the carboxylated intermediate by reduction with $NaBH_4$. A similar experiment was designed to stabilize the labile intermediate after a brief period in acid. However, treatment of the acid quenched reaction with buffered $NaBH_4$ interferred with subsequent extraction of $^{32}P_i$; destruction of the $NaBH_4$ with glucose or acid did not alleviate the interference. Another approach to examine the stability of the liberated intermediate was to enzymatically convert it to the final products of the carboxylation reaction. Five seconds after acid treatment, an additional 0.5 ml of activation buffer with or without 4.12 mg of purified spinach carboxylase was added to the acid quenched solution (Table 1). The addition of a second aliquot of enzyme effectively prevented the liberation of P_i. These results indicate that the labile intermediate was stable for 5 s in acid. Furthermore the liberated intermediate was apparently a substrate for the enzyme catalyzed reaction, since addition of buffer alone did not prevent the release of P_i. The relatively stable nature of the intermediate suggests that P_i release was not from the ene-diol(ate) which should have eliminated P_i or tautomerized to the stable keto form within 5 s.

Table 1. The Stability of the Phosphate Labile Intermediate[a].

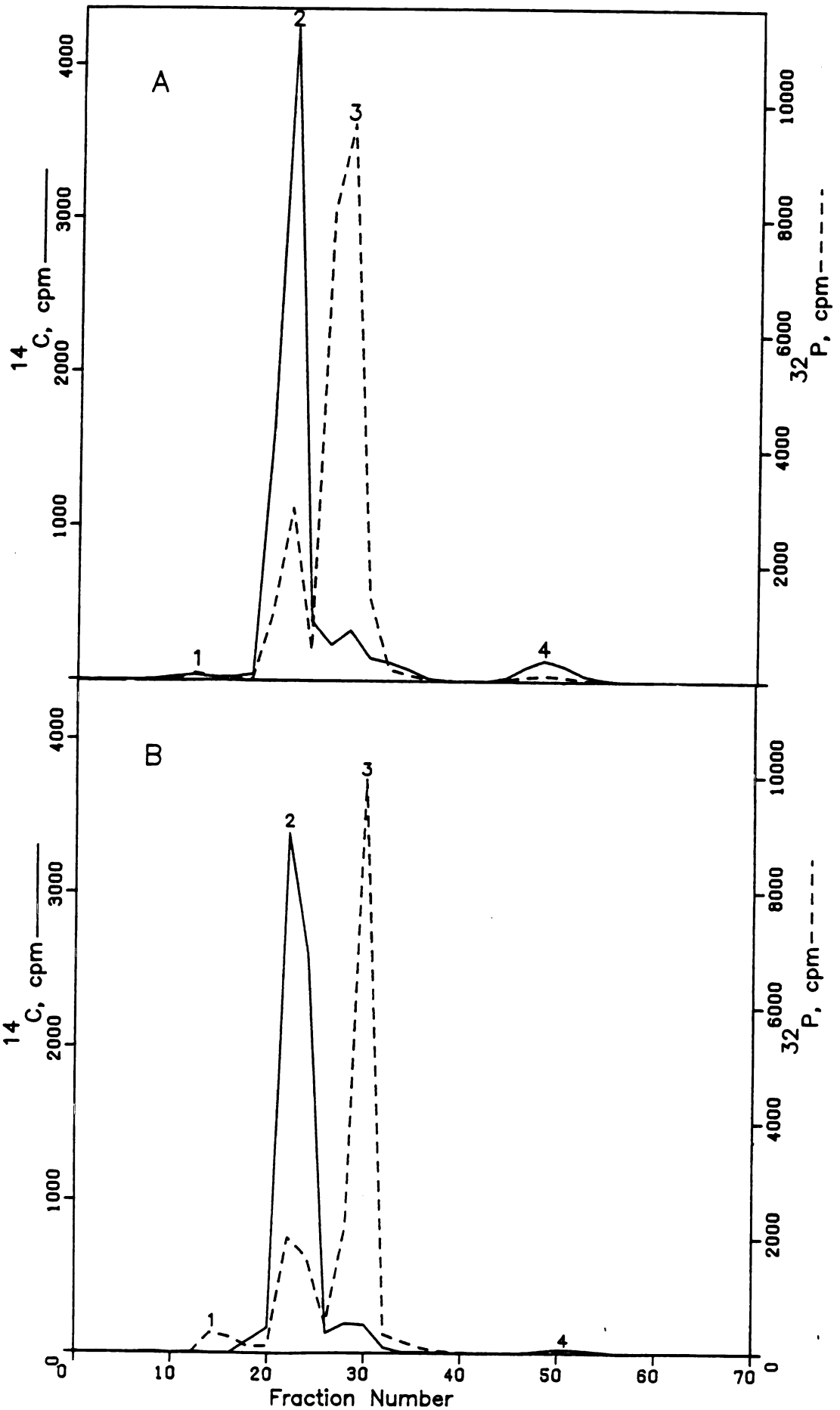
Post Quench Treatment	Pi released, expressed as % of the active sites	% Ribulose-P_2 Remaining
None	5.0	93.5
Activation Buffer (No Enzyme)	4.4	90.7
Activated Enzyme	0	0

[a]Ribulose-P_2 and ribulose-P_2 carboxylase were mixed for approximately 150 ms and quenched in trichloroacetic acid (final concentration of 0.08 N). The post quench solution was added after 5 s, and contained 250 mM Bicine-Na^+ (pH 8.5), 1 mM dithiothrieitol, 20 mM $MgCl_2$, 10 mM $NaHCO_3$ with or without 8.23 mg ml^{-1} of purified spinach carboxylase. P_i and unreacted ribulose-P_2 were determined after 1h at 22°C.

To establish the lability of the carboxylated intermediate, the reductive stabilization technique of Schloss and Lorimer (1982) was used. In our experiment, [1-^{32}P]ribulose-P_2 and $^{14}CO_2$ were mixed with activated enzyme for approximately 150 ms and quenched with HCl. After 5 s or 1 h at 22 C, the acid quenched solution was reduced with $NaBH_4$ and chromatographed on Dowex 1-Cl. The elution profiles obtained after $NaBH_4$ reduction after 5 s or 1 h are shown in Figure 2A and 2B respectively. Peak 2 co-chromatographed with glycerate-3-P prepared enzymatically with ribulose-P_2 and carboxylase. The ratio of ^{32}P:^{14}C in peaks 2 and 4 was the same, and the radiospecific activities indicated that each peak contained compounds which incorporated C from CO_2 and P from ribulose-P_2 with a ratio of 1:1. Peak 4 chromatographed similarly to the lactone of carboxyarabinitol-P_2. Thus peak 4 was due to the reduction product of the carboxylated intermediate. The ^{14}C observed in peak 4 of Figure 2A represented 4.2 nmol of carboxylated intermediate which was trapped from 112 nmol of active sites. Peak 3 was mostly pentitol-P_2, the reduction product of unreacted ribulose-P_2. This peak also contained a small amount of ^{14}C which may be due to the incomplete lactonization of carboxyarabinitol-P_2 by the procedure described by Schloss and Lorimer (1982). Considerable correction for the spill of ^{32}P counts into the ^{14}C channell was required for the large number of counts from ^{32}P in peak 3. Therefore, the ^{14}C counts observed in the peak 3 fractions were subject to considerable error.

A comparison of Figures 2A and 2B indicates that the carboxylated intermediate was trapped only in the sample in which reduction was initiated 5s after denaturing the enzyme with acid. After 1 h, only a

Figure 2. Resolution of the Carboxylated Intermediate from the Ribulose-P_2 Carboxylase Reaction after Acid Denaturation and Reduction. Activated ribulose-P_2 carboxylase (112 nmol active sites) was mixed with a ribulose-P_2 solution for approximately 150 ms and delivered to a quench solution of 2.1 ml of 0.2 N HCl. Five ml syringes were used in this experiment to accomodate 1.75 ml of enzyme and ribulose-P_2 solutions. The reaction mixture contained 70 mM Bicine-Na^+ (pH 8.2), 10 mM $NaH^{14}CO_3$ (0.7 $\mu Ci \cdot \mu mol^{-1}$), 20 mM $MgCl_2$, 1 mM dithiothreitol, 2.65 $mg \cdot ml^{-1}$ spinach ribulose-P_2 carboxylase and 40 μM [1-^{32}P]ribulose-P_2 (0.5 $\mu Ci \cdot \mu mol^{-1}$). In Figure 2A, 10 ml of a solution containing 0.1 M $NaBH_4$ and 0.1 M $NaHCO_3$ was added 5 s after acid denaturation. In Figure 2B, the $NaBH_4$ solution was added after 1 h. Subsequent sample preparation and chromatography is described by Schloss and Lorimer (1982). Briefly this involved removal of borate, lactonization of carboxyarabinitol-P_2 and chromatography on Dowex 1-Cl (X8) with a linear gradient of 0-0.8 M LiCl prepared in 5 mM HCl. Aliquots (0.5 ml) were counted for ^{32}P and ^{14}C.


A
B
1
2
3
4
14C, cpm
32P, cpm
0
1000
2000
3000
4000
6000
8000
10000
0
10
20
30
40
50
60
70
Fraction Number

Ftgure 2.

Figure 3. Inhibition of Ribulose-P_2 Carboxylase by the $NaBH_4$ Reduction Product of the Carboxylated Intermediate. A) Aliquots (0.1 ml) of fractions of peak 4 from Figure 2 were incubated at pH 9.0 for 12 h at 22°C to hydrolyze the lactones and then added to 0.05 nmol of activated ribulose-P_2 carboxylase protomers. After 2 h incubation, the enzyme was assayed for carboxylase activity in a volume of 1.0 ml by the addition of 1 mM ribulose-P_2. The specific activity of the enzyme was 1.8 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein. The solid line is for the gradient fractions in Figure 2A (reduction after 5 s) and the dashed line is for fractions of the gradient shown in Figure 2B (reduction after 1 h). B) The ^{14}C in each fraction from Dowex chromatography is shown. The solid line is from Figure 2A (reduction with $NaBH_4$ after 5 s) and the dashed line is from Figure 2B (reduction after 1 h).

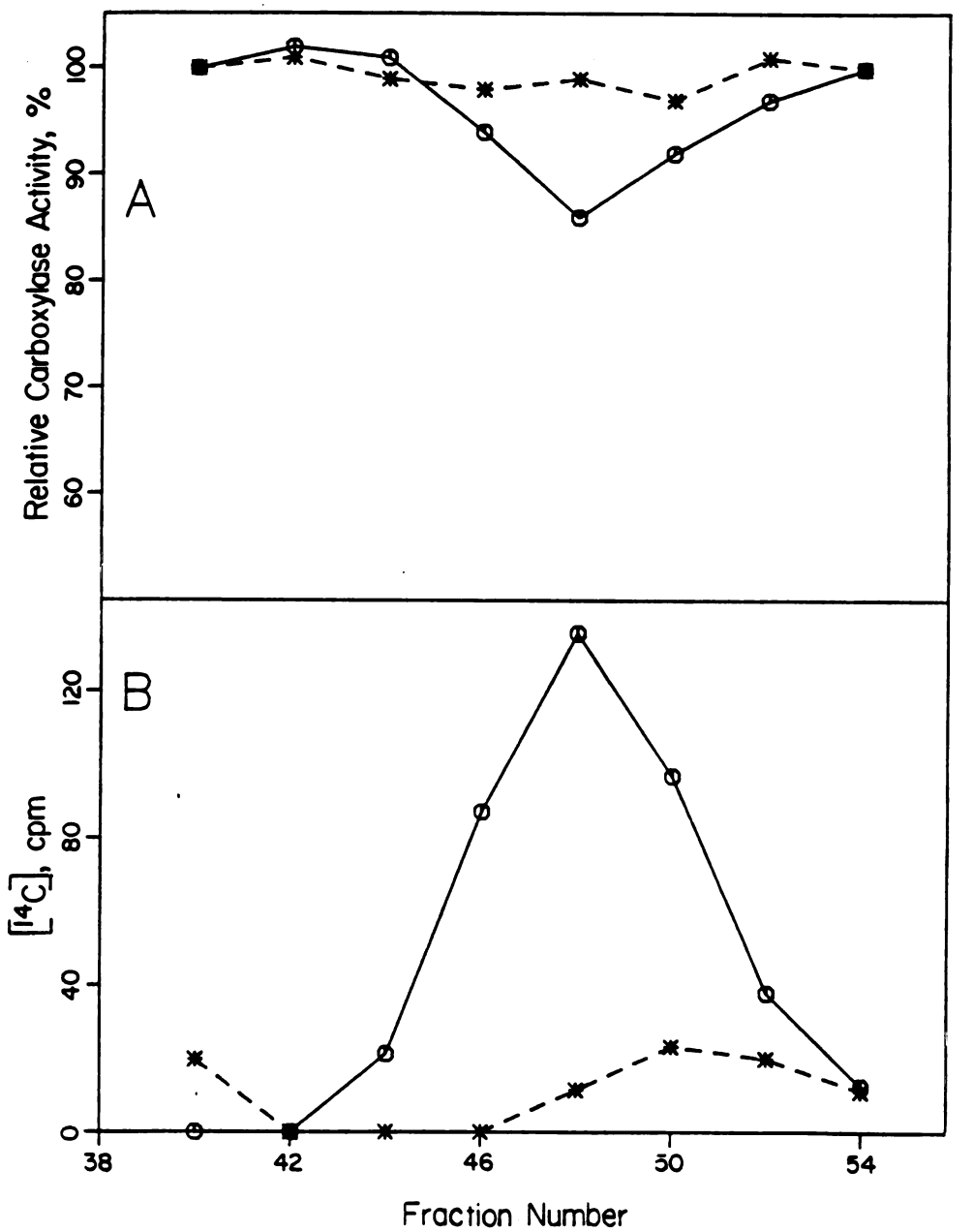

Relative Carboxylase Activity, %
100
90
80
70
60
A
B
[14C], cpm
120
80
40
0
38
42
46
50
54
Fraction Number

Figure 3.

trace of the carboxylated intermediate was trapped. In Figure 2B, the loss of ^{32}P counts due to the carboxylated intermediate (peak 4) after 1 h incubation was accompanied by an increase in the ^{32}P counts due to P_i (peak 1). Further evidence that the carboxylated intermediate could be trapped by reduction after 5 s in acid, but not after 1 h, is presented in Figure 3. Aliquots of fractions from peak 4 (Fig. 2A and 2B) were incubated with purified spinach ribulose-P_2 carboxylase under conditions in which carboxyarabinitol forms an essentially irreversible complex with the enzyme (Pierce et al., 1980). Substantial enzyme inhibition was observed only by aliquots from the gradient presented in Figure 2A, in which reduction was initiated after 5 s. Essentially no inhibition was observed by aliquots from the gradient presented in Figure 2B in which reduction was initiated after 1 h.

Absence of Paracatalytic Inactivation of Ribulose-P_2 Carboxylase/Oxygenase. Aldolase catalyzes the formation of a carbanion intermediate of dihydroxyacetone-P which is oxidized by hexacyanoferrate (III) and results in the 2 electron oxidation of the carbohydrate and commensurate paracatalytic inactivation of the enzyme (Christen, 1977; Christen et al., 1976). Similar studies were used to study the mechanism of ribulose-P_2 carboxylase/oxygenase. The ene-diol or carbanion intermediate should have the electronic propensity to reduce strong oxidants such as hexacyanoferrate (III) and a preliminary report suggests that the enzyme from Pseudomonas facilis does show paracatalytic inactivation (Hsu and Kuehn, 1978). Hexacyanoferrate (III) and DCPIP were tested for reduction by activated ribulose-P_2 carboxylase/oxygenase under conditions that favored

carboxylase (10 mM $NaHCO_3$) or oxygenase (0.04 mM $NaHCO_3$) activities. No dye reduction could be observed spectrophotometrically and no inhibition of dioxygen consumption was observed upon the addition of these oxidants to an oxygenase assay. A possible explanation is that the active intermediate might be compartmentalized away from the oxidant. The experiment was repeated under various conditions that might have partially denatured the enzyme: 50°C; 0.2 M NaCNS; 0.2 M guanidine HCl; 0.8 M urea; and 1% Triton-X-100. Oxidant reduction could not be observed under any of these conditions. Hexacyanoferrate (III) dependent paracatalytic inactivation of ribulose-P_2 carboxylase activity was also tested and no time dependent inactivation was observed with the enzyme from spinach leaves.

Paracatalytic Inhibition of Fructose-P_2 Aldolase. Since aldolase catalyzes the formation of a reactive carbanion and a reactive enediol(ate) or carbanion are proposed to be involved in the reaction mechanisms of ribulose-P_2 carboxylase/oxygenase, aldolase was tested to determine if the carbanion intermediate had any propensity to undergo carboxylation or oxygenation. No carboxylase activity could be observed when aldolase (1.8 units) and dihydroxyacetone-P (1.7 mM) were incubated with $NaH^{14}CO_3$ (1.5 μCi, 56 mCi/mmol), either carrier free or with 10 mM $NaHCO_3$. No oxygenase activity could be observed by O_2 consumption under these conditions. Hexacyanoferrate (III) was readily reduced under these conditions. In the course of these experiments, it was observed that upon the addition of hexacyanoferrate (III) (0.5 mM) to aldolase (2 units) and dihydroxyacetone-P (1.7 mM), O_2 consumption ensued at approximately 3% of the rate of hexacyanoferrate

reduction. Dioxygen consumption ceased when hexacyanoferrate (III) was consumed. Hexacyanoferrate (II) was not responsible for O_2 consumption since addition of this compound did not result in O_2 consumption. The O_2 consuming process was not associated with the paracatalytic inactivation phenomenon, since the rate of inactivation of aldolase by hexacyanoferrate (III) in the presence of fructose-P_2 was essentially the same under atmospheres of 100% dinitrogen or dioxygen. These results are consistent with previous results of Christen et al. (1976) where they observed that anaerobiosis did not effect the rate of aldolase inactivation.

Discussion

Acid denaturation of the ribulose-P_2 carboxylase reaction liberated the carboxylated intermediate which may be stabilized by reduction and isolated by anion exchange chromatography. The reduction products of the carboxylated intermediate were isolated by this procedure and identified by Schloss and Lorimer (1982) as a mixture of 2 and 4 carboxyarabinitol-P_2. The compounds which were isolated after reduction are characterized to have the chromatographic properties of CABP by anion exchange chromatography (Fig. 2) and possess the potent inhibitory properties of CABP in an enzyme assay (Fig. 3), as was observed by Schloss and Lorimer (1982). In addition, the C and P incorporated into the carboxylated intermediate from CO_2 and ribulose-P_2 was in a ratio of 1:1, which is consistent with the molecular formula of the carboxylated intermediate. The stoichiometry of the carboxylated intermediate which was trapped per enzyme active

site was 0.04, in reasonable agreement with the value of 0.07 obtained by Schloss and Lorimer. These data confirm the results and interpretation of Schloss and Lorimer that the carboxylated intermediate has the threo stereochemistry (analogous to the arabinitol configuration). The carboxylated intermediate has consequently been identified as 2-carboxy-3-keto-D-threo-pentitol-1,5-bisphosphate. Since the products of the carboxylation reaction are 2 molecules of D(-)glycerate-3-P, hydrolysis of the carboxylated intermediate must occur with inversion of the stereochemistry at carbon 2 as discussed by Pierce et al. (1980).

Acid denaturation of the ribulose-P_2 carboxylase reaction liberates an intermediate which has a labile phosphate ester. Since β-elimination of P_i readily occurs from ene-diol(ate)s, this form of ribulose-P_2 was initially assumed responsible for the phosphate release phenomenon. However several observations suggest that the source of the inorganic phosphate is from the carboxylated intermediate. First the ene-diol of dihydroxyacetone-P was rapidly labile, with a half life of 12 to 17 ms (Iyengar and Rose, 1981a). In contrast, the labile species from the ribulose-P_2 carboxylase reaction was stable for 5 s in acid (Table 1), which was considerably more stable than might be expected for an ene-diol. Secondly, the carboxylated intermediate was stable for 5 sec, but not for 1 h under acidic conditions (Fig. 2A and 2B). Therefore both the carboxylated intermediate and the species with a labile phosphate ester were stable for 5 s in acid, but not for 1 h. The instability of the carboxylated intermediate was unexpected. The mechanism of the degradation of the carboxylated intermediate is not known, but could involve

decarboxylation to the ene-diol with subsequent β-elimination of the carbon 1 phosphate ester. Finally the stoichiometries of the carboxylated intermediate trapped and phosphate released to enzyme active sites were both about 0.04. Therefore the intermediate responsible for the release of phosphate is probably the carboxylated intermediate, although this has not been unequivocally demonstrated by these results.

The carboxylated intermediate appears to be a substrate for ribulose-P_2 carboxylase/oxygenase, since treatment of the acid quenched reaction with fresh enzyme converted the labile intermediate to a stable form. Similarly, triose-P isomerase was capable of trapping the ene-diol of dihydroxyacetone-P and the enzyme acted catalytically to convert the ene-diol to a stable form (Iyengar and Rose, 1981b). The ability of ribulose-P_2 carboxylase to bind the intermediate is not unexpected since the enzyme readily binds carboxyarabinitol-P_2 and other carboxylated pentitol-P_2.

The carboxylated intermediate accounts for approximately 4% of the bound substrate, and very little of the ene-diol(ate) form of the substrate appears to accumulate. Perhaps this is an indication that the carboxylated intermediate exists at a lower energy level than the ene-diol(ate) in the free energy profile of the carboxylase reaction. Since only a small fraction of bound substrate exists as the ene-diol(ate) or carboxylated intermediate, the major form of enzyme-bound substrate probably exists as ribulose-P_2 and glycerate-3-P.

No P_i was liberated in the normal course of catalysis by ribulose-P_2 carboxylase/oxygenase. Triose-P isomerase exhibits an

apparent phosphatase activity, although P_i elimination occurs at a low rate (0.1 s^{-1}) compared to catalysis (4 x 10^3 s^{-1}) (Iyengar and Rose, 1981b). This frequency is easily observed with triose-P isomerase since the reaction is readily reversible. The carboxylase reaction is irreversible, and if P_i liberation occurs at a frequency similar with that observed with triose-P isomerase, it would have been below the level of detection.

The observation that O_2 consumption occurs at about 3% of the rate of hexacyanoferrate (III) reduction by the aldolase-dihydroxyacetone-P carbanion is consistent with the oxidation occurring with a free radical intermediate (Christen et al., 1976). Since hexacyanoferrate (III) is a univalent oxidant, it is probable that the oxidation involves two single electron oxidations of the carbanion. Dioxygen is known to react readily with carbon based radicals. It is possible that the mechanism of dioxygen consumption in this system is the single electron oxidation of the carbanion to the radical, followed by reaction with dioxygen to form the peroxy radical. The reaction with oxygen is apparently not involved in the paracatalytic inactivation phenomenon, since rates of inactivation were not effected by equilibration with atmospheres of 100% dinitrogen or dioxygen.

The oxygenase activity may be an unavoidable consequence of the carboxylase mechanism (Lorimer and Andrews, 1973b). Perhaps the strongest evidence for this is the ubiquitous coincidence of the two activities, even in the ribulose-P_2 carboxylase enzyme from obligate anaerobes. Carbanions have been observed in certain cases to undergo reactions with both carbon dioxide and dioxygen (Lorimer and Andrews, 1973b). The experiments with dihydroxyacetone-P and aldolase indicated

that the carbanion intermediate of dihydroxyacetone-P does not readily undergo a reaction with carbon dioxide or dioxygen, even though the carbanion is easily accessible to solutes such as hexacyanoferrate. Therefore, it seems that something more than mere carbanion formation is required for reaction with oxygen or carbon dioxide. Although the role of metal ions in catalysis is not known it has been suggested that the metal ion in the carboxylase reaction may act to stabilize the carboxyl group of the six carbon intermediate (Pierce et al., 1980). Divalent metal ions are also known to stabilize O_2^- (Sawyer and Gibian, 1979) and this may relate to the conditions required for oxygenase and carboxylase activities and explain the modified kinetics in the presence of various divalent metal cations (Wildner and Henkel, 1978; Christeller, 1981).

CHAPTER 2

INVESTIGATIONS ON THE MECHANISM OF RIBULOSE-P_2 OXYGENASE.

Ribulose-P_2 carboxylase/oxygenase is an unusual oxygenase since the enzyme is an internal monooxygenase, yet does not require a transition metal or organic cofactor for activity. An early report indicated that ribulose-P_2 carboxylase contained one mol of copper per mol of holoenzyme (Wishnick et al., 1969), but results from other laboratories indicate only trace quantities of copper associated with the enzyme (Chollet et al., 1975; Johal et al., 1980; Lorimer and Andrews, 1973a). McCurry (1979) reported that 2 mol iron per mol of holoenzyme were present in several preparations of ribulose-P_2 carboxylase. Dialysis against 100 mM DTT removed 65% of the iron from the protein with no loss in carboxylase or oxygenase activity. Other investigators have searched for organic cofactors such as flavins (Chollet et al., 1975) and a variety of other organic cofactors (R. Gee, personal communication), but no more than a trace of these compounds could be observed.

The anomaly that ribulose-P_2 carboxylase/oxygenase lacks a transition metal or organic cofactor could be explained by the oxygenase reaction proceeding by a free radical mechanism. This chapter records my studies on the mechanism of ribulose-P_2 oxygenase and potential reaction mechanisms are presented and discussed.

Materials and Methods.

Ribulose-P_2 carboxylase/oxygenase from spinach leaves was purified, stored, activated and assayed as described in Chapter 1. Ribulose-P_2 was synthesized and purified as described in Chapter 1. The copper chelate of lysine, $Cu(lys)_2$, was synthesized as described by Stevens and Bush (1950). Trypsin and chymotrypsin were obtained from Millipore. Other reagents were obtained from Sigma or were of analytical reagent quality. Fluorescence measurements were made with an Aminco-Bowman Spectrophotofluorometer.

Results.

The Presence and Stoichiometry of Flavin in Ribulose-P_2 Carboxylase/Oxygenase. Purified ribulose-P_2 carboxylase/oxygenase exhibited a fluorescence excitation spectrum characteristic of a flavin (excitation maxima at 380 and 470 nm) when fluorescence emission was monitored at 525 nm (Fig. 4). The emission spectrum showed only a shoulder, with no peak resolved. Activated enzyme gave similar results with no apparent changes in fluorescence intensity. Enzyme which was denatured with SDS (Fig. 5), digested with trypsin and chymotrypsin (data not shown) or hydrolyzed with 2.0 N HCl at 100 C (data not shown) showed excitation and emission spectra which were clearer and better resolved. A distinct emission maximum at 520 nm and excitation maxima at 380 and 450 nm are shown in

Figure 4. The Fluorescence Spectra of Native Ribulose-P_2 Carboxylase/Oxygenase.

Enzyme (13.8 mg/ml) was prepared in 0.1 M Bicine-Na^+ at pH 8.0 and scanned for fluorescent excitation with emission at 520 nm and scanned for emission with excitation at 380 nm. Similar spectra were obtained with enzyme activated with 10 mM $NaHCO_3$ and 10 mM $MgCl_2$.

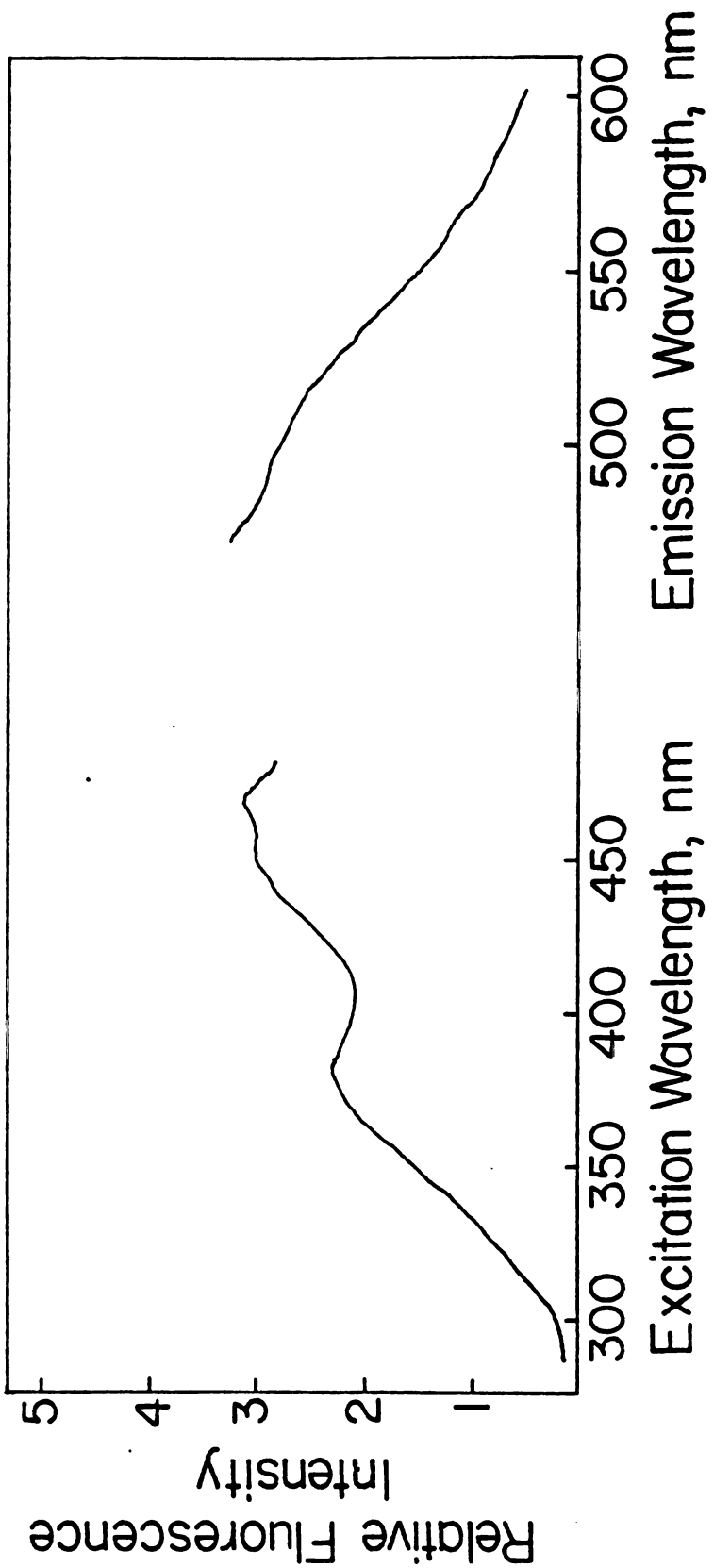

Relative Fluorescence Intensity
1
2
3
4
5
300
350
400
450
500
550
600
Excitation Wavelength, nm
Emission Wavelength, nm

Figure 4.

Figure 5. The Fluorescence Spectra of Denatured Ribulose-P_2 Carboxylase/Oxygenase.

Enzyme (12.1 mg/ml) was denatured by heating at 100 C for 2 min in the presence of 0.1 M Bicine-Na^+ (pH 8.0), 0.8% (w/v) SDS and 8% (v/v) 2-mercaptoethanol. Fluorescence scans were obtained for excitation with emission monitered at 525 nm and for emission with excitation at 450 nm.

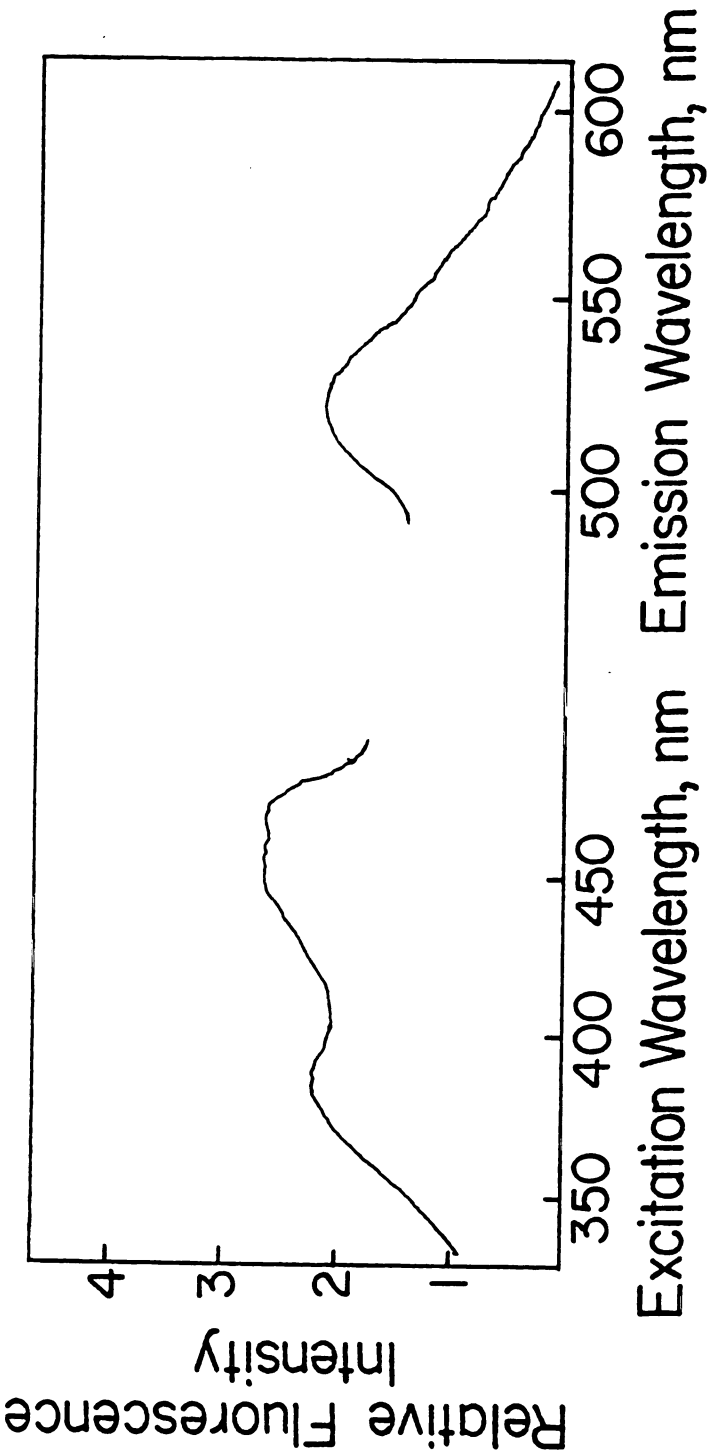

Relative Fluorescence Intensity
1
2
3
4
350
400
450
500
550
600
Excitation Wavelength, nm
Emission Wavelength, nm

Figure 5.

Figure 6. The Fluorescence Spectra of Flavin Mononucleotide.

Flavin mononucleotide (10 μM) was prepared in 0.1 M sodium formate (pH 3.2) and scanned for fluorescence as described in Figure 2.

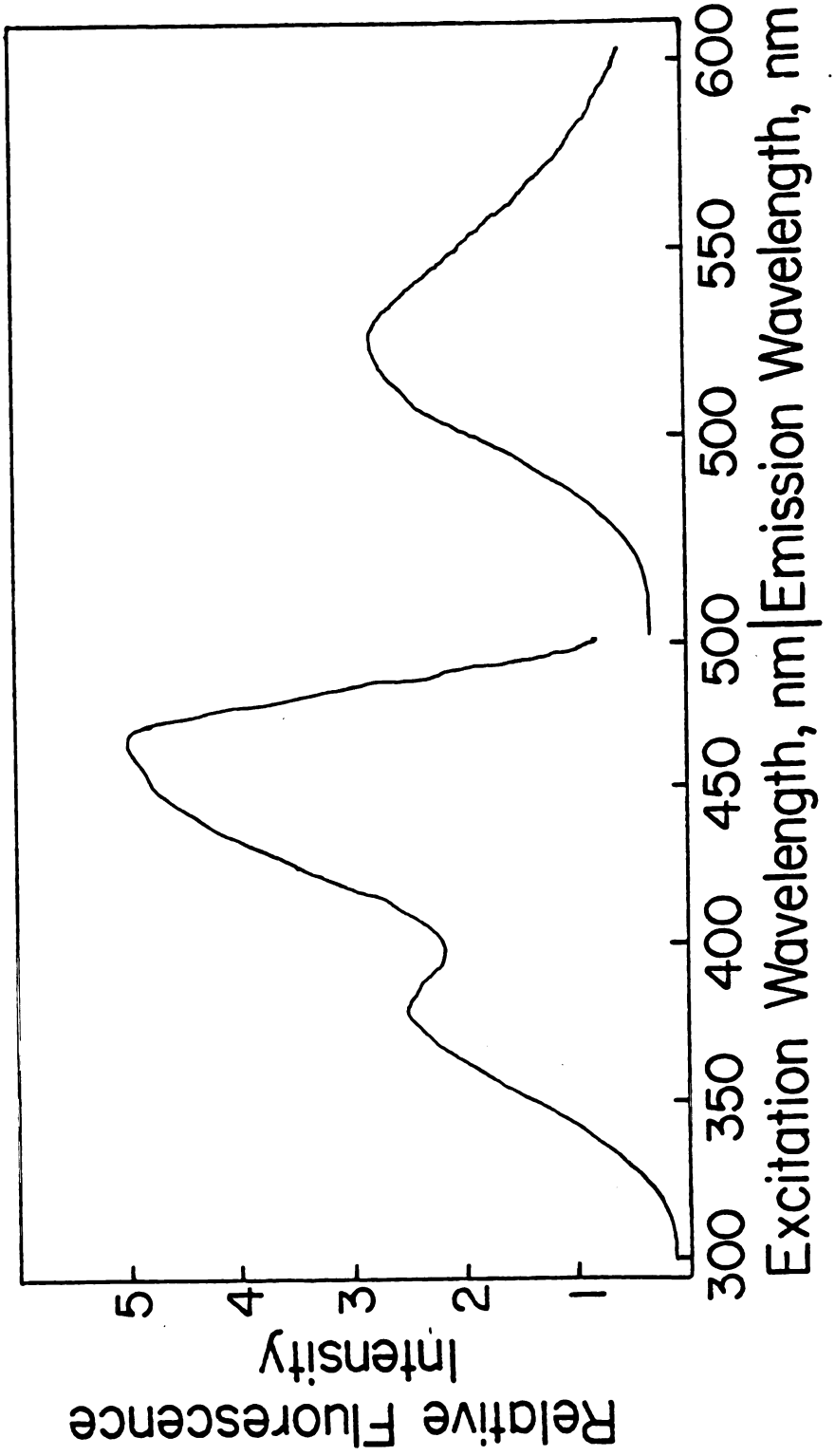

Relative Fluorescence Intensity
1
2
3
4
5
300
350
400
450
500
500
550
600
Excitation Wavelength, nm
Emission Wavelength, nm

Figure 6.

Figure 5. These spectra are very similar to those observed with authentic flavins (Fig. 6).

A fluorescent compound was isolated from ribulose-P_2 carboxylase/oxygenase by digestion of 50 mg of enzyme with 5% (w/w) of trypsin and chymotrypsin for 36 h at 22 C. This preparation was fractionated by chromatography on Bio-Gel P-4 which was eluted with distilled water. A fraction with fluorescence spectra similar to those in Figure 6 was recovered. This fraction was lyophilized and a sample was applied to a cellulose thin layer plate. The plate was developed with 80% (w/v) phenol:H_2O in the first dimension and 2:1:1.4 (v/v/v) butanol:propionic acid:water in the second dimension. Riboflavin, flavin mononucleotide and flavin adenine dinucleotide are readily resolved by this method. The unknown fluorescent compound co-chromatographed with riboflavin under this protocol. This compound co-chromatographed with riboflavin in 6 other solvent systems (R. Gee, personal communication).

The stoichiometry of the fluorescent compound was assessed by using the internal standard method described by Koziol (1971). Ribulose-P_2 carboxylase/oxygenase (12.1 mg/ml) was denatured with SDS and the relative fluorescence intensity was measured. A standard curve was generated by addition of riboflavin to the denatured enzyme and measurement of the relative fluorescent intensity. The standard curve and the fluorescence of denatured carboxylase is pre-

Figure 7. Quantitation of Riboflavin by Fluorescence of Ribulose-P_2 Carboxylase.

Enzyme (22 μM holoenzyme) was denatured with SDS as described in Figure 5 and the relative fluorescence intensity (excitation 450 nm, emission 525 nm) was determined and is indicated by the dashed line. The standard curve of riboflavin fluorescence was generated by addition of small aliquots of a riboflavin solution to the denatured enzyme. The increase in fluorescence intensity is plotted as a function of the concentration of riboflavin added. The fluorescence of the denatured enzyme is equivalent to 0.28 μM riboflavin. This analysis indicates that this carboxylase preparation contained 0.013 mol riboflavin per mol of holoenzyme.

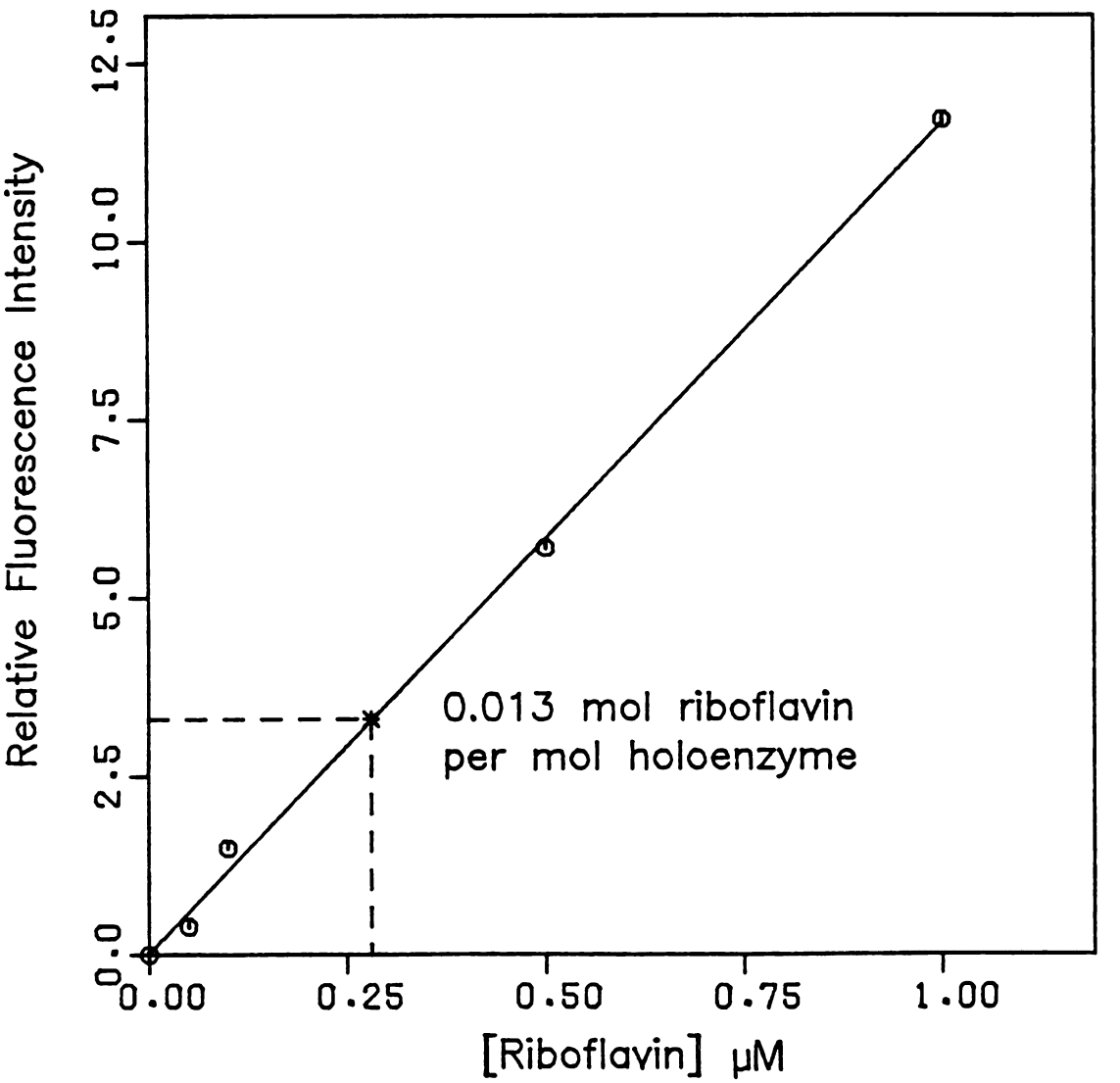

Relative Fluorescence Intensity
0.0
2.5
5.0
7.5
10.0
12.5
0.00
0.25
0.50
0.75
1.00
[Riboflavin] μM
0.013 mol riboflavin
per mol holoenzyme

Figure 7.

sented in Figure 7. The stoichiometry of riboflavin to holoenzyme is exceedingly small; these data indicate that only 0.013 mol of riboflavin were present per mol of holoenzyme. The flavin seems to be a trace contaminant of purified ribulose-P_2 carboxylase/oxygenase.

Attempts to Observe Intermediates in the Ribulose-P Oxygenase Reaction. Ribulose-P_2 oxygenase has been proposed to proceed by a radical mechanism which may involve a carbon based radical derived from ribulose-P_2, superoxide and 2-hydroperoxy-3 keto-pentitol-P_2 (Lorimer, 1981a). The presence of these intermediates was tested by several techniques.

Superoxide is a reactive species of oxygen generated by one electron reduction of O_2. NBT^{2+} will oxidize O_2^- and this reaction may be followed spectrophotometrically at 560 nm. Addition of NBT^{2+} to a ribulose-P_2 oxygenase reaction did not result in dye reduction and NBT^{2+} did not inhibit O_2 consumption in an oxygenase assay. A possible explanation for these results is that the active intermediate was compartmentalized from the oxidant in the enzyme catalytic site. The experiment was repeated under the following conditions which may partially denature the protein: 50 C; 0.2 M NaCNS; 0.2 M guanidine HCl; 0.8 M urea; and 1% Triton-X-100. Dye reduction was not observed under any of these conditions.

Amino acid chelates of copper have superoxide dismutase activity and these have been used to implicate the involvement of O_2^- in the reaction mechanism of diamine oxidase (EC 1.4.3.6) (Younes and Weser, 1978). The copper chelate of lysine was synthesized and demonstrated to exhibit potent inhibition of O_2^- dependent cytochrome c reduction. The copper chelate of lysine was a poor inhibitor of both ribulose-P_2 carboxylase and oxygenase activities and did not inhibit either activity selectively (data not shown).

Discussion.

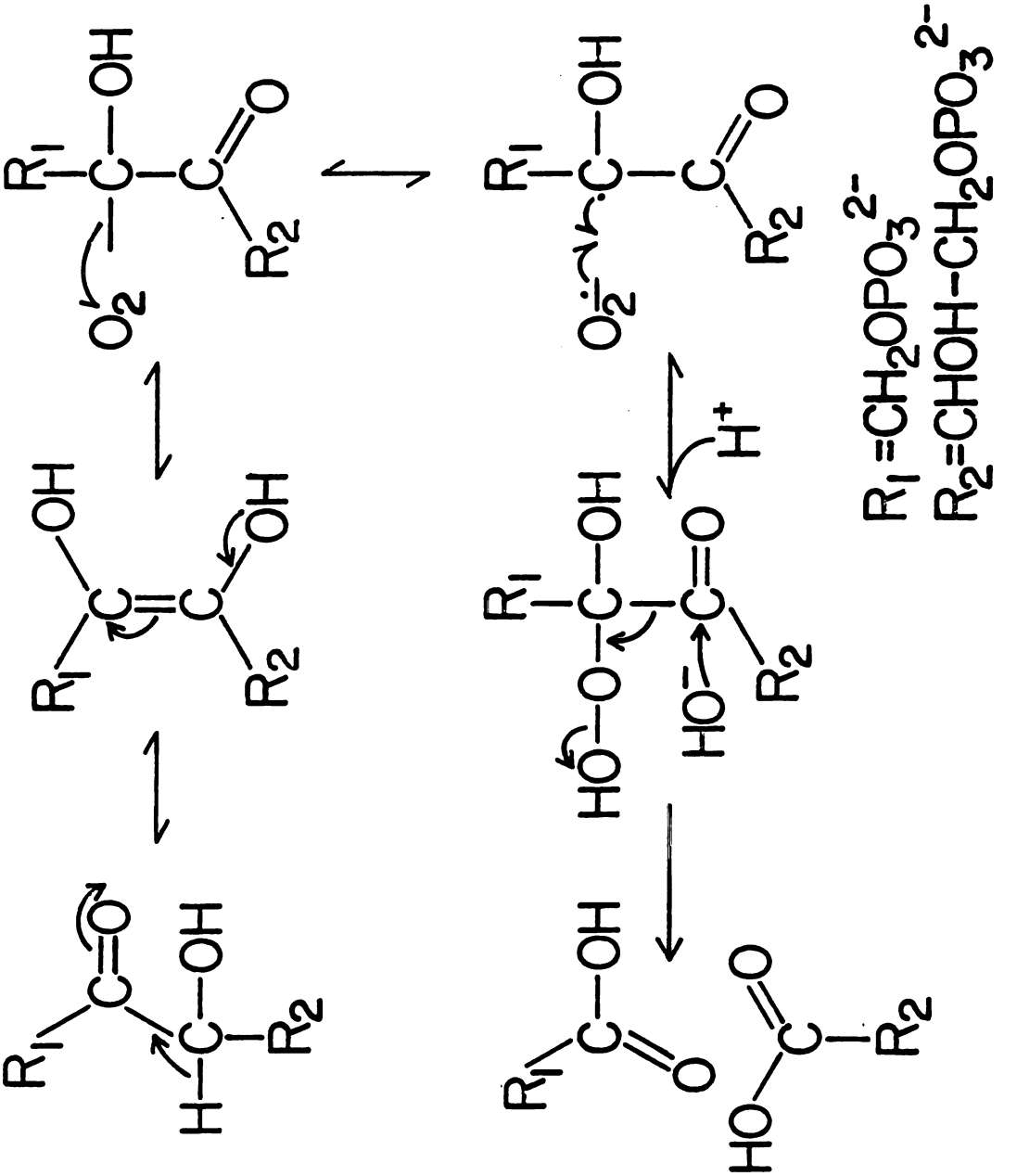
A compound which co-chromatographs with riboflavin on two dimensional chromatography and exhibits the fluorescent spectra characteristic of a flavin has been isolated from purified spinach ribulose-P_2 carboxylase/oxygenase. The quantity of the compound present in the enzyme preparation was assessed by fluorescence spectroscopy using riboflavin as the standard. The stoichiometry was 0.013 mol flavin per mol holoenzyme. Therefore this compound was present at far less than 8 mol per mol of holoenzyme, which would be expected if the flavin were a required cofactor for the oxygenase reaction. Riboflavin is probably a contaminant of the purified enzyme, which is present in trace amounts. Purified ribulose-P_2 carboxylase from bean and pea leaves also binds indole-3-acetic acid with a K_D of about 10^{-6} M

and stoichiometry of 1 mol indole-3-acetic acid per mol of holoenzyme (Wardrop and Poyla, 1980). The significance of flavin or auxin binding to ribulose-P_2 carboxylase remains unknown. These may represent related phenomenon since the isoalloxazine ring of flavin is similar in structure to the indole ring of indole acetic acid.

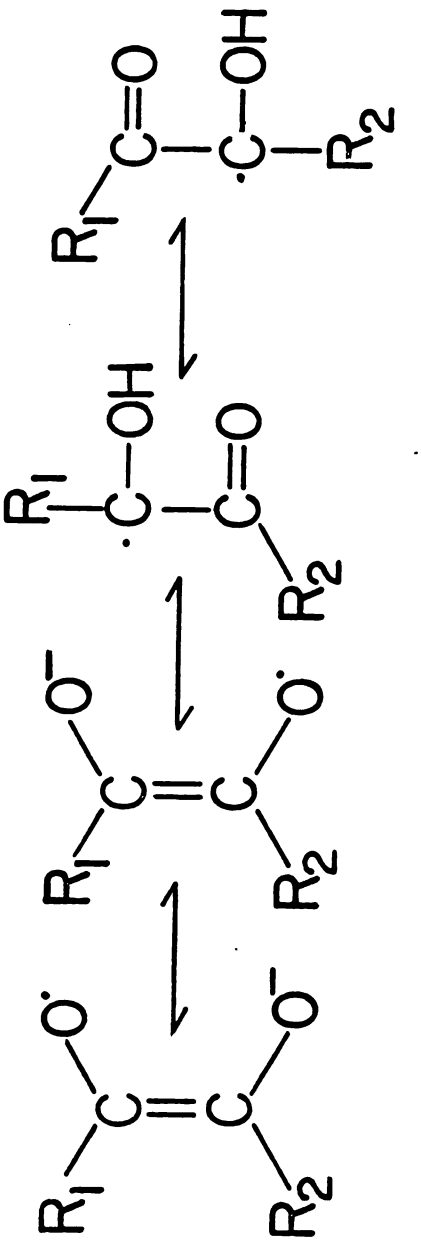
No evidence was obtained for a free radical mechanism for ribulose-P_2 oxygenase. Neither NBT^{2+} or $Cu(lys)_2$ were effective in interrupting the oxygenase reaction. Previous investigators have reported NBT^{2+} reduction by ribulose-P_2 oxygenase and inhibition of O_2 consumption by NBT^{2+} (Bhagwat and Sane, 1978b); I have been unable to repeat these results. Bhagwat and Sane (1978a) also reported that superoxide dismutase was a selective inhibitor of ribulose-P_2 oxygenase. This observation could not be repeated in several laboratories (McCurry, 1979; Lorimer et al., 1973a). The copper chelate of lysine is a small compound with potent superoxide dismutase activity, and I have been unable to demonstrate selective inhibition of ribulose-P_2 oxygenase with this reagent.

All the experimental approaches utilized in these studies suffer from the handicap that unequivocal conclusions may not be drawn. A reactive intermediate may be compartmentalized in the enzyme catalytic site, or may be too short-lived or labile to be observed by these methods.

The mechanism of ribulose-P_2 oxygenase remains a mystery. Iron, copper and organic cofactors are not present in ribulose-P_2 carboxylase/oxygenase at a stoichiometry indicative of a cofactor. In view of the absence of electron delocalizing cofactors, it seems obligatory that ribulose-P_2 oxygenase must proceed through a radical mechanism. Two mechanisms for ribulose-P_2 oxygenase have been proposed (Lorimer, 1981a; Lorimer and Andrews, 1981). Scheme 2 shows a possible mechanism for ribulose-P_2 oxygenase. The enediol(ate) may tautomerize to the carbanion and this species could reduce O_2 by one electron, indicated by the single headed arrow. The intermediates of this reaction would be a carbon based radical and superoxide radical. These two radicals may recombine to form 2-peroxy-3-keto-pentitol-P_2. This putative intermediate of the oxygenase reaction may decompose by addition and elimination of hydroxyl ion. This mechanism involves the reaction of a triplet and a singlet to form two doublets, followed by reaction to form two singlets, essentially as discussed with the reduction of dioxygen by reduced flavin in the literature review. Thus ribulose-P_2 is proposed to react in a manner analogous to flavin. Ribulose-P_2 is incapable of forming resonance structures as highly stabilized as the flavin semiquinone, although the resonance stabilization of the semidione radical is considerable (Russel and Strom,



Scheme 2.

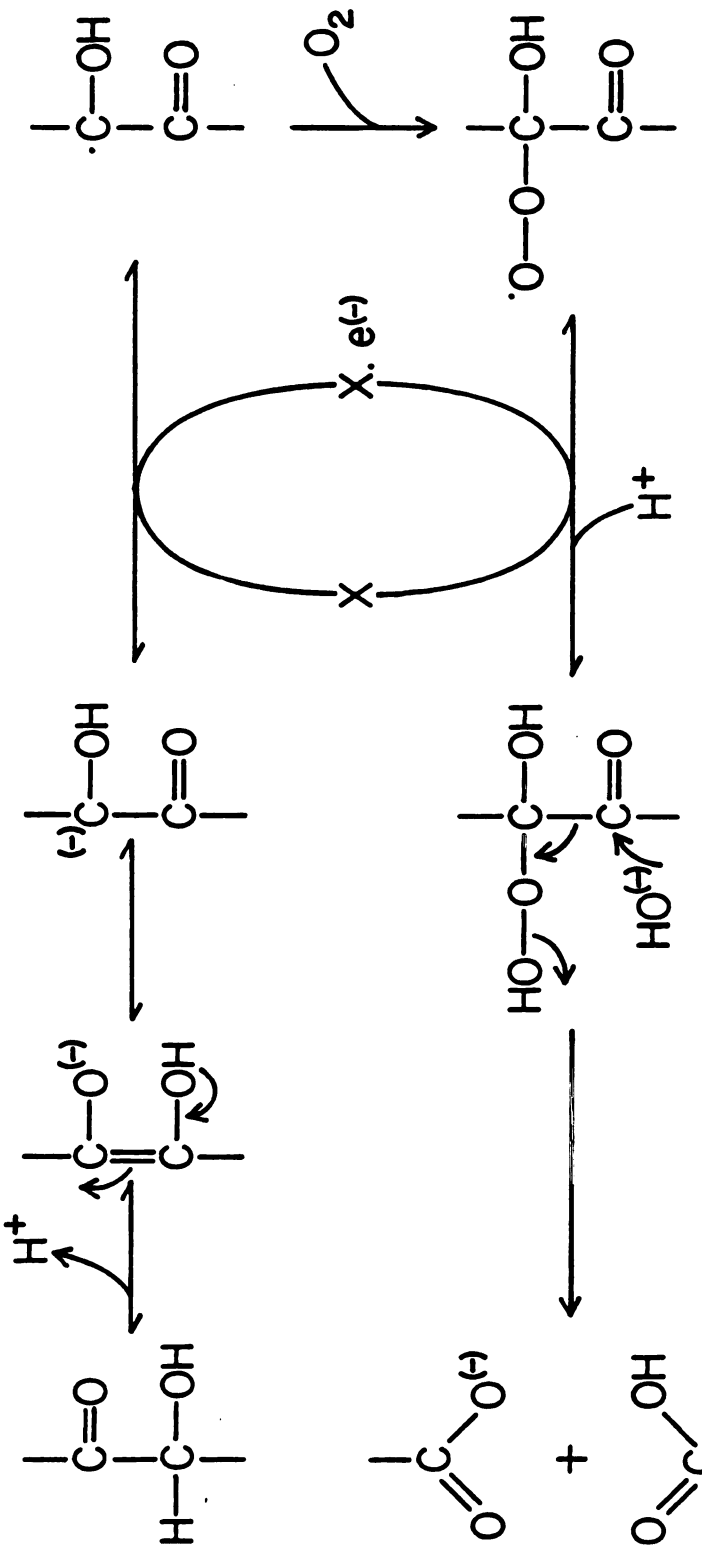


Scheme 3,

1964; Russel, 1968) (Scheme 3).

A second mechanism proposed for ribulose-P_2 oxygenase is analogous to lipid peroxidation (Scheme 4). In this mechanism the enzyme oxidizes ribulose-P_2 by one electron, yielding a carbon-based radical of ribulose-P_2. This could react with O_2 to yield a peroxy radical intermediate. The peroxy radical may be reduced by one electron from the enzyme yielding the same 2-peroxy-3-keto-pentitol-P_2 as in Scheme 2.

Although this mechanism is possible in terms of the oxygen chemistry, we must consider what functional group might participate in this sort of catalysis. The mechanism involves the one electron oxidation of ribulose-P_2, and concomitant one electron reduction of an amino acid residue. Several amino acid residues, such as cysteine or tyrosine, may form radicals on oxidation; none of the amino acid side chains readily form radicals on reduction. This presents a problem to the mechanism proposed in Scheme 4. The enzyme would have to exist in the native state with an amino acid residue oxidized by one electron in a radical form to be poised for the catalytic cycle. A possible resolution of this might be the involvement of a disulfide bond in catalysis (Eq 3). However, no disulfides have been observed in ribulose-P_2 carboxylase/oxygenase (Sugiyama et al., 1968b; Mulligan, unpublished). Thus, although either mechanism is possible and no data exist on which to discriminate between



Scheme 4.

these mechanisms, the mechanism depicted in Scheme 4 seems less likely since one electron reduction of an amino acid residue seems chemically unreasonable.

$$-S\text{-}S \longrightarrow -S^{-}\ {}^{+}S- + \text{Ribulose-}P_2 \longrightarrow -S^{-}\ {}^{\bullet}S- + {}^{\bullet}\text{Ribulose-}P_2 \qquad \text{(Eq.}$$

CHAPTER 3

STIMULATION AND INHIBITION OF RIBULOSE-P_2 CARBOXYLASE/OXYGENASE BY GLYOXYLATE

Ribulose-P_2 carboxylase/oxygenase catalyzes the first reaction of each of the two cycles of photosynthetic carbon metabolism. The ribulose-P_2 carboxylase reaction fixes CO_2 and initiates the reductive photosynthetic carbon cycle in which CO_2 is reduced to the level of carbohydrate by a series of reactions in the chloroplasts. The ribulose-P_2 oxygenase reaction consumes O_2 and initiates the oxidative photosynthetic carbon cycle. This pathway proceeds by a series of reactions in the peroxisomes, mitochondria and chloroplasts and is partially responsible for the evolution of CO_2 and consumption of O_2 in the light.

The partitioning of carbon between the reductive and oxidative photosynthetic carbon cycles is mediated by ribulose-P_2 carboxylase/oxygenase. The known mechanism for modifying the flux of carbon between the cycles is by changing the availability of the gaseous substrates, CO_2 and O_2. It has been speculated that intermediates of the oxidative photosynthetic carbon cycle may regulate the relative magnitudes of the oxidative and reductive pathways by a feedback mechanism (Oliver and Zelitch, 1977). Oliver and Zelitch (1977) reported that glyoxylate was a potent inhibitor of CO_2 fixation in isolated spinach chloroplasts, however, these authors and others (Hatch and Jensen, 1980) did not

observe inhibition of ribulose-P_2 carboxylase by glyoxylate. The effect of glyoxylate on purified spinach ribulose-P_2 carboxylase/oxygenase has been examined in this chapter.

Materials and Methods

Ribulose-P_2 carboxylase/oxygenase was purified, stored, activated and assayed as described in Chapter 1. Ribulose-P_2 was synthesized and purified as described in Chapter 1. CABP was prepared as previously described (Pierce et al., 1980).

Sodium 1-[^{14}C]-glyoxylate (6.98 mCi/mmol) was obtained from Amersham and solutions were lyophilized for storage when not in use. Sodium glyoxylate was obtained from Sigma, and solutions were prepared fresh daily. Other chemicals were of analytical reagent quality.

Enzyme was activated for 30 min at 30 C, and glyoxylate was added to the desired concentration and incubated at 30 C. Unless otherwise indicated, the interaction of the unactivated enzyme was studied in 0.1 M Bicine-Na^+ (pH 8.2), 0.2 mM EDTA and CO_2 in equilibrium with the atmosphere (approximately 0.7 mM HCO_3^-). Sodium glyoxylate was usually added at 5 or 10 mM and standard carboxylase assays were performed, except the duration of the assay was 15 s to minimize activation or inhibition during the assay.

Results

Inhibition of Ribulose-P_2 Carboxylase/Oxygenase by Glyoxylate. Inhibition of ribulose-P_2 carboxylase by glyoxylate follows psuedo first-order kinetics with a fast initial phase and a slower second phase (Fig. 8A). Rapid inactivation was observed with the activated enzyme, and gluconate-6-P, a competitive inhibitor with respect to ribulose-P_2, was exceedingly effective in protecting the enzyme from glyoxylate inhibition. Bovine serum albumin at 5-fold the concentration of carboxylase was ineffective in protecting the carboxylase from inhibition. These data indicate that the interaction of glyoxylate with ribulose-P_2 carboxylase was specific and that occupation of the catalytic site precluded inhibition.

A double reciprocal plot of glyoxylate concentration versus the psuedo first-order rate constant for inhibition of carboxylase acitvity indicates rate saturation with a maximal rate constant for inactivation of 7×10^{-3} s^{-1} and K_{inact} (concentration giving half the maximal rate of inactivation) for glyoxylate of 3.3 mM (Fig. 8B). These results indicate the formation of an inhibitor-enzyme complex prior to enzyme inactivation (Meloche, 1967) (Eq. 4).

$$E + I \rightarrow EI \rightarrow EI^{*} \qquad \text{(Eq. 4)}$$

Figure 8. Time course of Inhibition of Ribulose-P_2 Carboxylase Activity by Glyoxylate under Various Conditions.

A). Time course following enzyme activity over a 4 h period with enzyme (2 mg/ml) treated under the following conditions 1) activated enzyme incubated with 1 mM gluconate-6-P prior to the addition of 1 mM glyoxylate (o); 2) activated enzyme with no glyoxylate (control) (Δ); 3) activated enzyme in the presence or absence of BSA (10 mg/ml) and 1 mM glyoxylate (▲); 4) activated enzyme and 2 mM glyoxylate (□) b) activated enzyme and 10 mM glyoxylate,(■). Aliquots were removed at the times indicated after the addition of glyoxylate and assayed in the standard assay mixture.
B). Time course over the first 3 min with enzyme (2 mg/ml) treated with 1 mM (▲), 2 mM (Δ), 5 mM (□) and 10 mM (■) glyoxylate. The inset is a double reciprocal plot of the rate of inactivation versus glyoxylate concentration.

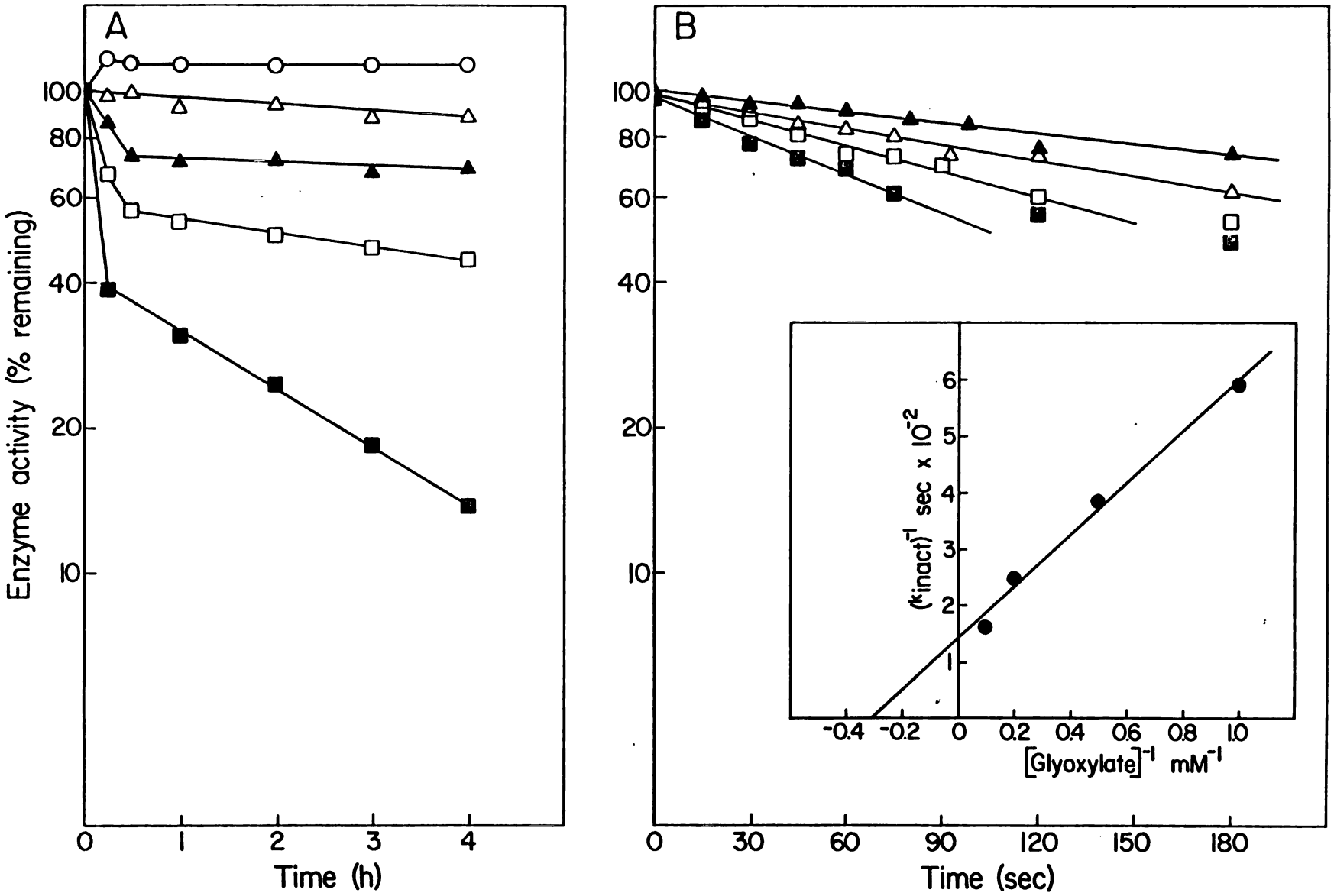

A
B
Enzyme activity (% remaining)
100
80
60
40
20
10
0
1
2
3
4
Time (h)
0
30
60
90
120
150
180
Time (sec)
$(k_{inact})^{-1}$ sec x 10^{-2}
6
5
4
3
2
1
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1.0
$[Glyoxylate]^{-1}$ mM^{-1}

Figure 8.

Kinetic analysis is analogous to Michaelis-Menten where k_{inact} is the observed rate of inactivation, $k_{max\ inact}$ is the maximal rate of inactivation and K_{inact} is the inhibitor concentration at 1/2 the maximal rate of inactivation (Eq. 5).

$$k_{inact} = \frac{k_{max\ inact} \cdot I}{K_{inact} + I} \qquad \text{(Eq. 5)}$$

Further evidence that the enzyme forms a reversible complex with glyoxylate was indicated by the competitive inhibition with respect to ribulose-P_2 (Fig. 9A). Kinetic analysis of enzyme which was exposed to glyoxylate only during catalysis (not activation) indicated glyoxylate was essentially competitive with respect to ribulose-P_2 with a K_I of 2 mM. Exposure of the enzyme to glyoxylate during activation resulted in a dramatic decrease in the Vmax (Fig. 9B). This response was due to enzyme inactivation which was irreversible in the time frame of the enzyme assay.

Inhibition of ribulose-P_2 carboxylase and oxygenase activities was assessed after 2 h incubation of enzyme with various concentrations of glyoxylate (Fig. 10). Both carboxylase and oxygenase activities were equally inhibited and glyoxylate was not selective in inhibition of either activity.

Figure 9. Inhibition of Ribulose-P_2 Carboxylase by Glyoxylate.

Activated enzyme (2 mg/ml) was assayed for 20 s with variable ribulose-P_2 (0.1 to 1.0 mM) and variable glyoxylate (0, 2.5, 10 mM). A). Kinetic parameters obtained when glyoxylate was exposed to enzyme only during the assay. B). Kinetic parameters obtained when glyoxylate was included in the enzyme activation and assayed at the same concentration.

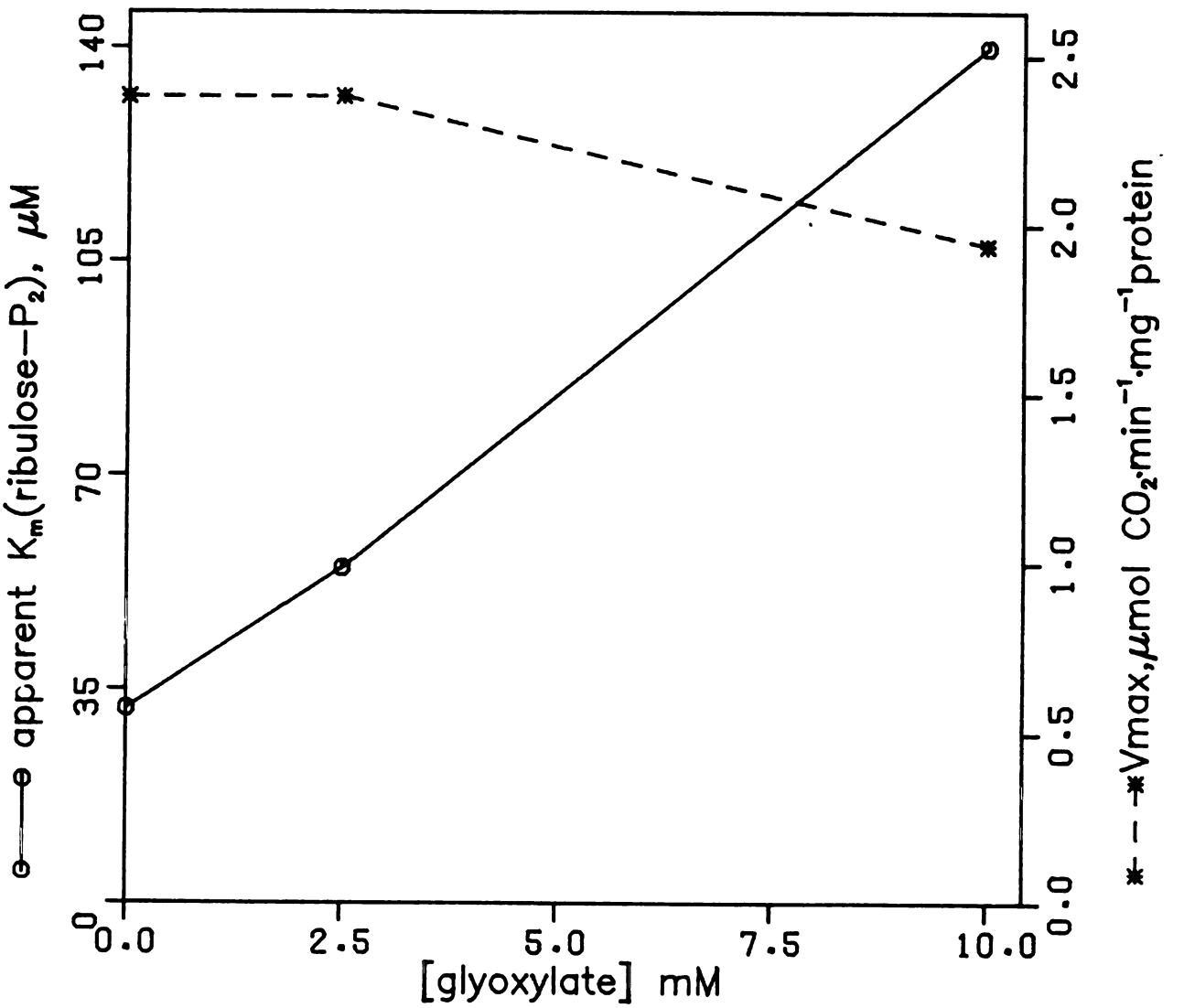

⊖—⊖ apparent K_m(ribulose–P_2), μM
✱ – –✱ Vmax, μmol $CO_2 \cdot min^{-1} \cdot mg^{-1}$ protein
0
35
70
105
140
0.0
0.5
1.0
1.5
2.0
2.5
0.0
2.5
5.0
7.5
10.0
[glyoxylate] mM

Figure 9A.

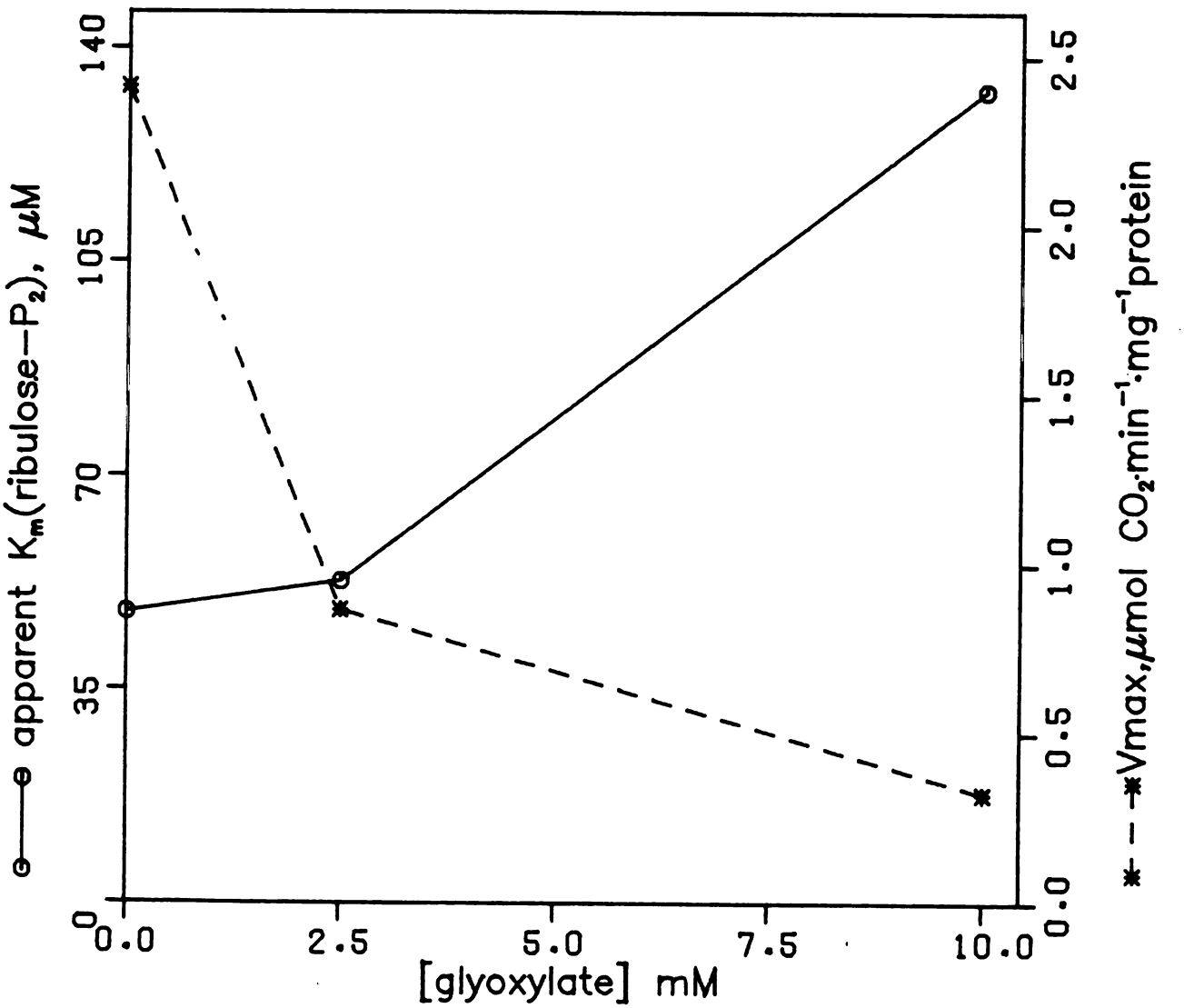

⊖—⊖ apparent K_m(ribulose–P_2), μM
0
35
70
105
140
– – Vmax, μmol CO_2·min^{-1}·mg^{-1}protein
0.0
0.5
1.0
1.5
2.0
2.5
0.0
2.5
5.0
7.5
10.0
[glyoxylate] mM

Figure 9B.

Figure 10. The Inhibition of Carboxylase and Oxygenase Activities as a Function of Glyoxylate Concentration. Activated enzyme (2 mg/ml) was incubated at each glyoxylate concentration for 2 h at 30 C. Carboxylase (o) and oxygenase (*) activities were assayed in the presence of glyoxylate at a concentration equal to that in the incubation.

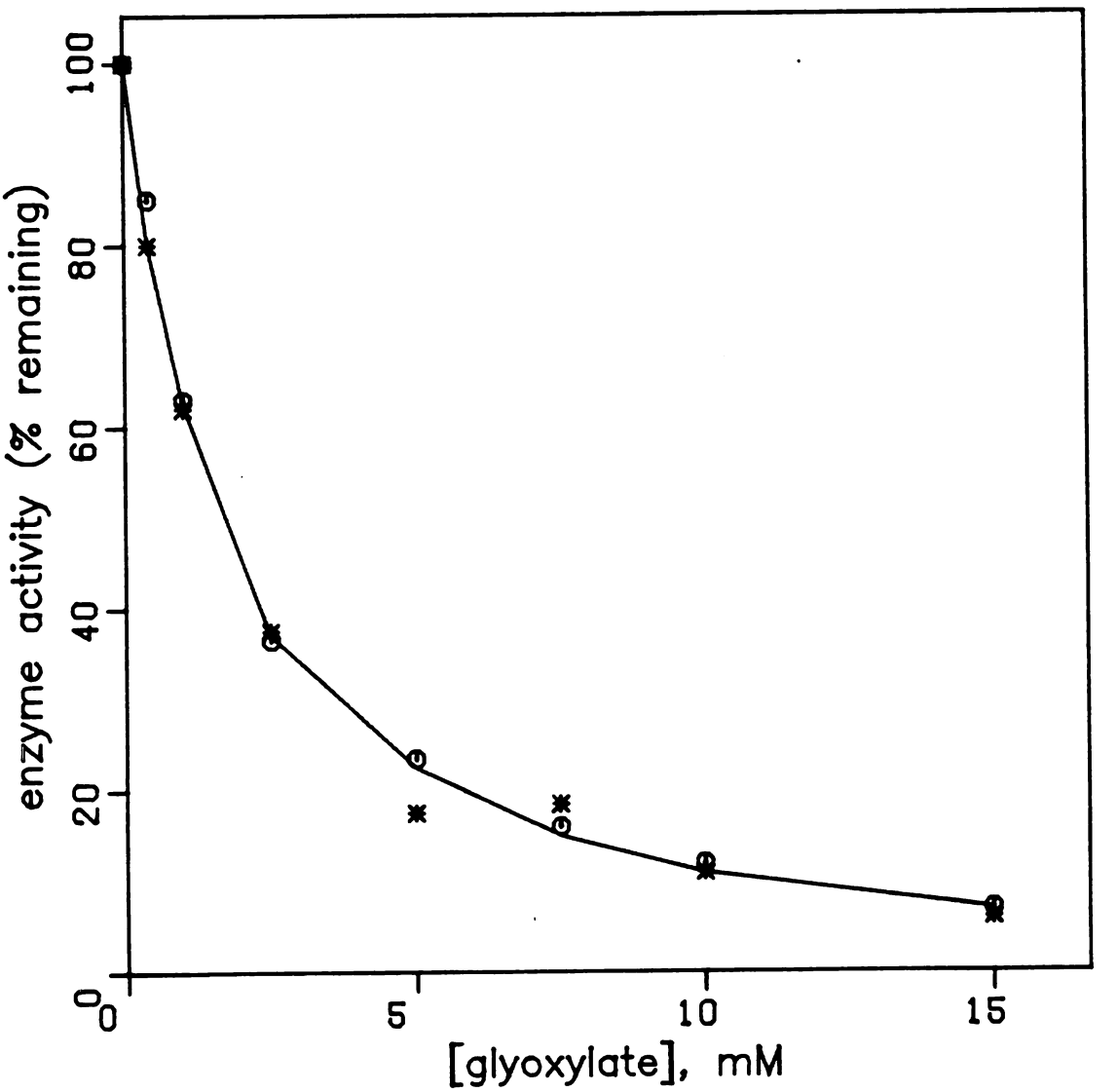

enzyme activity (% remaining)
100
80
60
40
20
0
0
5
10
15
[glyoxylate], mM

Figure 10.

Figure 11. The Reversibility of Glyoxylate Inhibition. Enzyme (10 mg/ml) was treated with 10 mM glyoxylate for 3 h at 30 C. Aliquots (10 μl) were taken and diluted (1:100) into 0.99 ml of assay buffer. Recovered enzyme activity was determined at timed intervals after dilution by initiating the reactions with ribulose-P_2. Control assays were run in the presence of 0.1 mM glyoxylate (equivalent to glyoxylate carried over with enzyme into the assay).

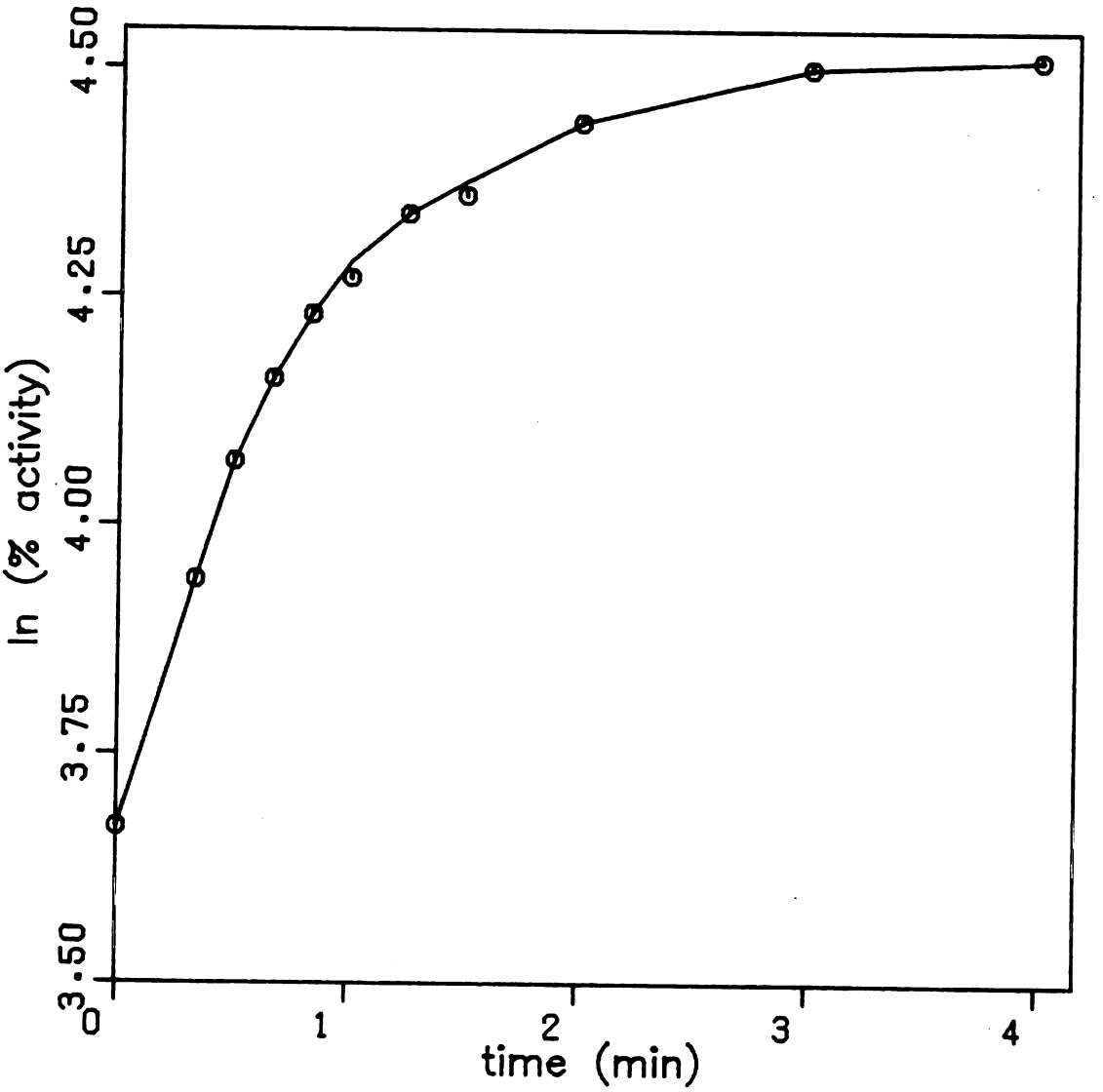


Figure 11.

The kinetics of dissociation of glyoxylate from the enzyme complex was assessed by inhibition of carboxylase with 10 mM glyoxylate for 3 hours, 100 fold dilution of the enzyme into inhibitor-free activation buffer and assay of carboxylase activity with time (Fig. 11). Reactivation followed psuedo first-order kinetics with a rate constant for reactivation of 1.2×10^{-2} s^{-1} and corresponding half-life of 60 s. Since reactivation was slow compared to the time frame of an enzyme assay (15-30 s), glyoxylate has been treated kinetically as an irreversible inhibitor. Figure 11 demonstrates that glyoxylate is actually a slowly reversible inhibitor.

The Stimulation of the Unactivated Enzyme by Glyoxylate. Addition of glyoxylate to unactivated carboxylase initially stimulated carboxylase activity which was followed by time-dependent inactivation (Fig. 12). The stimulation of activity could be observed if 1,3 or 10 mM $NaHCO_3$ were included prior to glyoxylate addition. Some stimulation of carboxylase activity could also be observed if 20 mM $MgCl_2$ was included prior to glyoxylate addition. If HCO_3^- and $MgCl_2$ were added together to activate the enzyme, only time dependent inhibition was observed. Addition of glycolate to the unactivated enzyme did not cause any change in the carboxylase acitvity, indicating that this was not a nonspecific response to an organic acid. Enzyme activated

Figure 12. Stimulation of Ribulose-P_2 Carboxylase Activity By Glyoxylate.

The indicated concentration of glyoxylate was added to a carboxylase solution (2 mg/ml) in 0.1 M Bicine-Na^+ (pH 8.2), 0.2 mM EDTA and air levels of CO_2. Carboxylase activity was determined by assay of 10 μl aliquots at the indicated times. Glyoxylate concentrations are as follows; O, 0 mM; Δ, 5 mM; X, 10 mM; *, 20 mM; and ◊, 5mM glyoxylate with 20 mM $MgCl_2$.

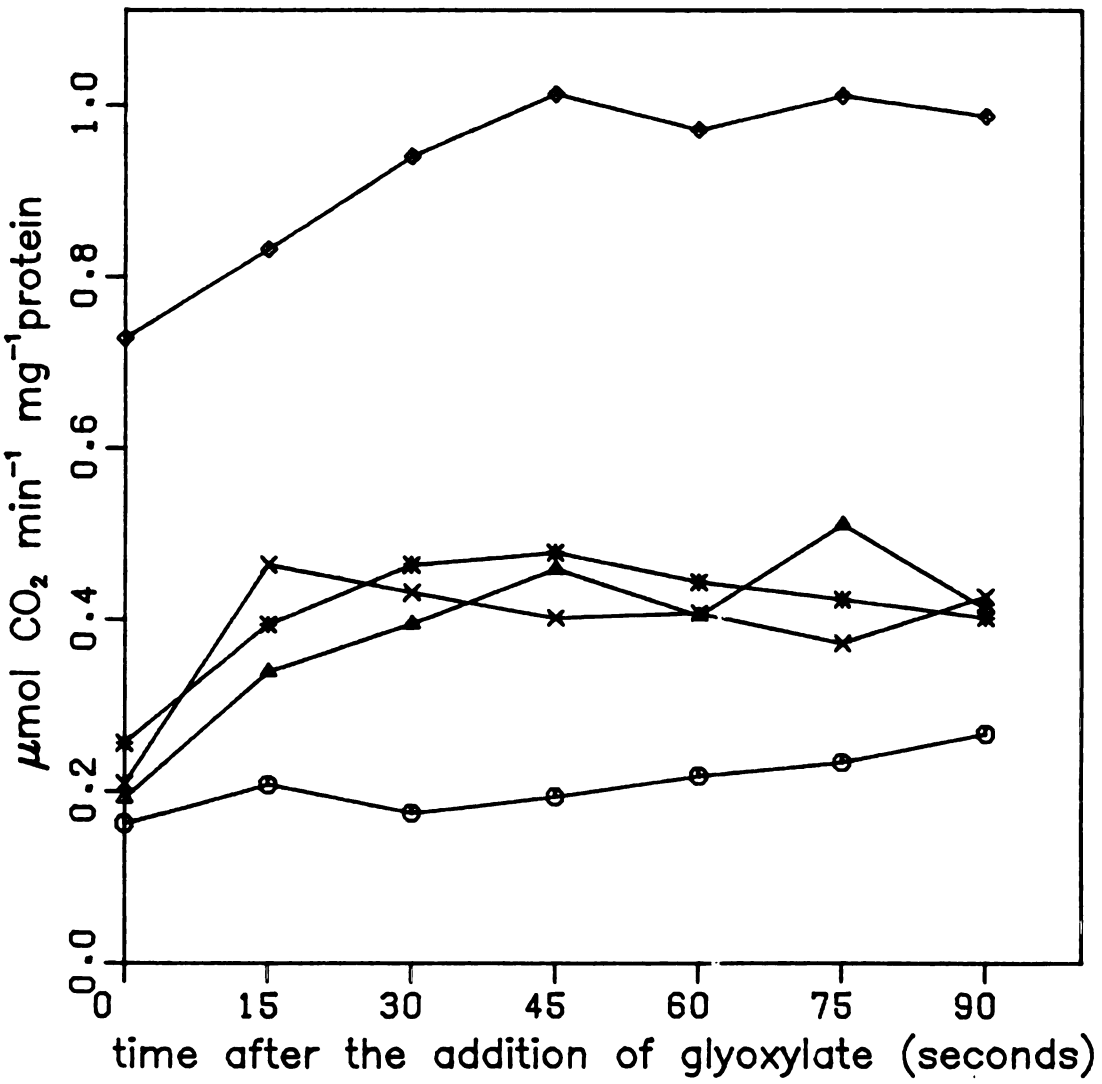

μmol CO_2 min^{-1} mg^{-1}protein
0.0
0.2
0.4
0.6
0.8
1.0
0
15
30
45
60
75
90
time after the addition of glyoxylate (seconds)

Figure 12.

with 3.3 mM $MgCl_2$, $MnCl_2$ or $CoCl_2$ showed similar glyoxylate-dependent carboxylase stimulation. The inhibition phase which occurred after addition of glyoxylate to the unactivated enzyme exhibited biphasicity with psuedo first-order rate constants of 1×10^{-3} s^{-1} and 8×10^{-5} s^{-1}.

The interaction between unactivated enzyme and glyoxylate was compared with the interaction with the effector, gluconate-6-P. Unactivated enzyme was exposed to 1 mM gluconate-6-P or 10 mM glyoxylate and carboxylase activity was measured over time (Fig. 13). The interactions of glyoxylate and gluconate-6-P with the enzyme were dissimilar. Glyoxylate elicted a rapid increase in carboxylase activity in the presence or absence of $MgCl_2$; gluconate-6-P treatment slightly inhibited carboxylase activity in the absence of $MgCl_2$ and resulted in a slow activation of the activity in the presence of $MgCl_2$. The enzyme activity after 1 h was 0.085 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein for carboxylase incubated with gluconate-6-P and 1.30 for carboxylase treated with gluconate-6-P and 20 mM $MgCl_2$.

The interaction of glyoxylate with unactivated enzyme was probed by examination of the binding of CO_2, CABP and glyoxylate (Table 2). Glyoxylate did not stimulate or inhibit CO_2 or CABP incorporation into the quaternary complex of enzyme-CO_2-Mg^{2+}-CABP. In addition, CABP was ineffective in trapping glyoxylate beyond that bound in the absence of CABP. Gluconate-6-P stimulated a large increase in bound

Figure 13. The Effect of Gluconate-6-P and Glyoxylate on Unactivated Ribulose-P_2 Carboxylase.

In this experiment, CO_2 depleted reagents were prepared by using freshly boiled water and gassing all solutions with N_2 during handling and for storage. Glyoxylate (10 mM) (□) or 6-P-gluconate (1 mM) (*) was added to 2 mg/ml ribulose-P_2 carboxylase in 0.1 M Bicine-Na^+ (pH 8.2) and 0.2 mM EDTA with or without 20 mM $MgCl_2$. Carboxylase activity was assayed with 20 μg of protein at the indicated times. Solid lines are treatments in the absence of $MgCl_2$. Dashed lines are for treatments which contained 20 mM $MgCl_2$.

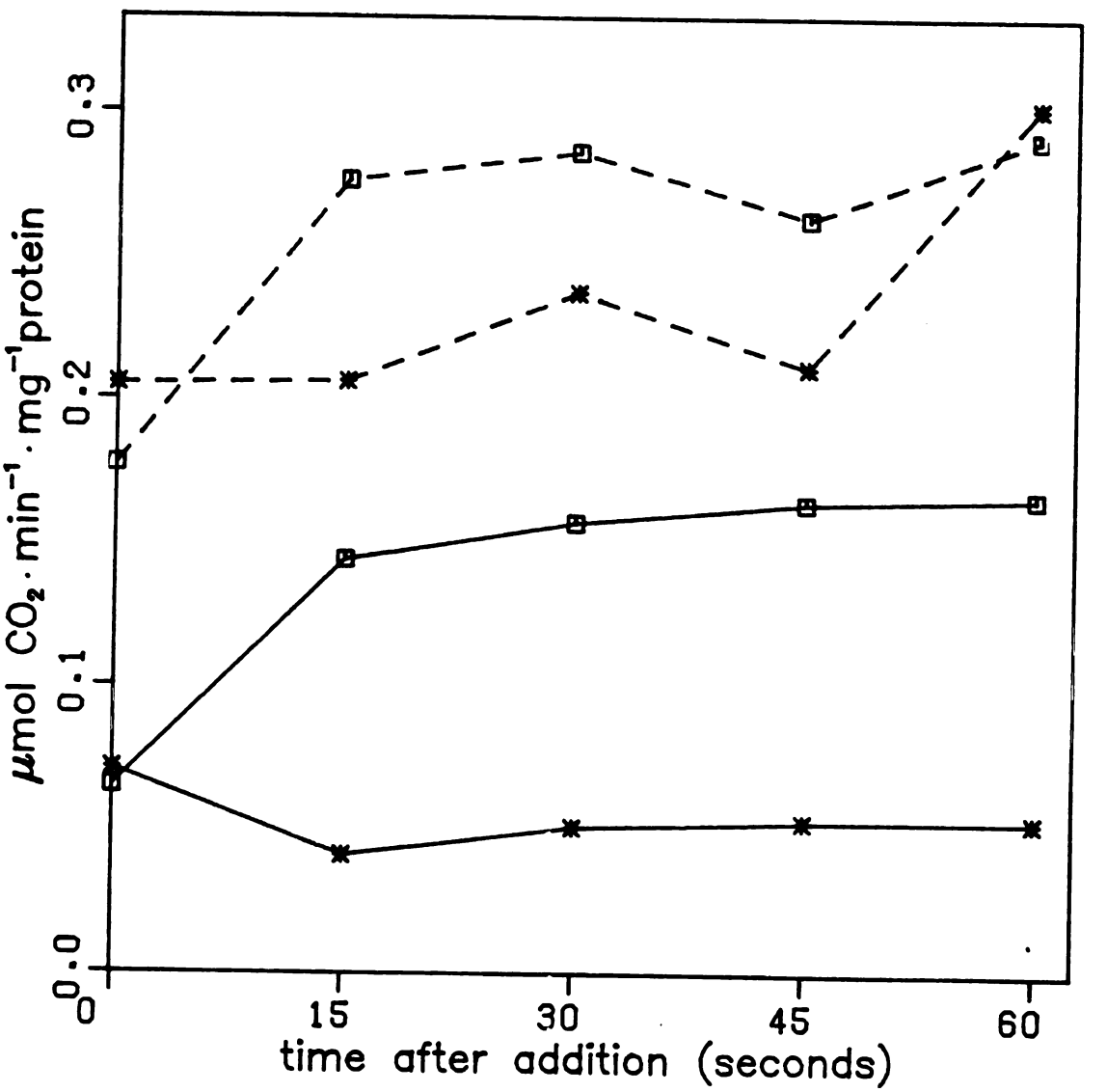

0.3
0.2
0.1
0.0
μmol $CO_2 \cdot min^{-1} \cdot mg^{-1}$ protein
0
15
30
45
60
time after addition (seconds)

Figure 13.

Table 2. Binding of CO_2, CABP and Glyoxylate by Partially Activated Ribulose-P_2 Carboxylase.

Ribulose-P_2 carboxylase (1 mg protein, 14.55 nmol protomer) was incubated with 0.1 M Bicine-Na^+ (pH 8.2), 0.2 mM EDTA and 1 mM $NaHCO_3$. Sodium glyoxylate (10 mM), gluconate-6-P (1 mM) and $MgCl_2$ (20 mM) were added where indicated. After 45 s, 40 nmol of CABP was added, and $MgCl_2$ was added to a concentration of 20 mM. The protein solutions were incubated for 45 min, and the radioisotope was diluted 10 fold in radiospecific activity. The loosely bound ligands were allowed to exchange for 30 min (5 h for CABP binding). The protein was then precipitated with 20% polyethylene glycol 4000 and 20 mM $MgCl_2$ prepared in 50 mM Bicine-Na^+ (pH 8.2) and 0.1 mM EDTA. The precipitate was collected by centrifugation (20 min, 48,000 x g) and washed twice by suspension in the polyethylene glycol and $MgCl_2$ media and centrifugation. The protein was dissolved in 50 mM Bicine-Na^+ (pH 8.2), 0.1 mM EDTA and 5 mM $NaHCO_3$ for scintillation counting. The radioactively labelled species is indicated (species bound) for each determination. Glyoxylate is abbreviated as Glx.

Additions to Activation	Trapping Condition	Radiolabelled Species Bound	mol bound per mol protomer
10 mM Glx	CABP,Mg^{2+}	CO_2	0.11
none	CABP,Mg^{2+}	CO_2	0.13
10 mM Glx	CABP,Mg^{2+}	CABP	0.11
none	CABP,Mg^{2+}	CABP	0.10
10 mM Glx	CABP,Mg^{2+}	Glx	0.34
10 mM Glx	none	Glx	0.32
10 mM Glx 20 mM $MgCl_2$	CABP,Mg^{2+}	CO_2	0.38
20 mM $MgCl_2$	CABP,Mg^{2+}	CO_2	0.35
20 mM $MgCl_2$ and 1 mM 6-P-gluconate	CABP,Mg^{2+}	CO_2	1.08

CO_2 due to carbamate formation. Glyoxylate interacts with ribulose-P_2 carboxylase in a manner which is unlike the effector gluconate-6-P and apparently will not substitute for carbamate formation as judged by CABP binding or CABP trapping of glyoxylate.

Discussion

Glyoxylate is a slowly reversible inhibitor of ribulose-P_2 carboxylase/oxygenase. The fully activated enzyme is inhibited by glyoxylate at about an order of magnitude faster than the unactivated form. Activation by CO_2 and Mg^{2+} does result in conformational changes in the protein and this could alter the accessibility or reactivity of an amino acid residue. The interaction of ribulose-P_2 carboxylase with glyoxylate appears to be a specific interaction based on several criteria: the presence of excess bovine serum albumin offered no protection against glyoxylate inhibition; gluconate-6-P, a competitive inhibitor with respect to ribulose-P_2, protected carboxylase activity from inhibition by glyoxylate; glyoxylate was a competitive inhibitor with respect to ribulose-P_2; inactivation was psuedo first-order with rate saturation; and complete inactivation corresponded to 1 mol of glyoxylate per mol of protomer when incorporation was corrected for nonspecific incorporation (Cook, personal communication). The interactions of glyoxylate with ribulose-P_2 carboxylase satisfy the criteria which

Hartman et al. (1978) proposed to establish a compound as an affinity label.

Glyoxylate was irreversibly incorporated into the protein after reduction with $NaBH_4$ and SDS gel electrophoreses of the protein derivitized with 1-^{14}C-glyoxylate demonstrated that the large subunit was labelled (Cook, unpublished observation). In addition, correlation of enzyme inhibition and glyoxylate incorporation indicated that complete loss of enzyme activity occurred with derivitization of a single lysyl residue per protomer. These data indicate that glyoxylate forms a Schiff-base with a lysyl residue which is probably in the region of the catalytic site. This hypothesis has recently been confirmed by amino acid sequencing of the derivitized peptide after proteolysis (C.M. Cook, F.C. Hartman and N.E. Tolbert, personal communication). Lysyl residue 175 was covalently modified by glyoxylate after reduction with $NaBH_4$. This residue has been derivitized by pyridoxal-P (Spellman et al., 1979) and various affinity ligands (Schloss et al., 1978b). This lysyl residue exhibits enhanced nucleophilicity toward carbonyl containing reagents and reactivity toward active site probes. The reactive nature of this residue may indicate that it has some catalytic function such as proton abstraction during the enolization of ribulose-P_2.

The biphasic nature of glyoxylate inhibition was unexpected. Although the incorporation of glyoxylate

correlated with the observed inhibition of activity, the reason for the change in the rate of inactivation was not evident. The extent of incorporation which occurred before the change in rate varied with the glyoxylate concentration. The biphasic rates of inhibition could be due to: cooperativity between subunits (e.g. negative allosterism); 2 distinct residues which are derivitized; or 2 conformers of the enzyme which show different rates of inactivation with glyoxylate. Since lysyl 175 was specifically derivitized, it seems unlikely that more than one amino acid residue was involved. The enzyme exhibits Michaelis-Menten kinetics, and cooperativity between subunits with substrate binding or catalysis has not been observed. However, it is possible that the subunits act cooperatively with inhibition by glyoxylate. Different forms of the activated enzyme may also exist, however there is no indication of this from the literature.

Glyoxylate caused a small stimulation of the activity of the unactivated protein. The mechanism responsible for this stimulation is not known, although glyoxylate behaved in a manner clearly unlike an effector, gluconate-6-P. Perhaps glyoxylate interacted with the unactivated protein in a manner that simulates the molecular changes of activation. One hypothesis proposed during this investigation was that glyoxylate formed a Schiff-base with lysyl 201, the residue that reacts with CO_2 to form the lysyl-carbamate during

activation. The glyoxylate adduct of lysyl 201 might be an analogue of the carbamate, and consequently glyoxylate might alleviate the CO_2 requirement of activation. This sort of interaction could explain the results, but one would predict that glyoxylate should stimulate CABP incorporation into the tight binding complex and this was not observed. The mechanism of glyoxylate stimulation of the unactivated enzyme is unknown. Glyoxylate did not act as an effector in stimulation of carbamate formation. Glyoxylate did not stimulate CABP incorporation into a tight-binding complex with the enzyme, which might be indicative of Schiff-base formation with lysyl 201.

Glyoxylate has been demonstrated to be a slowly reversible inhibitor or ribulose-P_2 carboxylase. The inhibition was not selective against the carboxylase or oxygenase activities. The inhibition exhibited by glyoxylate was due to Schiff-base formation with lysyl 175, a reactive catalytic site amino acid residue. Ribulose-P_2 carboxylase is weakly sensitive to glyoxylate as a competitive inhibitor (K_I = 2 mM) or as an affinity label (K_{inact} = 3.3 mM). Since glyoxylate is a peroxisomal metabolite and glyoxylate reductase ($NADP^+$) activity exists in the chloroplast (Chapter 4), glyoxylate should not accumulate in the chloroplast. These facts, together with the lack of selective inhibition of ribulose-P_2 carboxylase and oxygenase activities, indicate that glyoxylate does not serve any physio-

logical role in the regulation of photosynthesis or photorespiration as mediated by ribulose-P_2 carboxylase/oxygenase.

Acknowledgement

This chapter represents my contribution to a collaboration with Cathy M. Cook. Figures 8 and 10 represent experiments performed by Cathy M. Cook. They have been included in the thesis for clarity and I wish to thank her for allowing me to use them.

CHAPTER 4

THE EFFECT OF GLYOXYLATE ON PHOTOSYNTHESIS BY INTACT CHLOROPLASTS

The partitioning of carbon between the reductive and oxidative photosynthetic carbon cycles is mediated by ribulose-P_2 carboxylase/-oxygenase. The known mechanism for modifying the flux of carbon between the cycles is by changing the availability of the gaseous substrates, CO_2 and O_2. It has been speculated that intermediates of the oxidative photosynthetic carbon cycle may regulate the relative magnitudes of the oxidative and reductive pathways by a feedback mechanism (Oliver and Zelitch, 1977; Oliver, 1980). Oliver and Zelitch (1977) reported that glyoxylate was a potent inhibitor of CO_2 fixation in isolated spinach chloroplasts, but that glyoxylate did not inhibit ribulose-P_2 carboxylase activity. Glyoxylate is an inhibitor of purified ribulose-P_2 carboxylase/oxygenase in vitro (Chapter 3; Cook and Tolbert, 1982). Glyoxylate is a competitive inhibitor with respect to ribulose-P_2, and in addition forms an adduct with an amino acid residue of ribulose-P_2 carboxylase/oxygenase. Glyoxylate does not selectively inhibit the carboxylase or oxygenase activities. The inhibitory nature of glyoxylate suggested that it might exert some regulatory effect on photosynthesis. In view of these possibilities we

have examined the effect of glyoxylate on photosynthesis by intact chloroplasts.

Materials and Methods

Chloroplasts. Intact chloroplasts were isolated by the procedure of Nakatani and Barber (1977) from spinach leaves (Spinacia oleracea L.) which were obtained at a local market. Chloroplasts were suspended in a photosynthetic media composed of 330 mM sorbitol, 20 mM Bicine, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 50 mM sodium pyrophosphate which was adjusted to pH 8.0 with NaOH. The intactness of the chloroplast preparations was typically 70% as determined by the ferricyanide permeability procedure (Nakatani and Barber, 1977). Chlorophyll was determined spectrophotometrically in 80% acetone (Arnon, 1949).

Photosynthetic CO_2 Fixation. An aliquot of chloroplasts was suspended in photosynthetic media containing 1 mM ribose-5-P, 0.5 mM ADP and 5 mM $NaHCO_3$. Photosynthetic CO_2 fixation was stimulated by the addition of ribose-5-P and ADP by 10-15%, but CO_2 fixation was not dependent on these compounds. The chloroplasts (approximately 100 $\mu g\ Chl \cdot ml^{-1}$) were placed in a flattened test tube in a glass waterbath at 22°C. Illumination from a slide projector was filtered through a solution of $CuSO_4$. Photosynthetically active radiation was measured with a Lambda Instruments L1-185 quantum meter as 2500 $\mu Einsteins \cdot s^{-1} \cdot m^{-2}$. Photosynthesis was determined by the rate of $^{14}CO_2$ fixation and was typically 50 $\mu mol \cdot mg^{-1} Chl \cdot h^{-1}$.

Assay of Ribulose-P_2 Carboxylase from Intact Chloroplasts. Assays were performed essentially as described by Bahr and Jensen (1978). A 0.025 ml aliquot of chloroplasts (2.5 µg Chl) in photosynthetic media was added to 0.225 ml of the carboxylase assay media. The assay media contained 27.5 mM Bicine-Na^+ (pH 8.2), 22 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ ($\geq$0.4 µCi·µmol^{-1}), 1.1 mM dithiothreitol, and 0.55 mM ribulose-P_2. The carboxylase assay was incubated for 30 s at 22°C and terminated by the addition of 0.1 ml of 2 N HCl. The assay mixtures were dried at 95°C and analyzed for $^{14}CO_2$ incorporation into acid stable products by scintillation counting. The specific radioactivity was corrected to reflect the dilution of the isotope with unlabeled $NaHCO_3$ which was added with the chloroplast preparation. An enzyme unit was defined as the amount of enzyme which catalyzed the formation of 1 µmol of acid stable ^{14}C in 1 min.

Ribulose-P_2 Determination. This procedure was similar to that described by Sicher et al. (1979). Total ribulose-P_2 was determined by addition of a 0.1 ml aliquot of chloroplasts (7 to 10 µg Chl) to 0.9 ml of reaction media containing 27.5 mM Bicine-Na^+ (pH 8.2), 22 mM $MgCl_2$, 11 mM $NaH^{14}CO_3$ ($\geq$ 0.6 µCi·µmol^{-1}), 172 µg·ml^{-1} of purified spinach ribulose-P_2 carboxylase/oxygenase, 0.55 mM 2,6-dichlorophenol-indophenol and 0.44 mM carbonyl cyanide m-chlorophenylhydrazone. Ribulose-P_2 which was not in the chloroplast was determined by centrifugation (15 s, 11,000 x g) of the chloroplast suspension and determination of ribulose-P_2 in 0.1 ml of the supernatant solution. Determinations were terminated after 30 min with 0.2 ml of 2 N HCl and counted for ^{14}C incorporation as described above. Chloroplast ribulose-P_2 was calculated by

correcting the total ribulose-P_2 by the amount observed in the supernatant solution. The amount of ribulose-P_2 in the supernatant solution was usually less than 10% of the total ribulose-P_2.

Glyoxylate Reductase ($NADP^+$). This reductase was assayed in 50 mM potassium phosphate buffer (pH 6.2) and 0.17 mM NADPH. The rate of change in absorbance was measured at 340 nm and the enzymatic rate was calculated with the molar extinction coefficient for NADPH.

Paper Chromatographic Separation of Metabolites. Photosynthesis by chloroplasts was terminated by addition of formic acid to a final concentration of 0.67 N. The suspensions were diluted 10 fold with water, and centrifuged (10 min, 27,000 x g). The supernatant solutions were decanted and the volumes were reduced with a Buchler vortex-evaporator. Equivalent radioactivity was applied to each chromatogram (Whatman 4 paper). The chromatograms were developed by descending chromatography with 80% phenol in water (w/v) in the first dimension and with n-butanol:propionic acid:H_2O (2:1:1.4 by volume) in the second dimension. The chromatograms were dried briefly after chromatography in the second dimension and sprayed with 0.5 M $NaHCO_3$ to minimize loss of volatile acids. The location of radioactive metabolites was assessed by exposure of the chromatogram to x-ray film.

Fructose-P_2 Determination. A chloroplast preparation (5 ml, 100 $\mu g\ Chl \cdot ml^{-1}$) was allowed to photosynthesize for 10 min and then glyoxylate or formate was added to a concentration of 10 mM (equivalent volume of water added for the control). Photosynthesis proceeded for another 10 min and then the suspension was acidified with 2 ml of 2 N HCl. The suspension was centrifuged and an aliquot of D-[U-^{14}C]-fructose-P_2 (1 μCi, 239 $\mu Ci \cdot \mu mol^{-1}$) was added.

The fructose-P_2 was purified by chromatography on Dowex 1-Cl with a linear 0 to 0.5 M LiCl gradient prepared in 5 mM HCl. Fractions of the fructose-P_2 peak were pooled and the phosphate ester was precipitated as the barium salt in the presence of 50% ethanol (v/v). The barium salt of fructose-P_2 was dissolved by treatment with Dowex 50-H^+ and neutralized with 1 M triethanolamine. The amount of fructose-P_2 was enzymatically determined by conversion to glycerol-P. The change in absorbance at 340 nm was recorded after adding aldolase to a solution of fructose-P_2 with NADH, triose-P isomerase and glycerol-P dehydrogenase.

Materials. Ribulose-P_2 was synthesized and purified as described in Chapter 1. Ribulose-P_2 carboxylase/oxygenase was purified from spinach leaves and stored as described in Chapter 1. Sodium glyoxylate was obtained from Sigma and solutions were made fresh daily. Sodium 1-[^{14}C]-glyoxylate (6.98 mCi·mmol^{-1}) was obtained from Amersham and solutions were freeze-dried for storage when not in use. Paper chromatography of the 1-[^{14}C]-glyoxylate preparation indicated that it contained less than 1% [^{14}C]-glycolate.

Results

Activity and Activation of Ribulose-P_2 Carboxylase in Intact Chloroplasts. Glyoxylate at 10 mM inhibited CO_2 fixation by intact chloroplasts about 30%, and confirmed the results of Oliver and Zelitch (1977). Since ribulose-P_2 carboxylase/oxygenase is inhibited

by glyoxylate *in vitro*, the possibility that this mechanism accounted for the inhibition of CO_2 fixation was investigated. However the activation kinetics of ribulose-P_2 carboxylase in intact spinach chloroplasts during illumination or its activity upon chloroplast lysis did not change significantly with 10 mM of glyoxylate (Fig. 14). Typically 1 to 4 fold activation of the enzyme was observed after the chloroplasts were transferred into the light in the presence of 10 mM $NaHCO_3$. Frequently, there was a slight lag of about 1 min in the activation of ribulose-P_2 carboxylase when glyoxylate was included. When chloroplasts were incubated with 10 mM glyoxylate for 15 min in the light or dark without $NaHCO_3$ and then transferred into the light with the addition of $NaHCO_3$, substantial inhibition of ribulose-P_2 carboxylase activity was observed in the dark pre-incubated condition (Fig. 15). This was consistent with *in vitro* inhibition of ribulose-P_2 carboxylase by glyoxylate. However in the light there was a smaller effect of exogenous glyoxylate on ribulose-P_2 carboxylase activity. One explanation of the differential effect might be due to glyoxylate reduction by the chloroplast (see next section).

Ribulose-P_2 levels in illuminated chloroplasts were measured with or without glyoxylate (Fig. 16). The amount of ribulose-P_2 increased approximately two fold in the control, similar to results obtained by Sicher et al. (1979). However, in the chloroplast sample treated with 10 mM glyoxylate, the ribulose-P_2 level declined by about 40%. When the experiment in Figure 16 was repeated without addition of ribose-5-P and ADP, the initial level of ribulose-P_2 was 12 $nmol \cdot mg^{-1}$ Chl in the presence or absence of glyoxylate and then changed similarly to the data in Figure 16. These data indicate

Figure 14. The Effect of Glyoxylate on Ribulose-P_2 Carboxylase Activity in Chloroplasts. Chloroplasts were added to photosynthetic media (pH 8.0, 100 μg Chl·ml^{-1}, 10 mM $NaHCO_3$, with or without 10 mM glyoxylate) and illuminated. Aliquots were withdrawn and assayed for ribulose-P_2 carboxylase activity.

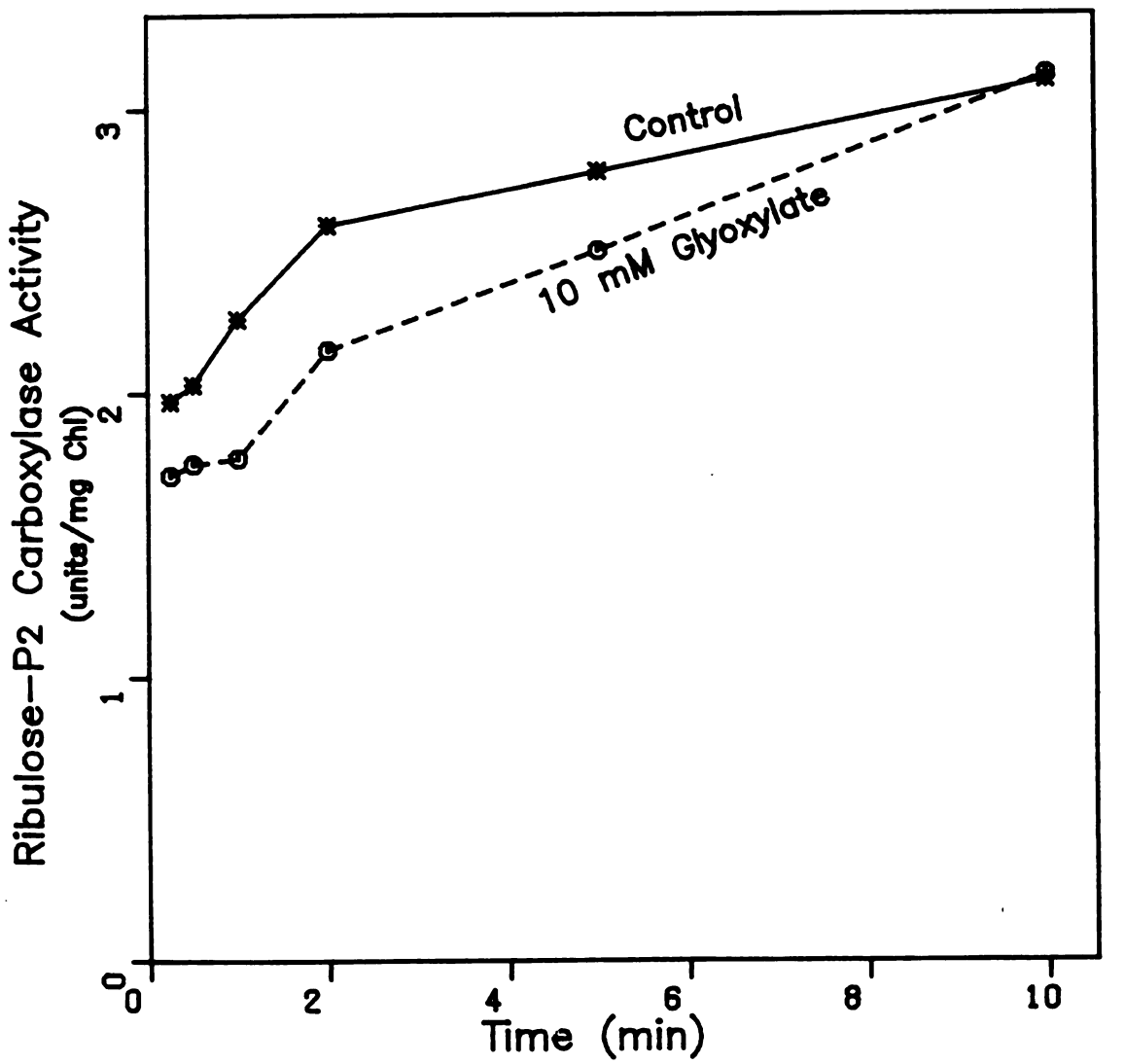

Ribulose-P2 Carboxylase Activity
(units/mg Chl)
0
1
2
3
0
2
4
6
8
10
Time (min)
Control
10 mM Glyoxylate

Figure 14.

Figure 15. The Effect of Preincubation with Glyoxylate and Illumination on Ribulose-P_2 Carboxylase Activity in Chloroplasts. Chloroplasts (100 μg Chl·ml^{-1} in photosynthetic media, pH 8.0) were incubated for 15 min at 25°C with or without illumination and 10 mM glyoxylate. The suspensions were then illuminated and $NaHCO_3$ added to a final concentration of 10 mM. Aliquots were withdrawn and assayed for ribulose-P_2 carboxylase activity.

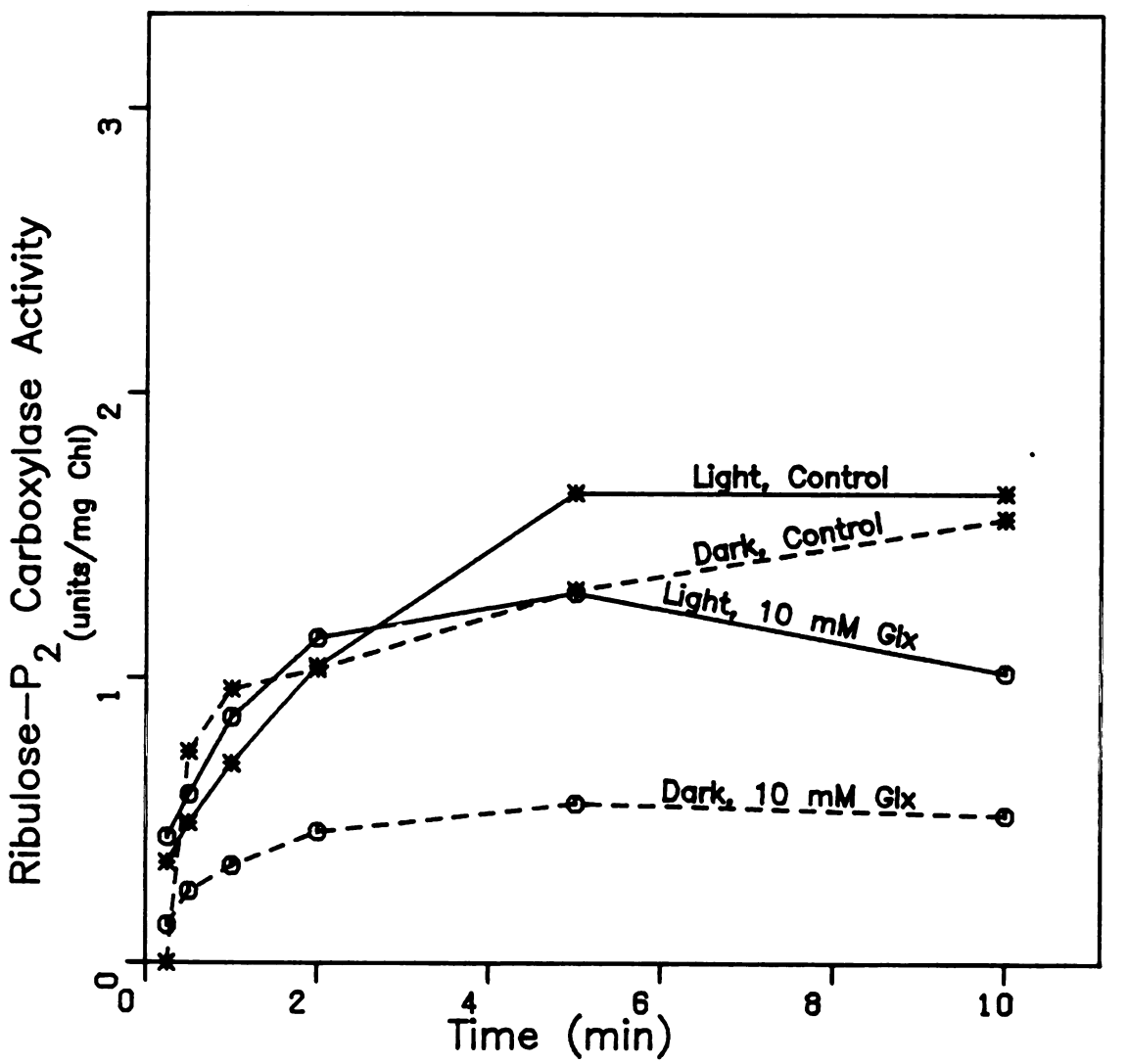

Ribulose–P2 Carboxylase Activity
(units/mg Chl)
0
1
2
3
0
2
4
6
8
10
Time (min)
Light, Control
Dark, Control
Light, 10 mM Glx
Dark, 10 mM Glx

Figure 15.

Figure 16. The Effect of Glyoxylate on Ribulose-P_2 Levels in Chloroplasts. Chloroplasts were suspended in photosynthetic media as described in the legend of Fig. 1 and illuminated with or without 10 mM glyoxylate. Aliquots were withdrawn and analyzed for chloroplast ribulose-P_2 as described in the methods. Similar results were obtained with or without ribose-5-P and ADP in the photosynthetic media.

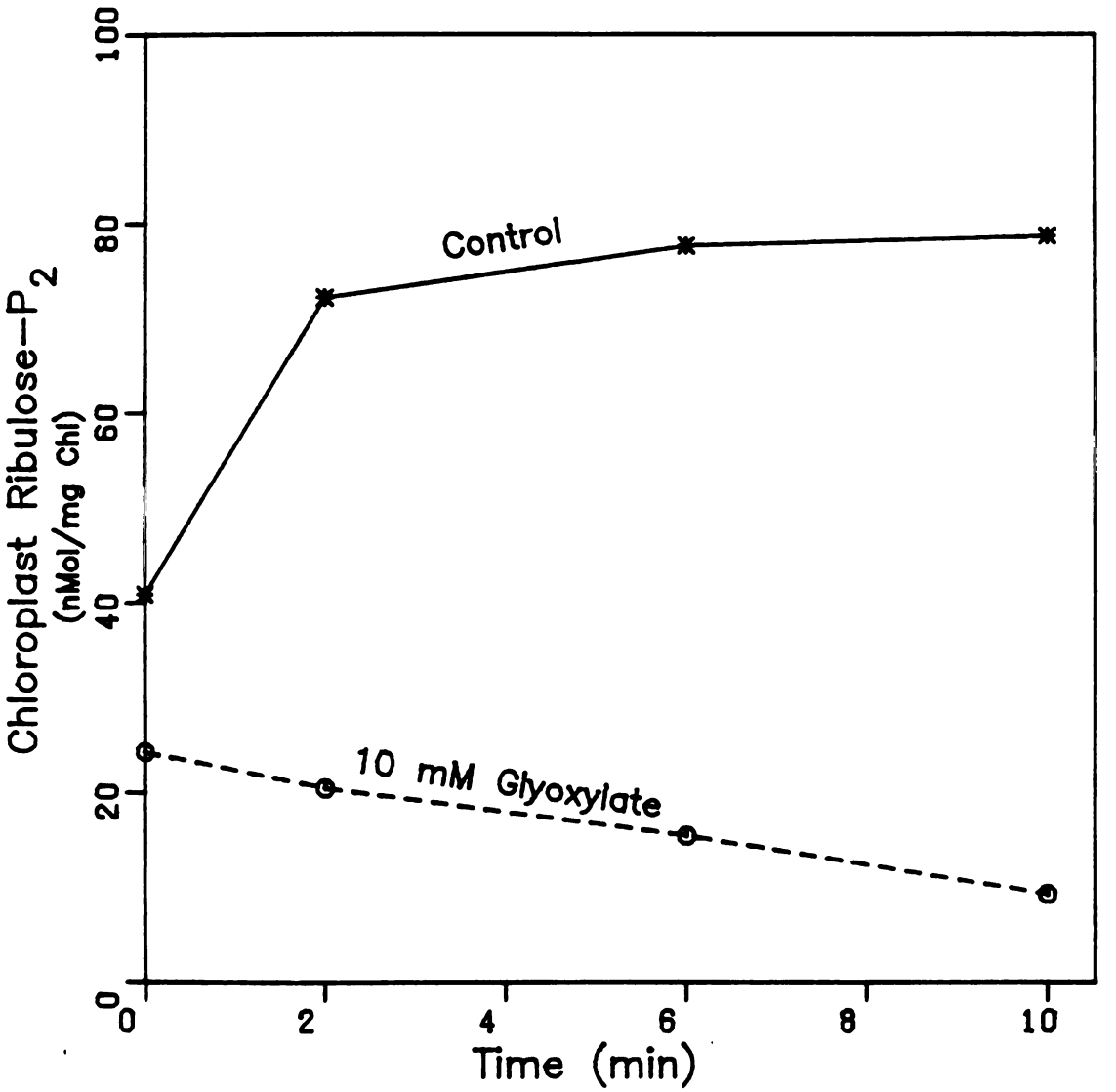

Chloroplast Ribulose–P2
(nMol/mg Chl)
100
80
60
40
20
0
Control
10 mM Glyoxylate
0
2
4
6
8
10
Time (min)

Figure 16,

that the activity of ribulose-P_2 carboxylase was not substantially inhibited in the light by glyoxylate, but rather the regeneration of ribulose-P_2 may have been inhibited by glyoxylate, consequently reducing the rate of CO_2 fixation in intact chloroplasts.

Glyoxylate Metabolism in Chloroplasts. Intact chloroplasts (104 μg Chl·ml^{-1}) were incubated with 0.57 mM 1-[^{14}C]-glyoxylate (2 μCi) for 20 min in photosynthetic media at pH 6 with or without illumination. Two-dimensional chromatography indicated the partial reduction of glyoxylate to glycolate by intact chloroplast preparations as has been observed previously by Kearney and Tolbert (1962) with chloroplasts prepared with concentrated salt solutions and Asada et al. (1965) with a reconstituted system. In the illuminated chloroplast preparation, 67% of the radioactivity was recovered as glycolate. This rate of glyoxylate reduction is equivalent to 11 μmol·mg^{-1} Chl·h^{-1}. When 1.0 mM $NaHCO_3$ was added to the chloroplast preparation for photosynthetic utilization of NADPH, similar results were obtained with 68% of the radioactivity recovered as glycolate. The chloroplast preparation which was not illuminated also reduced 40% of the glyoxylate to glycolate in 20 min. Reduction of glyoxylate by illuminated chloroplasts was also observed at pH 8. Only a trace (2% or less) of the radioactivity was found in glycine in these experiments.

Glyoxylate reductase ($NADP^+$) was present in the intact chloroplast preparations. This enzyme has previously been localized in spinach chloroplasts by isopycnic sucrose gradient centrifugation (Tolbert et al., 1970). The enzyme activity was completely soluble after chloroplast lysis by osmotic shock. No activity remained with

the membrane pellet after lysis and centrifugation. Kinetic studies with variable glyoxylate concentrations indicated a maximal rate of glyoxylate reduction of about 3 $\mu mol \cdot mg^{-1}$ $Chl \cdot h^{-1}$ and an apparent K_m(glyoxylate) of 140 μM. Substrate inhibition occurred at high glyoxylate concentrations ($\geq$ 500 μM). The rate of glyoxylate reduction observed spectrophotometrically (3 $\mu mol \cdot mg^{-1} Chl \cdot h^{-1}$) was somewhat less than the rate of glyoxylate reduction observed radiometrically (11 $\mu mol \cdot mg^{-1}$ $Chl \cdot h^{-1}$). Since glyoxylate reduction is presumably coupled to NADPH in the chloroplast, glyoxylate dependent O_2 evolution should occur in a manner similar to CO_2 or glycerate-3-P dependent O_2 evolution. However no glyoxylate dependent O_2 evolution was observed when intact chloroplast preparations were illuminated, perhaps because of the low activity of glyoxylate reductase.

The Effects of Salts of Weak Acids on Photosynthesis. Glyoxylate (10 mM) inhibited CO_2 fixation by intact chloroplasts about 30% when the pH of the incubation media was high (pH 8.0). Lactate, acetate and glycolate at 10 mM also inhibited CO_2 fixation under these conditions by 7, 9 and 19%, respectively. In addition formate was similarly inhibitory. Even sodium bicarbonate at 20 mM was inhibitory toward photosynthesis under these conditions, which Enser and Heber (1980) attributed to the uptake of CO_2, which resulted in the formation of the weak acid, carbonic acid. If the pH of the media was decreased to 6.0, these weak acids were much more inhibitory. The concentration of glyoxylate and glycolate which inhibited CO_2 fixation by 50% in intact chloroplasts at pH 6.0 were 50 and 150 μM, respectively (Fig. 17). The inhibition of photosynthesis by salts of these weak acids and the pH

Figure 17. The Effect of Glyoxylate and Glycolate on Photosynthesis by Intact Chloroplasts. Chloroplasts were suspended in photosynthetic media (pH 6.0, 1 mM $NaHCO_3$, 100 µg $Chl \cdot ml^{-1}$) which contained various concentrations of glyoxylate and glycolate. The rate of photosynthesis was determined over the linear portion of the $^{14}CO_2$ fixation time course.

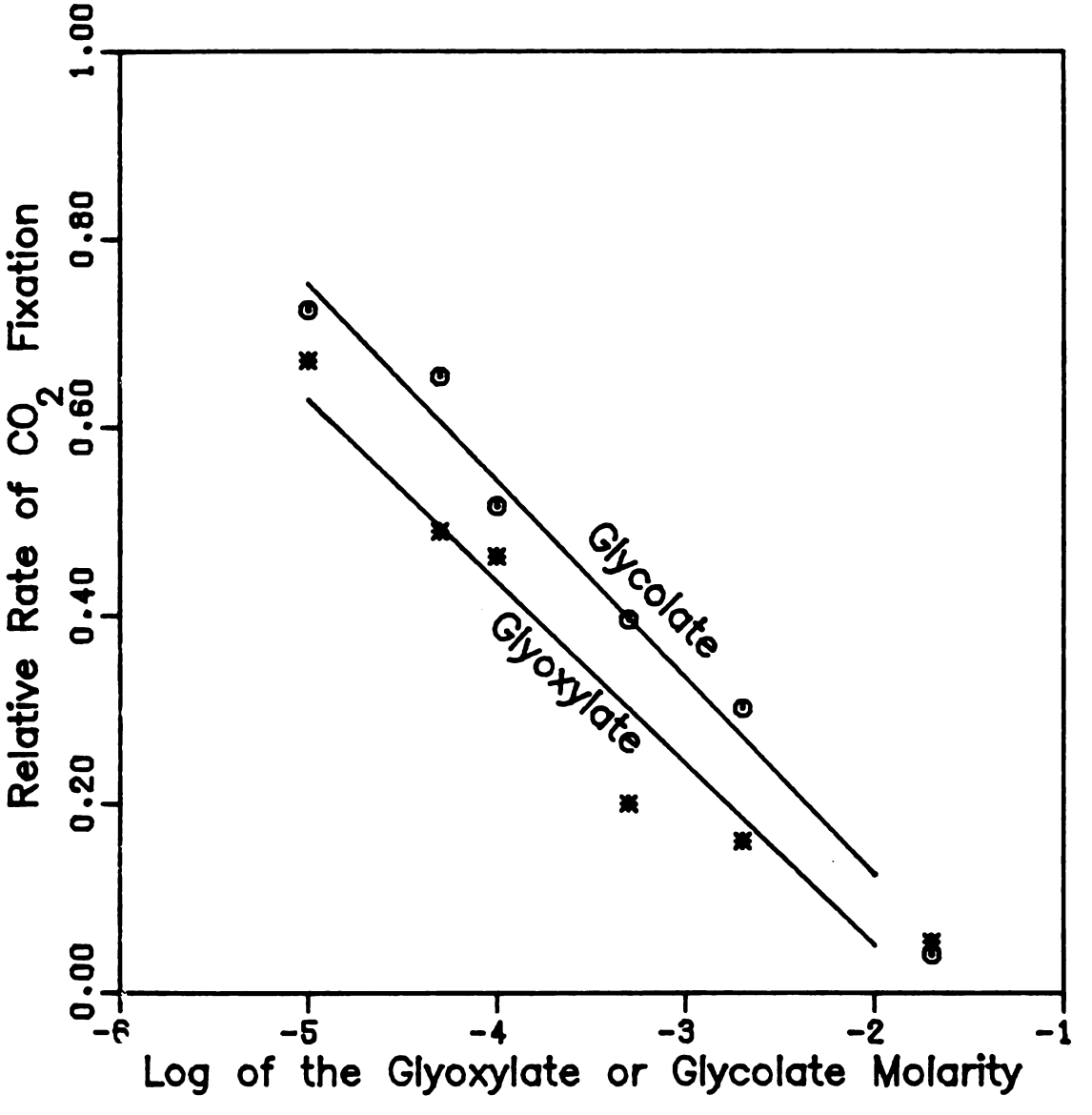

Relative Rate of CO_2 Fixation
0.00
0.20
0.40
0.60
0.80
1.00
-6
-5
-4
-3
-2
-1
Log of the Glyoxylate or Glycolate Molarity
Glycolate
Glyoxylate

Figure 17.

sensitivity toward inhibition suggested that these acids inhibited photosynthesis by a common mechanism, possibly by entering the chloroplast as the protonated acid and acidifying the stroma.

Effect of Glyoxylate Inhibition on the Products of Photosynthetic CO_2 Fixation. To determine whether some enzymatic step was inhibited by glyoxylate, intact chloroplasts were provided with $^{14}CO_2$ for 10 min to label the metabolites and then exposed to 10 mM glyoxylate in the light for an additional 10 min with the $^{14}CO_2$. The labeled products were separated by two-dimensional paper chromatography. Several compounds which migrated at the R_F of sugar bisphosphates contained a higher percentage of the ^{14}C when the chloroplasts were incubated with glyoxylate than in the untreated controls. The level of fructose-P_2 was measured enzymatically by coupling with aldolase and measuring NADH oxidation by glycerol-P dehydrogenase (see methods). After 10 min of photosynthesis, the concentration of fructose-P_2 was 70 nmol·mg^{-1} Chl in the control. When the chloroplasts were exposed to 10 mM glyoxylate or formate during photosynthesis, the level of fructose-P_2 increased to 200 or 190 nmol·mg^{-1} Chl, respectively.

Discussion

Glyoxylate inhibited photosynthesis in intact chloroplasts, as observed by Oliver and Zelitch (1977). The inhibition was not specific for glyoxylate, since salts of other weak acids such as lactate, glycolate, acetate and formate also inhibited photosynthesis. Also the inhibition of photosynthesis by glyoxylate was more severe at pH 6 than

at 8. The non-specific inhibition and pH dependence suggest that the mechanism of inhibition by the salts of these weak acids was in part the result of the permeability of the acid form to the chloroplast envelope and ionization in the alkaline environment of the stroma. The acid would equilibrate across the chloroplast envelope and the concentration of anions would distribute counter to the hydrogen ion gradient. Therefore the anion would accumulate in the alkaline compartment, resulting in a decrease of the pH of the compartment and concentration of the anion above the solution concentration. Acidification of the chloroplast stroma by 0.32 pH units was detected in the presence of 10 mM formate by Enser and Heber (1980).

The level of ribulose-P_2 in the chloroplast dropped after illumination in the presence of glyoxylate in contrast to the rise in ribulose-P_2 levels observed in the control. Thus the regeneration of ribulose-P_2 may be inhibited by glyoxylate, and chromatographic analysis indicated that fructose-P_2 accumulated. Increased fructose-P_2 levels in response to glyoxylate or formate treatment of intact chloroplasts has also been observed by others (Enser and Heber, 1980; Lawyer et al., 1983). In our experiments, fructose-P_2 levels were over 2-fold higher when photosynthesis in intact chloroplasts was inhibited by either glyoxylate or formate. These results suggested that fructose-P_2 phosphatase was a site of inhibition, but that this effect was not a specific response to glyoxylate. The activity of fructose-P_2 phosphatase is strongly effected by the pH and Mg^{++} concentration of its environment. Laing et al. (1981) reported that fructose-P_2 phosphatase activity decreased about 50% with a pH shift from 7.9 to 7.6.

Fructose-P_2 phosphatase is also activated by thioredoxin-dependent reduction (Buchanan, 1980) and inhibited by H_2O_2 oxidation of the sensitive sulfhydryl groups of the enzyme (Charles and Halliwell, 1981). Glyoxylate is known to form adducts with free sulfhydryl groups and free amines (Hamilton et al., 1979), and might also inhibit fructose-P_2 phosphatase directly or by interfering with thioredoxin-dependent activation. However the effect of salts of other weak acids on CO_2 fixation and fructose-P_2 levels suggests that the major inhibition is not related to the reactivity of carbon 2 glyoxylate, but rather to stromal acidification or anion accumulation.

Since glyoxylate is an inhibitor of ribulose-P_2 carboxylase/oxygenase in vitro, it was expected that inhibition would be observed in the intact chloroplast system. Glyoxylate inhibits isolated ribulose-P_2 carboxylase/oxygenase by two mechanisms. Glyoxylate is a competitive inhibitor with a K_I of about 2 mM, and in addition reacts slowly with an amino acid residue of ribulose-P_2 carboxylase/oxygenase to form an adduct (Chapter 3). Adduct formation results in severe inhibition. Under the conditions of the carboxylase assays performed in this paper, ribulose-P_2 was present at 20 times K_m and glyoxylate was present at 0.5 times K_i. Therefore only about 1% inhibition of ribulose-P_2 carboxylase could be expected due to glyoxylate as a competitive inhibitor in the assay. Inhibition of ribulose-P_2 carboxylase activity due to covalent adduct formation was tested under the condition of the assay, and was not substantial, except perhaps in the experiment in which chloroplasts were preincubated with 10 mM glyoxylate for 15 minutes in the dark.

The intact chloroplast preparations contained glyoxylate reductase ($NADP^+$) activity which was readily observed by spectrophotometric or radiometric assays. This enzyme has been partially purified and characterized from tobacco and spinach leaves (Tolbert et al., 1970; Zelitch and Gotto, 1962). Glyoxylate might inhibit photosynthesis by depletion of the NADPH pool by the activity of glyoxylate reductase. The rate of glyoxylate reduction was maximally at 20% of the rate of CO_2 fixation, which could account for the inhibition of photosynthesis. The role of glyoxylate reductase is unclear. There is still no clear evidence for a glycolate-glyoxylate shuttle. Perhaps the enzyme is responsible for the removal of glyoxylate from the chloroplast by reduction followed by excretion of glycolate. It may thus serve as a means of removing a reactive compound which may inhibit ribulose-P_2 carboxylase/oxygenase or some other enzyme required in chloroplast metabolism.

In our intact chloroplast system, ribulose-P_2 levels (measured enzymatically) decreased in response to glyoxylate treatment, in contrast to a control in which the ribulose-P_2 level doubled. However, Lawyer et al. (1983) observed increased ^{14}C labelling in ribulose-P_2 in intact chloroplasts after exposure to glyoxylate. Glyoxylate was approximately 50% inhibitory toward CO_2 fixation under their conditions (5 mM glyoxylate, pH 7.6), but only 30% inhibition was observed under our conditions (10 mM glyoxylate, pH 8.0). It is possible that little inhibition of ribulose-P_2 carboxylase occurs when glyoxylate is partially inhibitory to photosynthesis, but when glyoxylate inhibition is more severe, substantial carboxylase inhibition does occur. In addition, the photosynthetic media used by

the two groups were somewhat different. Our photosynthetic media contained pyrophosphate, which is slowly hydrolysed (Walker, 1971), in contrast to the media used by Lawyer et al. which contained 0.5 mM P_i. Thus the two chloroplast preparations may have had different metabolic conditions, since P_i is known to have dramatic effects on chloroplast metabolism.

The mechanism of glyoxylate inhibition of photosynthesis might involve stromal acidification, NADPH depletion or carboxylase inactivation. Our data indicate that carboxylase inactivation was not substantial. NADPH depletion due to glyoxylate reduction is a possible factor. The nonspecific response of photosynthesis to salts of weak acids suggests that stromal acidification probably was a factor. Regardless of the mechanism of glyoxylate inhibition of photosynthesis, it seem unlikely that glyoxylate serves any regulatory role *in vivo*, since glyoxylate synthesis is compartmentalized in the peroxisome and this metabolite should not accumulate in the chloroplast.

CHAPTER 5

THE EFFECT OF TRYPSIN ON RIBULOSE-P_2 CARBOXYLASE/OXYGENASE.

I became interested in correlating the kinetic parameters for the carboxylase reaction because of the apparent relationship between K_m (CO_2) and the type of photosynthetic carbon metabolism. The carboxylase from C_4 plants and other organisms that accumulate CO_2 tend to have a greater K_m (CO_2) than the enzyme from C_3 plants (Jordan and Ogren, 1981; Yeoh et al., 1980, 1981; Bird et al., 1982; Badger, 1980).

Energy is used in the formation of the Michaelis complex and tighter binding results in a decreased K_m. However, the energy used in binding must be overcome to attain the transition state. Therefore, high affinity of an enzyme for a substrate should result in a low K_m, a low k_{cat} and a high activation energy (Fersht, 1977). Therefore it is expected that an enzyme would tend to bind substrate weakly or moderately and consequently minimize the energy required to attain the transition state. Maximizing k_{cat}/K_m optimizes the use of energy in catalysis and Albery and Knowles (1976) have proposed that enzymes have evolved toward maximization of k_{cat}/K_m based on detailed kinetic studies of triose-P isomerase.

These concepts lead to interesting consequences when they are applied to ribulose-P_2 carboxylase in plants that accumulate CO_2 and those plants that do not. C_3 plants

have no known mechanism for the accumulation of CO_2. The only mechanism which is available to create a gradient of CO_2 for diffusion into the leaf is by removing CO_2 by fixation with ribulose-P_2 carboxylase. Therefore, evolution might result in a carboxylase with a low $K_m(CO_2)$ to minimize the steady-state concentration of CO_2 and maximize diffusion. Indeed, the K_m (CO_2) of ribulose-P_2 carboxylase from C_3 plants tends to be small. However, the penalty paid for a low K_m is a commensurate increase in the energy required to attain the transition state. Thus C_3 plant carboxylases may have evolved toward a reduced K_m (CO_2) and reduced k_{cat}. A C_3 plant might partially compensate for a low k_{cat} of the carboxylase by increased enzyme concentration. C_4 plants have a mechanism to concentrate CO_2 and the C_4 plant ribulose-P_2 carboxylase may have evolved toward a larger $K_m(CO_2)$ and k_{cat} than the C_3 plant enzyme. Ribulose-P_2 carboxylase from C_4 plants does exhibit a relatively large K_m (CO_2). I have attempted to correlate K_m (CO_2), k_{cat} and the activation energy of the carboxylase from several sources.

A technical problem which was anticipated in this study was that purified ribulose-P_2 carboxylase exhibits various specific activities from different organisms. The purified carboxylase from tobacco has been reported with specific activities varying between 0.1 to 0.5 (Brown et al., 1980; Kawashima et al., 1971; Kung et al., 1980; Koivuniemi et

al., 1980). The enzyme from other sources has likewise shown various specific activities (Bird et al., 1982). Few sources, such as spinach, are available which afford the purification of ribulose-P_2 carboxylase with specific activities of 2.0 to 2.3. Frequently inactivation due to protease digestion of carboxylase during purification has been proposed as an explanation of the poor specific activity of the enzyme (Gatenby and Cocking, 1978; Harris and Stern, 1977; Peoples and Dalling, 1978; Sugiyama et al., 1968b), and recently investigators have taken care to include protease inhibitors such as leupeptin in their enzyme extraction.

To eliminate the problem of variable specific activity dependent on enzyme preparation, enzyme active site concentration was assessed by the ability to bind the transition state analogue, CABP (Hall et al., 1981; Pierce et al., 1982). The ability to bind CABP correlates with ribulose-P_2 carboxylase activity when enzyme is denatured with heat (Hall et al., 1981). Unfortunately this procedure indicated widely different specific activities for various enzyme preparations. The effect of proteolysis on ribulose-P_2 carboxylase activity and CABP binding was consequently examined. CABP binds with high affinity to ribulose-P_2 carboxylase from spinach leaves after nearly total inactivation by proteolysis with trypsin. Therefore CABP binding may not be used as a measure of the catalytically

active sites where enzyme inactivation by protease digestion may be a factor.

MATERIALS AND METHODS

Ribulose-P_2 carboxylase/oxygenase was purified from spinach leaves from a local market by ammonium sulfate fractionation, molecular sieve chromatography and ion exchange chromatography, as described elsewhere (McCurry et al., 1982). The enzyme from Zea mays c.v. Pioneer 3780, Glycine max or Chlamydomonas reinhardtii was purified as described for the enzyme from spinach except for tissue homogenization. Extraction medium was as described for the preparation of spinach ribulose-P_2 carboxylase, except 0.4 mM dithiodiethyl-carbamate and 10 M leupeptin were included. Leaves from young maize plants (three weeks old) were cut into 1 cm squares and homogenized in an automated mortar and pestle (Torsion Balance Co, Clifton, NJ). Leaves from soybean were homogenized in a Waring blendor. Chlamydomonas cells were grown on 5% CO_2, collected by centrifugation and sonicated in a rosette cell. As judged by SDS-gel electrophoresis, the proteins were 95% pure (in the case of the maize enzyme) to essentially homogeneous for the enzyme from other sources.

Trypsin (292 units/mg protein) was obtained from Worthington and had been treated with N-p-toluenesulfonyl-L-phenylalanine chloromethyl ketone to inactivate

chymotrypsin. This enzyme was dissolved at 0.5 to 5 mg/ml in 1 mM H_2SO_4. Trypsin inhibitor, type II-0 from Sigma, was partially purified from chicken egg white.

Ribulose-P_2 was synthesized and purified as described in Chapter 1. CABP and 2'-^{14}C-CABP were prepared as described elsewhere (Pierce et al., 1980). Other chemicals were of analytical reagent quality and used without further purification.

Ribulose-P_2 carboxylase/oxygenase was stored, activated and assayed as described in Chapter 1. Carboxylase assays (30 s) were initiated with the addition of activated enzyme (4 to 60 g) to a vial containing 0.25 ml with 0.1 M Bicine-Na^+ (pH 8.2), 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM DTT, 0.5 mM ribulose-P_2 and 0.2 mM EDTA. Trypsin inhibitor was included in all assays at 150 times (w/w) the amount of trypsin included. Kinetic determinations required varying the concentration of $NaHCO_3$ (0.5 - 20 mM), the addition of carbonic anhydrase (0.010 mg/ml), maintenance of anaerobic conditions by gassing with N_2 and hermetically sealing the vial, and reduction of assay duration to 15 or 20 s. Carboxylase assays were terminated with the addition of 0.1 ml of 2 N HCl. The vials were dried at 95 C and analyzed for incorporation of acid stable ^{14}C by scintillation counting.

Ribulose-P_2 oxygenase activity was assayed by the addition of 100 μg of activated enzyme into the chamber of an

oxygen electrode containing 1 ml with 0.1 M Bicine-Na^+, 20 mM $MgCl_2$, and 0.5 mM ribulose-P_2.

CABP binding to ribulose-P_2 carboxylase was determined by a method described elsewhere (Pierce et al., 1982). Ribulose-P_2 carboxylase/oxygenase (1 mg, 14.5 nmol active sites) was added to a solution containing 29 nmol 2'-^{14}C-CABP (0.5 Ci/mol), trypsin inhibitor at 150 times (w/w) the amount of trypsin included, 20 mM $MgCl_2$ and 10 mM $NaHCO_3$. After 45 minutes at 22 C, 290 nmol of unlabelled CABP was added, and the enzyme was incubated for at least 6 hours at 22 C. The protein was precipitated with 20% (w/v) polyethylene glycol 4000 and 20 mM $MgCl_2$ and washed twice by resuspension of the protein in a solution of 20% polyethylene glycol and 20 mM $MgCl_2$. The protein was dissolved in 50 mM Bicine-Na^+ (pH 8.2) and 5 mM $NaHCO_3$ and counted for ^{14}C.

SDS-polyacrylamide gels were prepared with 15% acrylamide and 0.4% bis-acrylamide and electrophoresed with an LKB slab gel electrophoresis apparatus as described by Laemmli (1970). Gels were stained with 0.1% Coomassie Brilliant Blue R-250 in 50% ethanol and 10% acetic acid and destained with 17.7% (v/v) ethanol and 10% (v/v) acetic acid.

Peptides and protein were assayed by reaction with fluorescamine (Udenfriend et al., 1972). Sample was diluted to 1 ml with H_2O and 0.5 ml of 0.2 M borate-Na^+ buffer (pH

9.0) was added. Fluorescamine (0.5 ml, 0.2 mg/ml in acetone) was added with rapid mixing. The relative fluorescence was assessed on an Aminco fluoro-colorimeter with the appropriate filters (390 nm excitation, 475 nm emission).

RESULTS

The kinetic parameters obtained with ribulose-P_2 carboxylase from several sources are presented in Table 3. The enzyme from plants that have mechanisms for the accumulation of CO_2 (maize and Chlamydomonas) has a high Km (CO_2) compared to the enzyme from C_3 plants (spinach and soybean). The values reported here reflect the true Km(CO_2), since the determinations were performed under anaerobic conditions. The V_{max} value was calculated using a protein concentration based on the amount of CABP bound by a given volume of enzyme solution. Therefore, the protein concentration reflects only the fraction of the protein which tightly binds CABP. Using this technique, approximately 4-fold variation was observed in specific activity of ribulose-P_2 carboxylase between these organisms. There was no statistical difference in the enthalpy of activation among these proteins.

Table 3. Summary of Kinetic Parameters of Ribulose-P_2 Carboxylase from Various Sources.

	*Km(CO_2) (μM)	*V_{max} (μmol min^{-1} mg^{-1} protein)	ΔH^* (kcal/mol)
spinach	30.5 ± 2.2	2.87 ± 0.08	12.7 ± 0.87
soybean	19.6 ± 1.3	0.78 ± 0.05	12.1 ± 3.10
Maize	44.4 ± 6.3	1.49 ± 0.08	12.7 ± 0.49
Chlamydomonas	46.3 ± 3.3	3.23 ± 0.16	13.5 ± 1.54

* At 30 C, note $K_m(CO_2)$ has similar temperature dependence as k_{cat}; natural log $K_m[CO_2]$ = 10,800 (1/T) + const. for the spinach enzyme. ΔH^* was calculated from the slope of natural log [k_{cat}/T] versus 1/T over the range between 15 C and 35 C with kinetic analysis performed at 5 C intervals. Kinetic parameters were estimated with the aid of a computer program based on the method of Wilkinson (1969).

The validity of correlating active enzyme with CABP binding was examined by testing the effect of proteolysis, since this might reflect a process which occurs during protein purification. Digestion of activated ribulose-P_2 carboxylase/oxygenase with 0.5% (w/w) trypsin results in rapid loss of carboxylase activity (Fig. 18A). Ribulose-P_2 oxygenase activity declined similarly, and neither carboxylase or oxygenase activities were selectively inhibited (data not shown). The ability of the protein to bind the transition state analogue, CABP, was relatively insensitive to proteolysis by trypsin. After limited trypsin digestion, ribulose-P_2 carboxylase still bound 70 to 80% of the CABP

Figure 18. The Effect of Trypsin on Activity and CABP Binding by Ribulose-P_2 Carboxylase.

A. Activated ribulose-P_2 carboxylase (2 mg/ml) was treated with trypsin (0.01 mg/ml) at 30 C and assayed for carboxylase activity and the ability to bind CABP as proteolysis proceeded. The activity and ability to bind CABP recorded at time zero was assessed before the addition of trypsin. The theoretical activity was calculated by normalizing the observed (actual) activity by the fractional binding of CABP, as calculated by Hall et al. (1981).

B. The data presented in Figure 18A is plotted with CABP binding as a function of catalytic activity for comparison with Figure 3 of Hall et al. (1981).

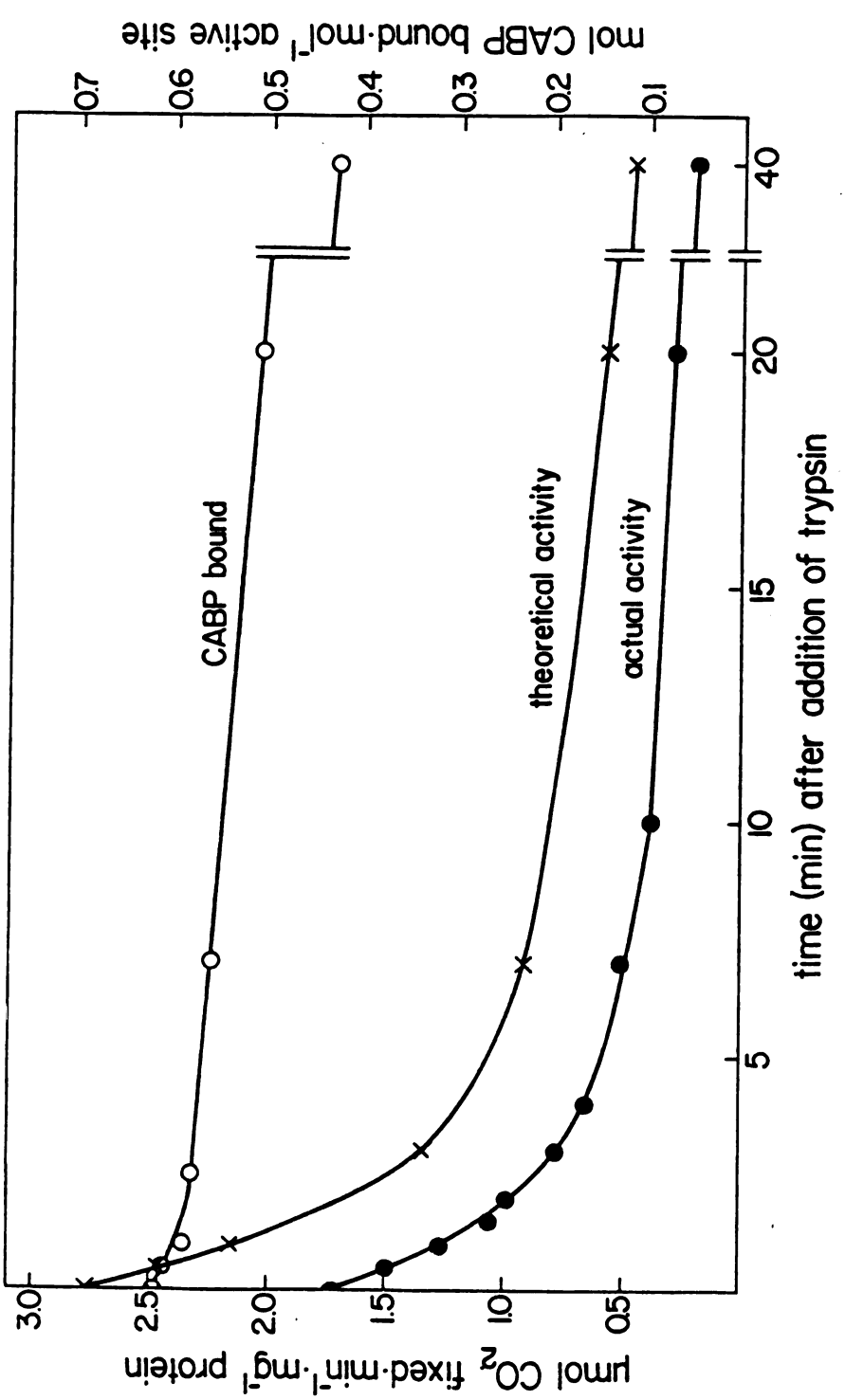

μmol CO_2 fixed·min^{-1}·mg^{-1} protein
3.0
2.5
2.0
1.5
1.0
0.5
mol CABP bound·mol^{-1} active site
0.7
0.6
0.5
0.4
0.3
0.2
0.1
5
10
15
20
40
time (min) after addition of trypsin
CABP bound
theoretical activity
actual activity

Figure 18A.

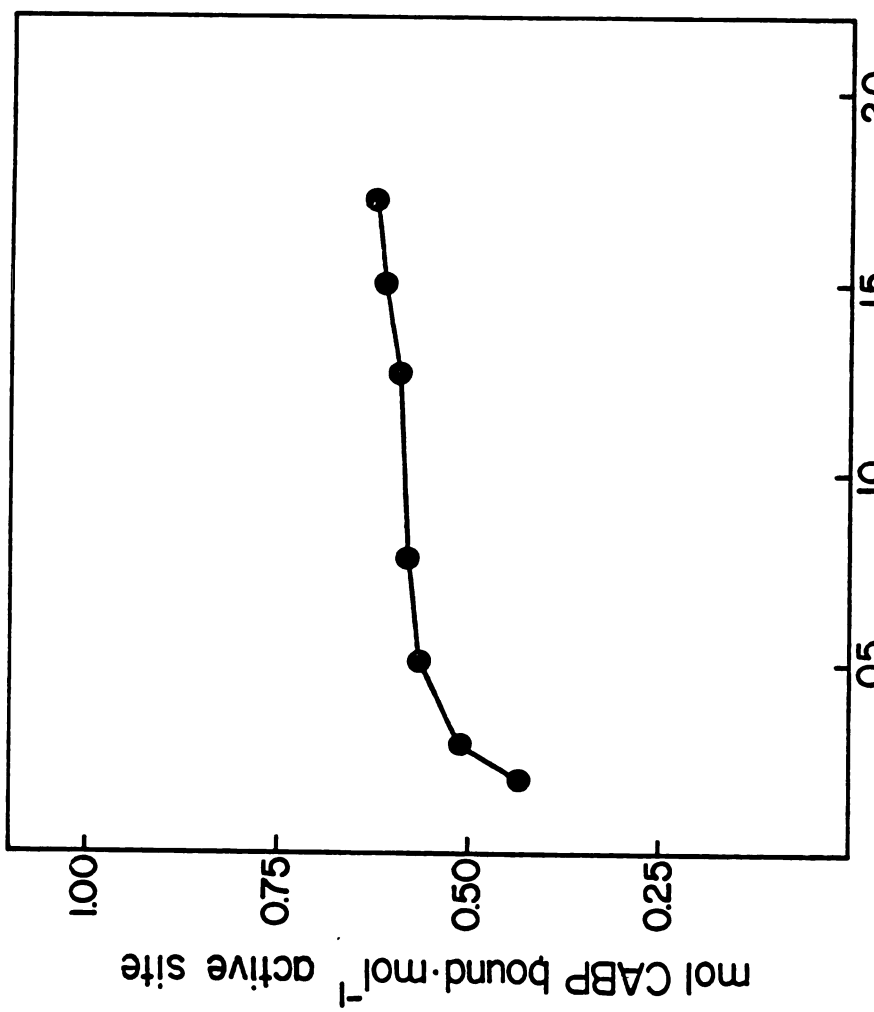

mol CABP bound·mol^{-1} active site
1.00
0.75
0.50
0.25
0.5
1.0
1.5
2.0
μmol CO_2 fixed·min^{-1}·mg^{-1} protein

Figure 18B.

which was bound before trypsin digestion; however, the carboxylase activity was only about 10% of the initial activity. The "theoretical specific activity" was obtained by dividing the observed specific activity by the fractional binding of CABP (mol CABP bound·mol^{-1} enzyme active site), which was calculated from the amount of CABP bound by a 1 mg aliquot of enzyme (14.5 nmol active sites). Trypsin inactivation had the effect of rapidly decreasing the theoretical specific activity.

This response was unexpected, since the binding of a transition state analogue was predicted to reflect the integrity of the catalytic site and therefore catalysis. It was shown by Hall et al. (1981) that CABP binding correlated very precisely with carboxylase activity when the protein was denatured with heat (Fig. 3; Hall et al., 1981). Their data indicated a theoretical specific activity of 2.8, which is consistent with the initial theoretical specific activity in Figure 18A. The CABP bound as a function of the catalytic activity of an enzyme solution after trypsin digestion is shown in Figure 18B, and may be compared with Figure 3 of Hall et al. (1981). CABP binding was independent of catalytic activity after inhibition with trypsin, unlike the rlationship of CABP binding to catalysis after heat denaturation. Only after severe ($\geq$ 80 %) inhibition of the enzyme activity by trypsin did CABP binding decrease.

CABP is known to bind to activated ribulose-P_2 carboxylase/oxygenase with a K_D of about 10 pM (Pierce et al., 1980b) and the half-life for release of CABP is 16 days. The half-life for release of CABP from trypsin digested ribulose-P_2 carboxylase/oxygenase was 10 days (Fig. 19). Therefore the trypsin inactivated enzyme retained the ability to tightly bind CABP, although this may be somewhat diminished from the native protein.

Electrophoresis of ribulose-P_2 carboxylase/oxygenase on SDS-polyacrylamide gels after trypsin digestion demonstrated rapid conversion of the large subunit (52,800 daltons) to two forms with molecular weights of approximately 51,300 and 49,300 daltons (Fig. 19; Lanes 1,2,3). Polyacrylamide gels prepared with 15% acrylamide were utilized in these experiments to improve the resolution of low molecular weight peptides. The small subunit appeared to remain intact during a 40 min digestion of the activated enzyme by trypsin.

Ribulose-P_2 carboxylase/oxygenase was digested with trypsin under various conditions (Fig. 19). The susceptibility to trypsin digestion increased with the following treatments: activated enzyme plus CABP; activated enzyme; unactivated enzyme. It appeared that activation and binding of the inhibitor cause a conformational change that decreased susceptibility to proteolysis, as observed by investigators with other proteins (Schimke, 1975). Investiga-

Figure 19. Dissociation of CABP from the Activated Enzyme CABP-Complex after Trypsin Digestion.

Activated ribulose-P_2 carboxylase (9 mg/ml) was treated with trypsin (0.045 mg/ml) for 20 min at 30 C. Assay of enzyme activity indicated that 13% of the initial activity remained. The enzyme solution was treated with trypsin inhibitor at 150 times (w/w) of the trypsin present and 2'-^{14}C-CABP (0.5 Ci/ mol) at twice the concentration of enzyme active sites present. After 45 min at 22 C, the radiospecific activity of the 2'-^{14}C-CABP was diluted by 10-fold. At various intervals after this point in time 0.75 or 1.0 mg aliquots of carboxylase protein were chromatographed on Sephadex G-50 (coarse). The protein concentration was analyzed by absorbance at 280 nm and 2'-^{14}C-CABP concentration analyzed by scintillation counting. The ratio of 2'-^{14}C-CABP to enzyme active sites was constant in the voided material and this ratio represents the fraction of enzyme active sites which retained 2'-^{14}C-CABP. The natural log of the percent of the enzyme active sites which retain 2'-^{14}C-CABP versus time gives a first-order rate constant for the dissociation of CABP from the activated enzyme CABP-complex of $2.93 (\pm 0.06) \times 10^{-3} \cdot h^{-1}$. This indicates a half-life of the activated enzyme CABP-complex of 10 days.

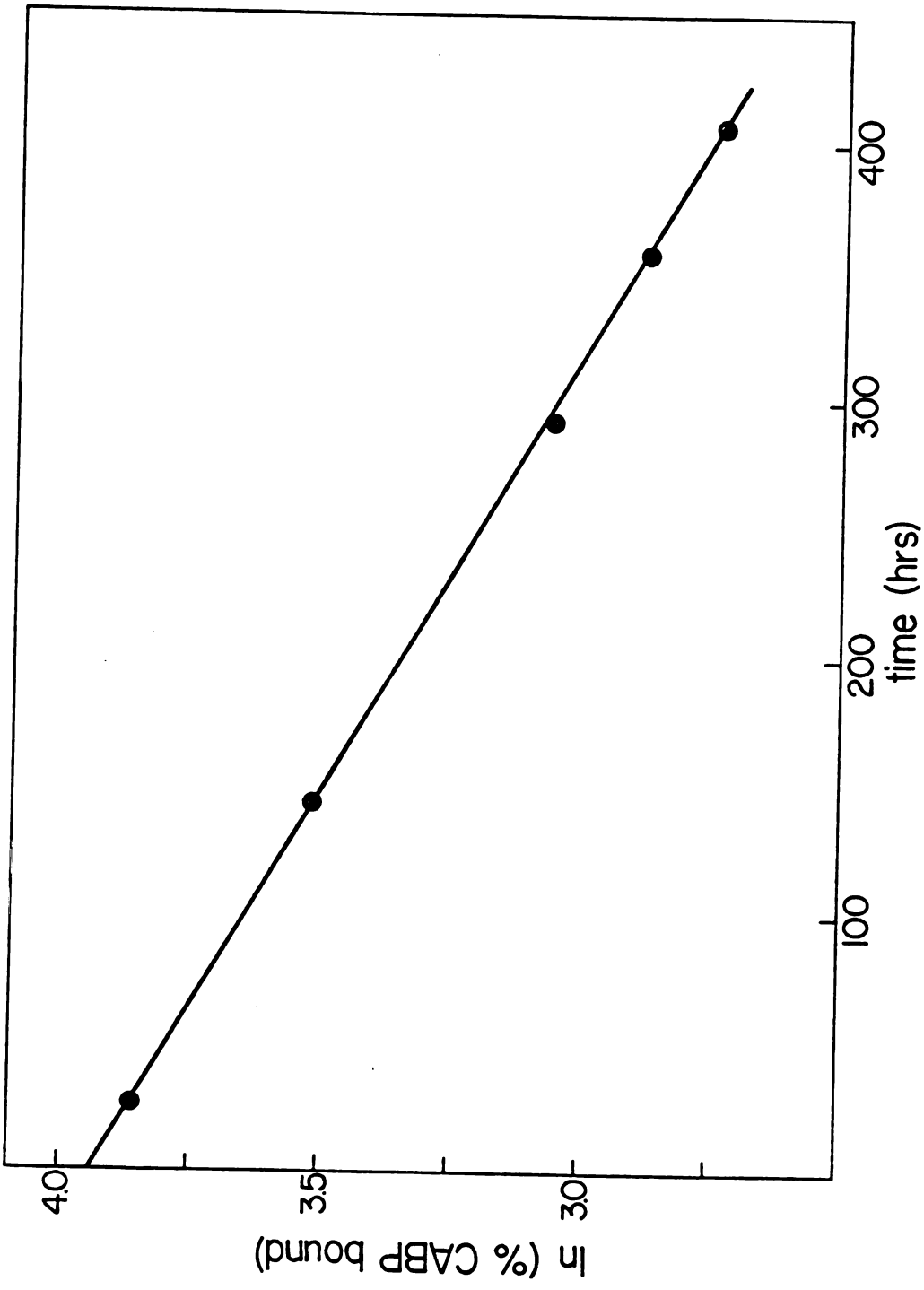


Figure 19.

Figure 20. SDS-Gel Electrophoresis of Ribulose-P_2 Carboxylase after Digestion by Trypsin.

Enzyme (2 mg/ml) was treated with trypsin (0.01 mg/ml) for 1 or 40 min. A sample was withdrawn and added to sample buffer composed of 62.5 mM Tris (pH 6.8), 1% SDS (w/v), 5% glycerol (v/v), 2.5% 2-mercaptoethanol and 0.0011% (w/v) bromophenol blue. The solution was boiled for two minutes and 7.5 μg of carboxylase protein was applied to a slab gel composed of a resolving gel with 15% acrylamide and stacking gel with 3% acrylamide. The protein was electrophoresed and stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% ethanol and 10% acetic acid and destained with 17% ethanol and 10% acetic acid. Lane 1 (numbered left to right) shows native carboxylase, with no trypsin digestion. Lanes 2 and 3 show activated ribulose-P_2 carboxylase digested with trypsin for 1 or 40 min, respectively. Lanes 4 and 5 show unactivated enzyme (in 0.1 M Bicine, pH 8.2, 0.2 mM EDTA) digested with trypsin for 1 or 40 min, respectively. Lanes 6 and 7 show activated enzyme incubated with 0.29 mM CABP for 45 min and digested with trypsin for 1 or 40 min, respectively.

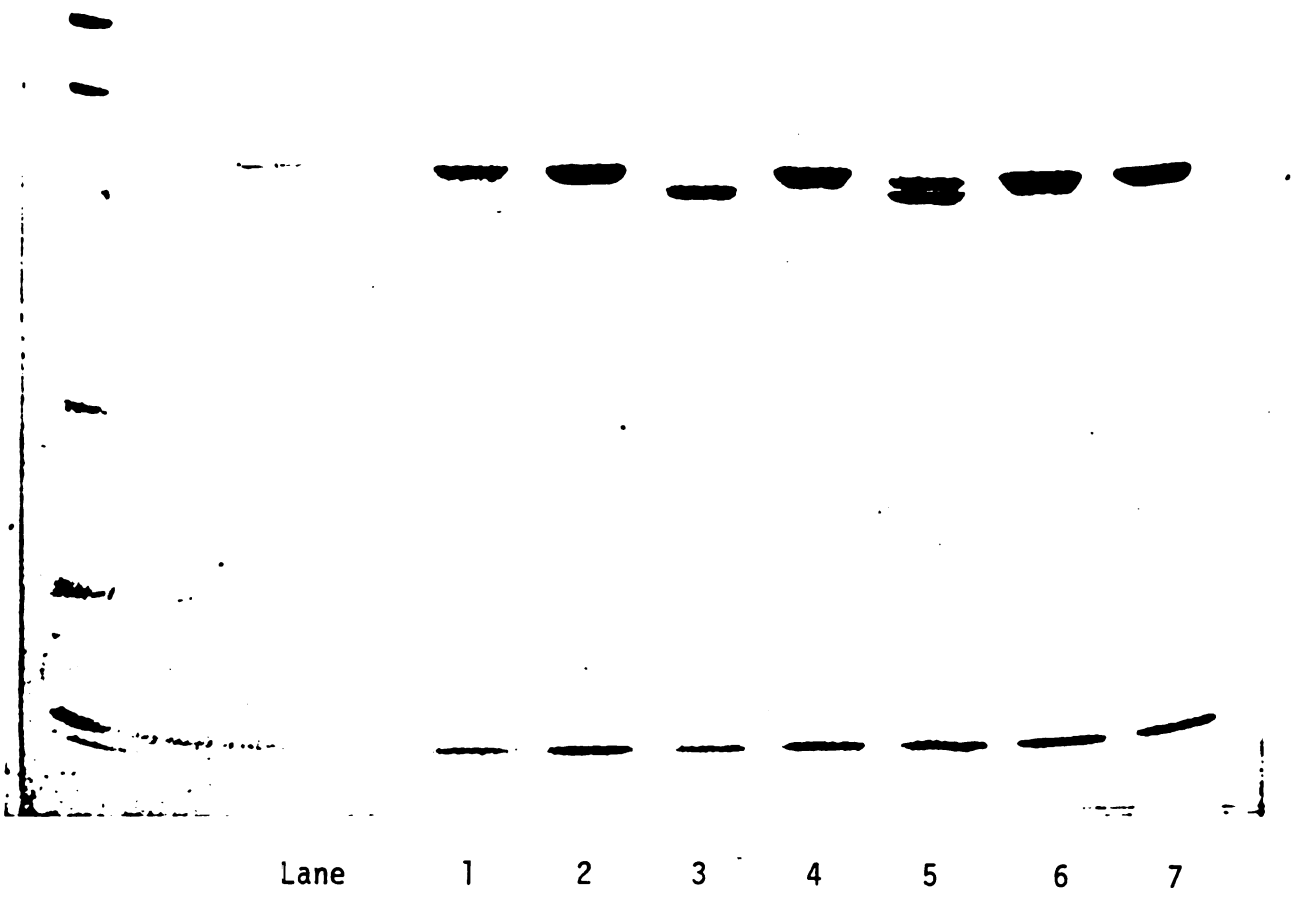


Figure 20.

Figure 21. Molecular Sieve Chromatography of Ribulose-P_2 Carboxylase after Digestion with Trypsin.

Activated ribulose-P_2 carboxylase (10.9 mg/ml) was treated with trypsin (0.055 mg/ml) for 40 min. Trypsin inhibitor was added at 3 times (w/w) of the trypsin present and 2.73 mg of carboxylase protein was chromatographed on Sephadex G-50 (coarse). The absorbance at 280 nm was determined spectrophotometrically and the fluorescence intensity after reaction with fluorescamine was determined.

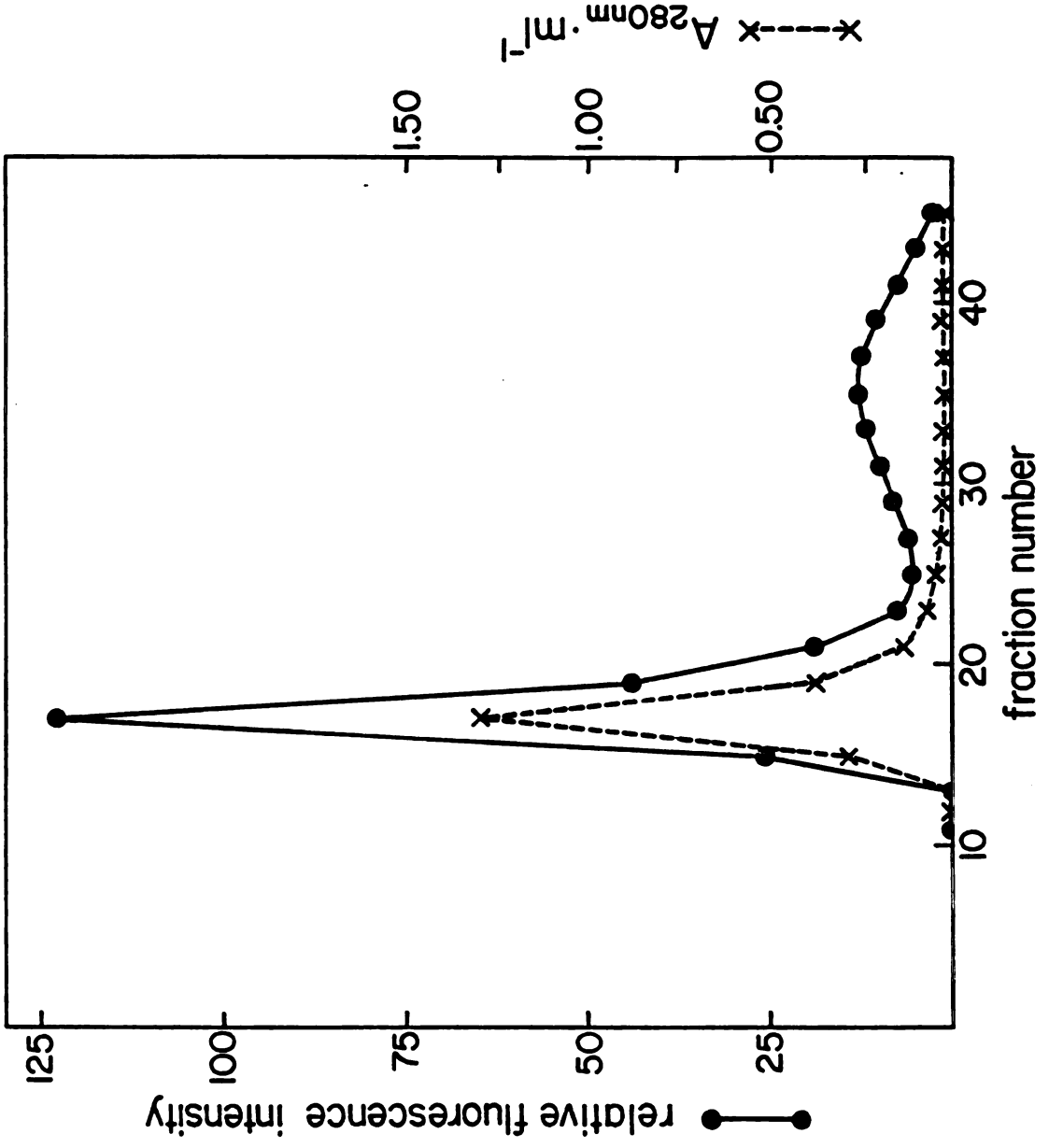

relative fluorescence intensity
125
100
75
50
25
fraction number
10
20
30
40
1.50
1.00
0.50
x---x $A_{280nm} \cdot ml^{-1}$

Figure 21.

tors have typically denatured proteins for complete digestion by a protease before peptide isolation (Schloss et al., 1978b).

Molecular sieve chromatography of ribulose-P_2 carboxylase after 40 min of digestion with trypsin resulted in the separation of two fractions. The first fraction voided on Sephadex G-50 and contained the bulk of the protein by absorbance at 280 nm or fluorescence after reaction with fluorescamine. A low molecular weight fraction eluted with the solvent, which possessed very little or no absorbance at 280 nm, but had considerable fluorescence after reaction with fluorescamine. The peptides which elute with the solvent should correspond to the fragments lost from the large subunit, and apparently contain few aromatic amino acid residues.

DISCUSSION

Ribulose-P_2 carboxylase/oxygenase has two or more sites near a terminus of the large subunit which are sensitive to trypsin. The peptides, which were cleaved by trypsin, were free in solution, since they may be separated from the enzyme by molecular sieve chromatography. Relatively little absorbance at 280 nm was detected in the peptides, suggesting that few aromatic amino acids were present.

Digestion of ribulose-P_2 carboxylase/oxygenase with trypsin rapidly inactivated the carboxylase and oxygenase

activity, but the stoichiometry of CABP binding was only slightly reduced. The dissociation of CABP from the enzyme-CABP complex after trypsin treatment was slow with a half-life of dissociation of 10 days. This value is somewhat smaller than that reported for the native enzyme (half-life of 16 days), although in either case binding is exceedingly tight. Thus proteolysis of ribulose-P_2 carboxylase by trypsin inhibits catalysis, although the protein still exhibits tight-binding of the transition state analogue. Presumably some enzyme catalyzed event is inhibited by proteolysis with trypsin, although the structure of the catalytic site is relatively intact. Therefore, CABP binding does not correlate with catalytic activity after proteolysis by trypsin.

A related observation was made with a mutant of Chlamydomonas reinhardtii which produced a modified ribulose-P_2 carboxylase protein (Spreitzer et al., 1982). The protein lacked catalytic activity, but still possessed the ability to bind CABP, although these investigators did not attempt to exchange loosely bound CABP in their study. CABP binding occurred in the absence of catalytic activity by this protein.

The sensitivity of ribulose-P_2 carboxylase/oxygenase to trypsin varied with the treatment of the protein. The amount of hydrolysis observed on SDS gels increased in the following order: activated enzyme plus CABP; activated en-

zyme; unactivated enzyme. Therefore, the susceptibility to trypsin appears to correlate with protein conformational changes, as observed in other studies (Schimke, 1975). A consequence of this relationship suggests that purification of ribulose-P_2 carboxylase under activating conditions and in the presence of a competitive inhibitor of ribulose-P_2 such as P_i, may render the enzyme less susceptible to proteolysis and result in improved activity of the purified enzyme preparation.

The binding of CABP has been used as a measurement of enzyme active sites by several investigators. This technique has proven instrumental in analysis of the activation status of the purified protein (McCurry et al., 1981). However, the practice of relating the CABP bound by an enzyme sample to the number of catalytic sites must be utilized with caution in view of the problems associated with proteolysis.

CHAPTER 6

NONENZYMATIC OXIDATION OF RIBULOSE-P_2 IN THE PRESENCE OF CYANIDE: AN ARTIFACT

The April 1, 1981 issue of Archives of Biochemistry and Biophysics contained an article on cyanide catalysis of the oxidation of aldehydes by dioxygen (Robertson et al., 1981). Since the proposed mechanism (Scheme 5) involves chemistry which is very similar to that proposed for ribulose-P_2 oxygenase, I initiated a study with ribulose-P_2 and cyanide. Cyanide dependent oxidation of ribulose-P_2 and the concomitant dioxygen consumption was ultimately shown to be an artifact. Perhaps the publication date should have been a clue, but I ended up playing the April fool.

Materials and Methods

DMPO and DETAPAC were obtained from Aldrich Chemical Company. Horseradish peroxidase, xanthine, xanthine oxidase, cytochrome c, Bicine, Dowex chelating resin, and the dimethyl ketal of dihydroxyacetone-P were obtained from Sigma Chemical Company. Catalase was obtained from the Millipore Corporation. Other chemicals were of reagent quality.

Ribulose-P_2 was synthesized and purified as described in Chapter 1. The dimethyl ketal of dihydroxyacetone-P was converted to the ketone as described in the instructions provided by Sigma. DMPO was purified by vacuum distillation. Trace metal contaminants were removed from buffers and reagents by the use of Dowex chelating resin, unless otherwise indicated.

MnSOD was purified from lyophilized E. coli strain B cells obtained from Sigma. The purification procedure of Keele, McCord and Fridovich (1970) was followed through fractionation with ammonium sulfate. This preparation was subjected to chromatography on Sepharose-4-B with a column buffer of 5 mM potassium phosphate (pH 7.8). The partially purified MnSOD was void of catalase activity and had a specific activity of 425 units per mg protein. MnSOD was assayed with xanthine and xanthine oxidase to generate O_2- and one unit of MnSOD activity was defined as the amount of enzyme activity that will reduce the rate of cytochrome c reduction by O_2- to 50% (McCord and Fridovich, 1969).

O_2 consumption was determined polarographically at 30°C with a Rank Brothers O_2 electrode (Cambridge, England).

EPR spectra were recorded with a Varian Century E112 spectrometer under the following conditions:3370 G magnetic field, 9.41208 GHz, 10 mW microwave power, 100 kHz modulation frequency, 2.5 G modulation amplitude, 1 second time constant, 4 minute scan time and ambient temperature (22°C).

Results

Sugar-P Dependent O_2 Consumption. The reaction of dioxygen with ribulose-P_2 or dihydroxyacetone-P in the presence of cyanide was examined by O_2 consumption. The initial rate was rapid, but ceased within a few minutes after the reaction was initiated by the addition of cyanide (Fig. 22a). The initial rate of O_2 consumption with dihydroxyacetone-P was about 5 times faster than that observed with ribulose-P_2 (conditions as described in Fig. 22). The total O_2 consumed was equivalent to about 3% of the ribulose-P_2 or 10% of the dihydroxyacetone-P included in the reaction.

The initial rate of O_2 consumption with dihydroxyacetone-P was dependent on the cyanide concentration up to 2 to 3 mM (Fig. 23). All reagents used in this, and other experiments, had metal contaminants removed by the use of Dowex chelating resin. No change in the rate of O_2 consumption was observed after the reagent solutions had been passed over the chelating resin. Therefore, trace metals were apparently not involved in the reactivity of dioxygen in this reaction.

The initial rate of O_2 consumption with ribulose-P_2 and cyanide was also examined as a function of pH (Fig. 24). The reaction was stimulated with increasing pH, but could be conveniently measured between pH 8 and 9, which would allow study of the reaction with the use of several enzymes.

Intermediates of Oxygen Reduction. The mechanism of the cyanide catalyzed oxidation of aldehydes was proposed by Robertson et al. (1981) to involve the univalent reduction of dioxygen to superoxide (O_2-) (Scheme 5). The reaction of aldehydes and cyanide with dioxygen was also found to generate a species which could reduce

Fig. 22. Dioxygen Consumption by Dihydroxyacetone-P in the Presence of Cyanide. a. The reaction mixture contained 1.16 mM dihydroxyacetone-P, 50 mM sodium phosphate (pH 8.5) and O_2 in equilibrium with the atmosphere. The reaction was initiated with the addition of 0.5 mM sodium cyanide. b. As in a, but catalase (100 units) was added after about 90% of the O_2 consumption had ensued.

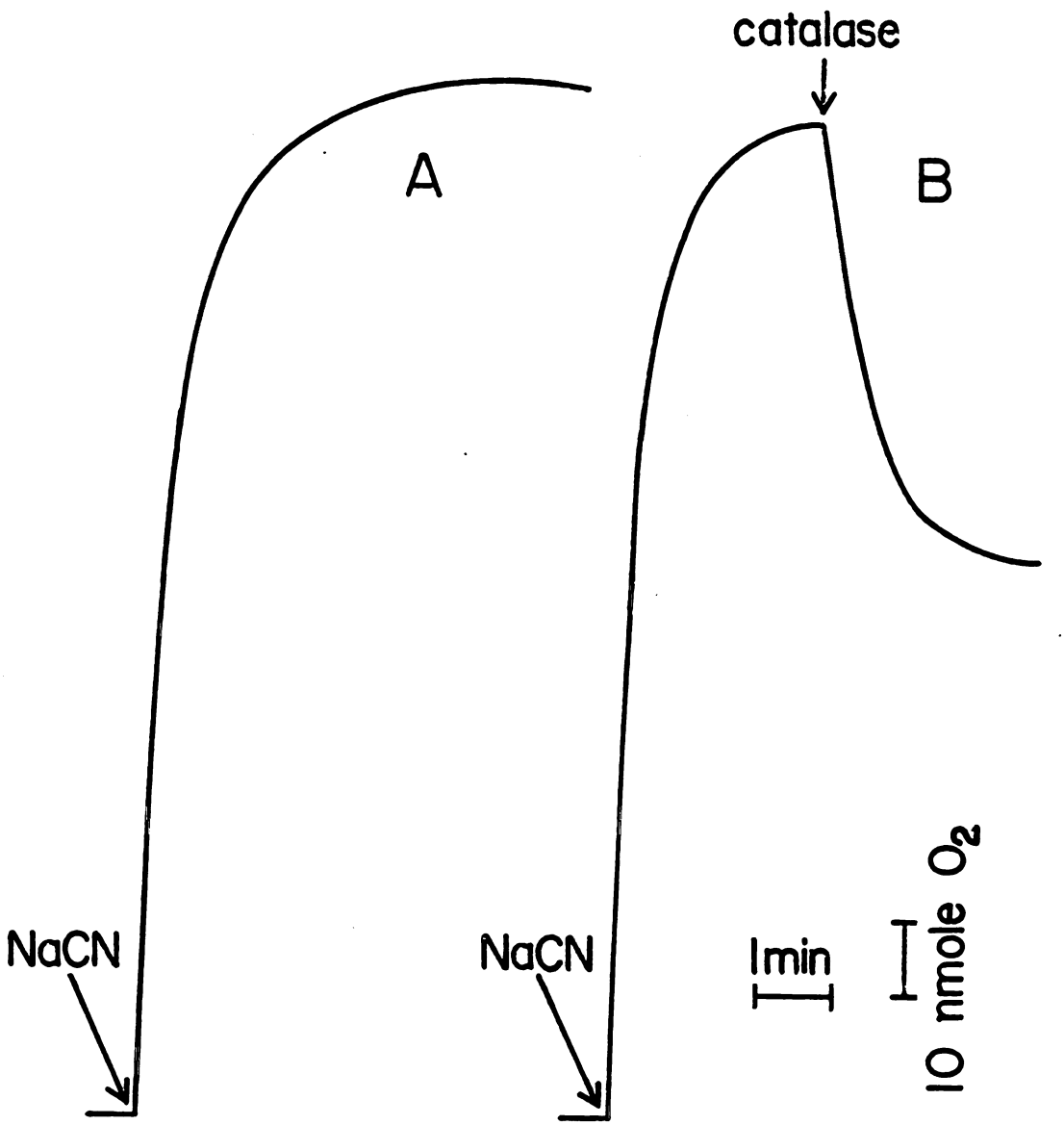


Figure 22.

Fig. 23. Initial Rate of Dihydroxyacetone-P Dependent O_2 Consumption as a Function of Cyanide Concentration. Reaction mixture contained 50 mM sodium phosphate (pH 8.5), 1.16 mM DHAP and indicated concentration of NaCN. Reactions were initiated with cyanide.

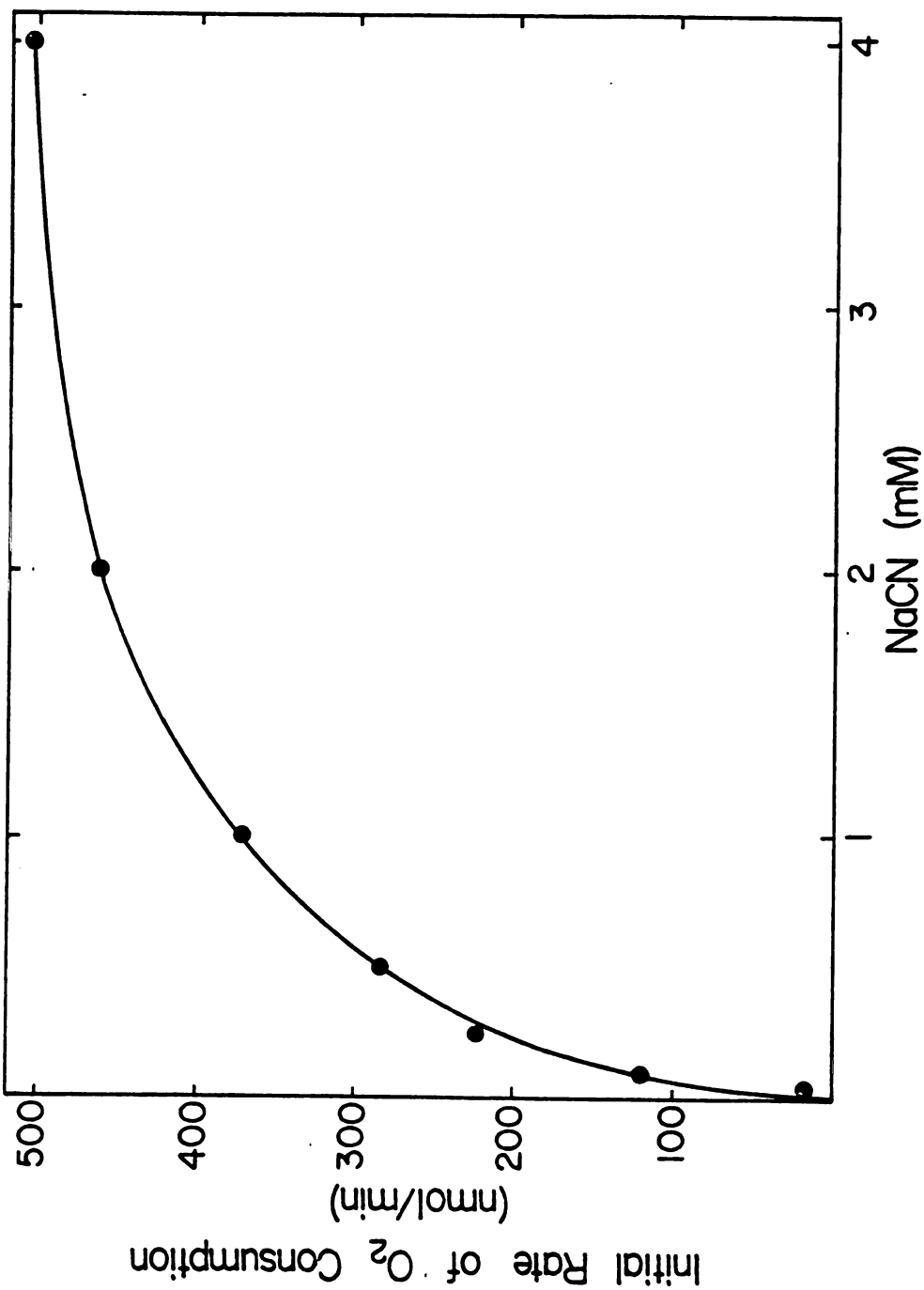

Initial Rate of O_2 Consumption
(nmol/min)
500
400
300
200
100
1
2
3
4
NaCN (mM)

Figure 23.

Fig. 24. Effect of pH on the Initial Rate of O_2 Consumption with Ribulose-P_2 and Cyanide. The reaction mixture was composed of 50 mM sodium phosphate, 50 mM sodium pyrophosphate, 0.6 mM ribulose-P_2 and 0.5 mM NaCN. Reactions were initiated with the addition of cyanide.

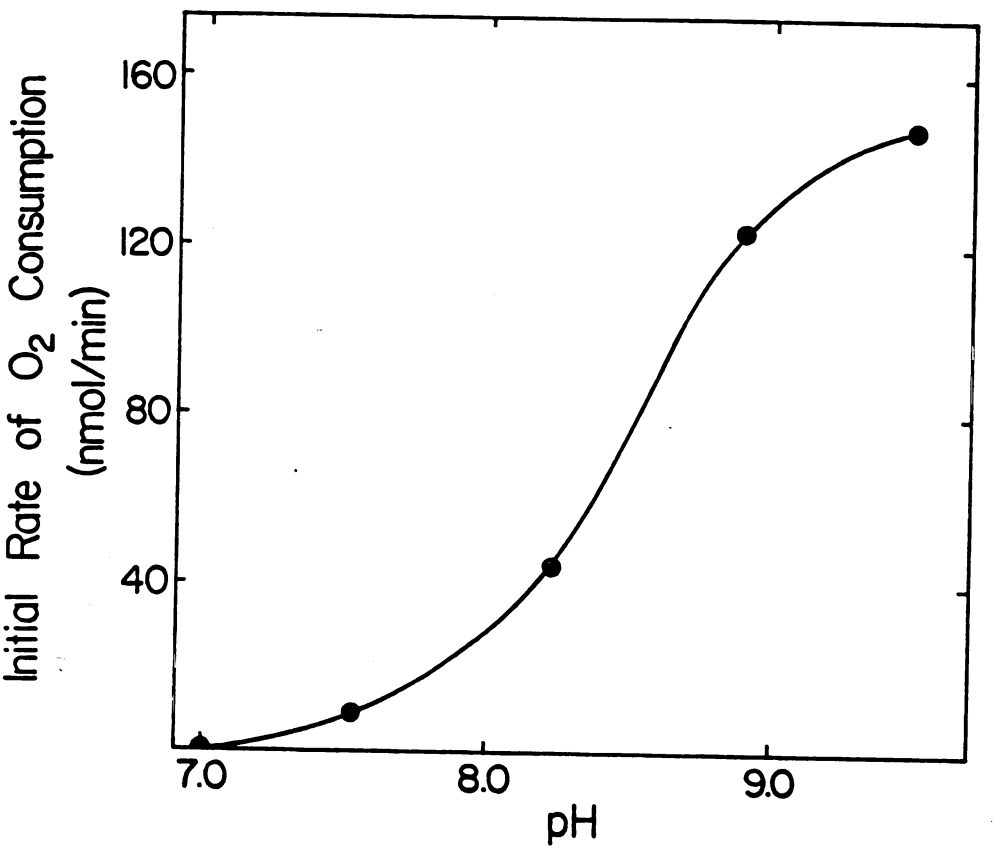
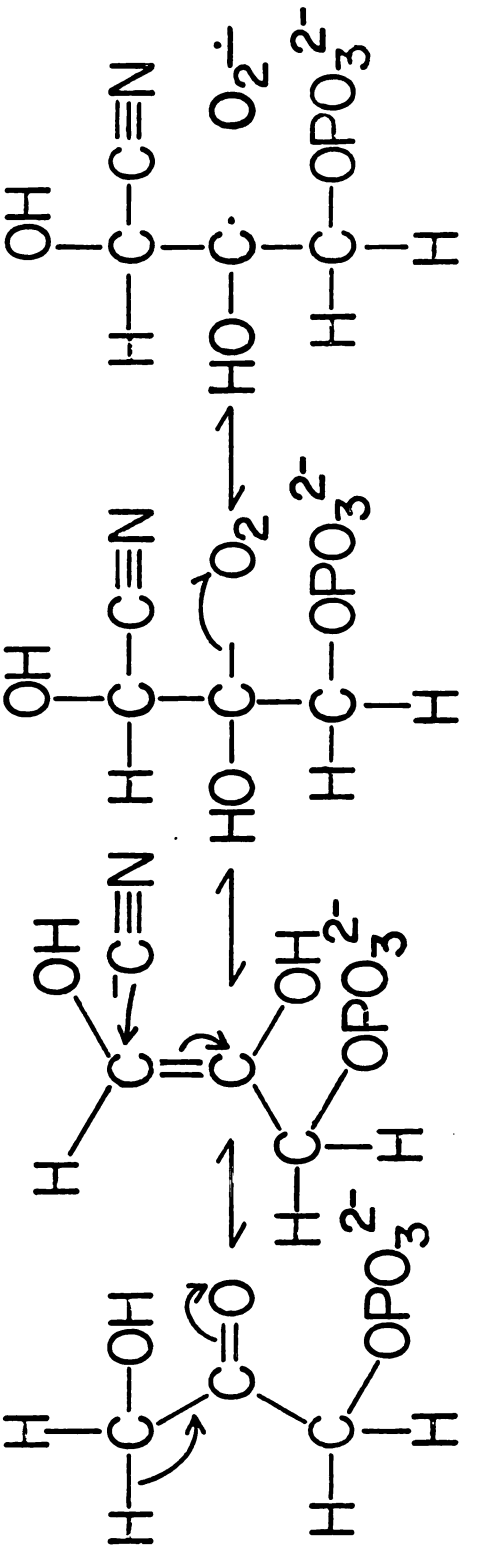

Initial Rate of O_2 Consumption (nmol/min)
160
120
80
40
7.0
8.0
9.0
pH

Figure 24.



Scheme 5.

ferricytochrome c or NBT^{2+}. Since the rate of cytochrome c or NBT^{2+} reduction was inhibited by MnSOD, the presence of O_2- was indicated.

The reaction of ribulose-P_2 and cyanide with O_2 resulted in the reduction of ferricytochrome c or NBT^{2+}. Also, the addition of cytochrome c or NBT^{2+}, which are reduced by O_2- to regenerate O_2, inhibited the rate of O_2 consumption. These observations (data not shown) confirmed the results of Robertson et al., and suggested that O_2- was involved in the mechanism of the reaction.

The role of O_2- in this reaction mechanism was corraborated by a study of the initial rate of O_2 consumption during the oxidation of ribulose-P_2 with cyanide in the presence of MnSOD. MnSOD was chosen for this study since it is not inhibited by cyanide, unlike the commercially available copper-zinc superoxide dismutase. The MnSOD preparation was void of catalase activity. Addition of increasing activity of MnSOD resulted in 49% inhibition of the initial rate of O_2 consumption with cyanide and ribulose-P_2 (Fig. 25) or dihydroxyacetone-P (data not shown). These results indicate that the initial product of O_2 consumption was O_2-, which could be enzymatically dismutated to H_2O_2 and O_2.

Since the spontaneous rate of dismutation of O_2- to H_2O_2 and O_2 is appreciable at pH 8.5 ($k_2=10^4$ $M^{-1}sec^{-1}$) (Fee and Valentine, 1977), the O_2- produced as a consequence of the oxidation of sugar-P in the presence of cyanide was unstable. One product of the dismutation, H_2O_2, was established in the reaction mixture by enzymatic analysis with catalase or peroxidase. Addition of catalase to the reaction, after about 90% of the O_2 consumption had ensued,

Fig. 25. Inhibition of the Initial Rate of O_2 Consumption by Manganese Superoxide Dismutase. The reaction mixture contained 50 mM Bicine-Na^+ (pH 8.5), 1.2 mM ribulose-P_2, 1 mM NaCN and the indicated activity of MnSOD. Inhibition by 0.75 or 1 unit of MnSOD was 49%.

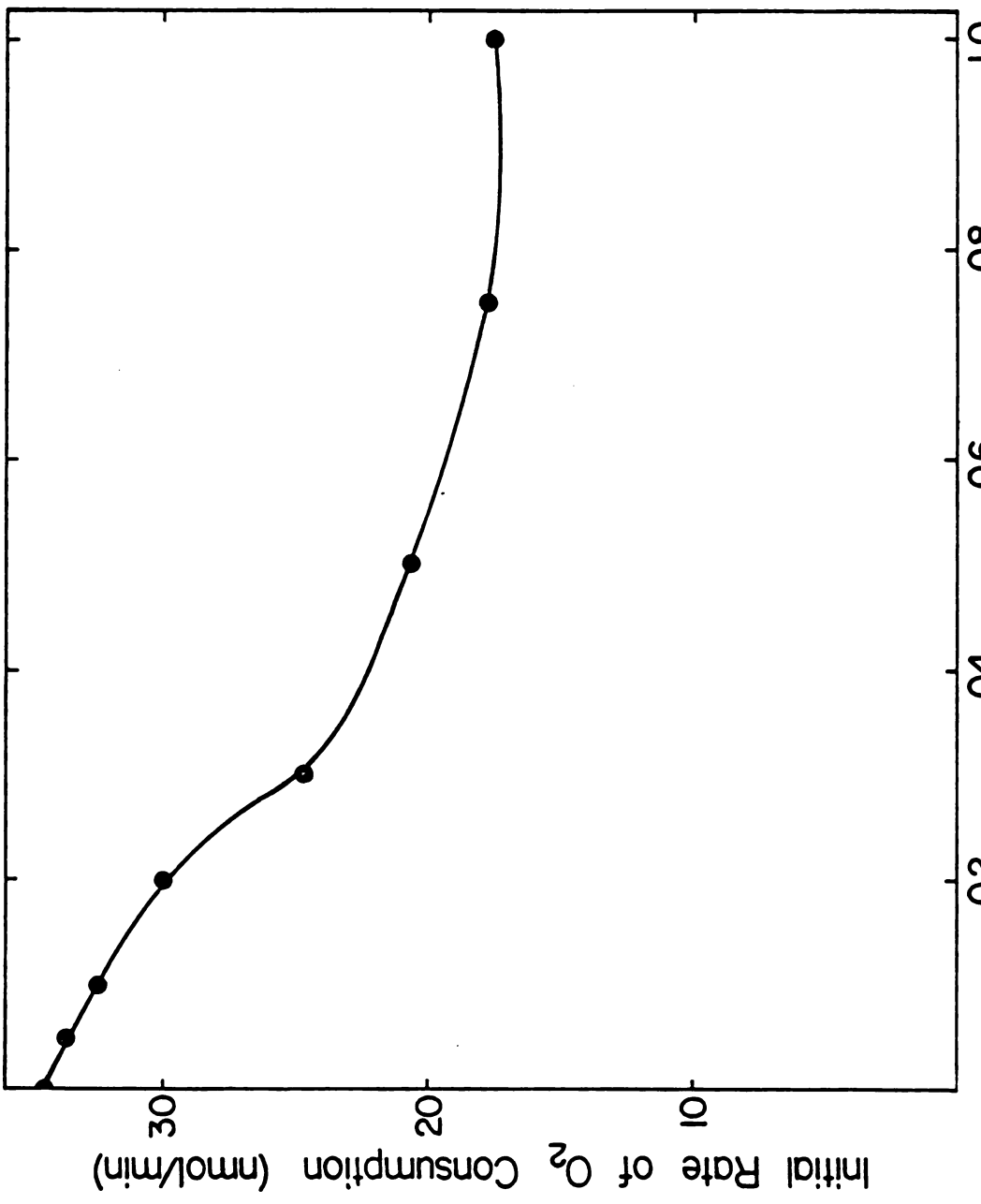

Initial Rate of O_2 Consumption (nmol/min)
10
20
30
0.2
0.4
0.6
0.8
1.0
Units of MnSOD Added

Figure 25.

Fig. 26. H_2O_2 Production by the Cyanide Catalyzed Oxidation of Ribulose-P_2. The reaction mixture (1 ml) contained 50 mM sodium phosphate (pH 8.5), 1.86 mM ribulose-P_2, 8 µg horseradish peroxidase, 0.3 mM guaiacol, 0.1 mM DETAPAC and 1 mM sodium cyanide. The reaction was initiated with the addition of cyanide, which is indicated by the arrow. Absorbance changes were recorded at 436 nm. The period of time between cyanide addition and the abrupt change in the absorbance was about 1.5 minutes.

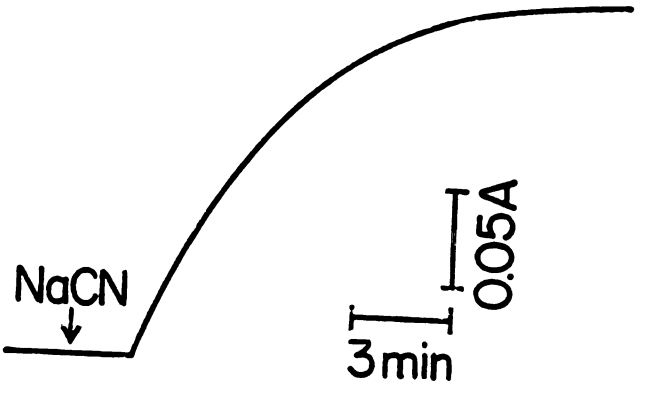


Figure 26.

resulted in the rapid release of about 40% of the O_2 consumed (Fig. 22b).

Further evidence for the formation of H_2O_2 was obtained through the use of an assay coupled with horseradish peroxidase. In this assay, guaiacol is oxidized by H_2O_2 in the presence of peroxidase to form tetraguaiacol, which may be measured spectrophotometrically. Hydrogen peroxide dependent guaiacol oxidation was observed with cyanide and ribulose-P_2 or dihydroxyacetone-P, but only after a lag of 1-2 minutes (Fig. 26). The abrupt change in the rate of product formation seemed unusual, since H_2O_2 should have been constantly formed from dismutation of O_2-. The possibility that O_2- was interefering with the measurement of tetraguaiacol formation was checked by addition of the xanthine and xanthine oxidase (for O_2- generation) to a solution containing tetraguaiacol. This resulted in the rapid loss of absorbance, indicating that tetraquaicol may be susceptible to reduction by O_2^-. Thus the lag in guaiacol oxidation by cyanide and ribulose-P_2 may be due to some interference by O_2-.

EPR Spin Trapping Studies. Direct evidence for the involvement of radicals formed in the reaction of ribulose-P_2 and cyanide with dioxygen was sought through the technique of EPR spin trapping. This technique involves the use of a reagent which may react with a labile radical species to form a relatively stable nitroxide radical (Eq. 4) (Evans, 1979). The spin trapping reagent used in this study, DMPO, is a useful compound for the identification of oxygen radicals, because the hyperfine splitting constants are quite sensitive to the trapped radical.

Fig. 27. Spin-trapping of ·OH Radical by DMPO. Reaction mixture contained 50 mM sodium phosphate (pH 8.5), 60 mM DMPO, 0.1 mM DETAPAC, 1.83 mM DHAP, and 1 mM NaCN. A 4 min scan with 4 x 10^4 gain was taken of this reaction mixture. The contents were removed from the cuvette and the reaction mixture was made 1 mM in $FeCl_3$. A second 4 min scan was taken with a gain of 1.6 x 10^4.

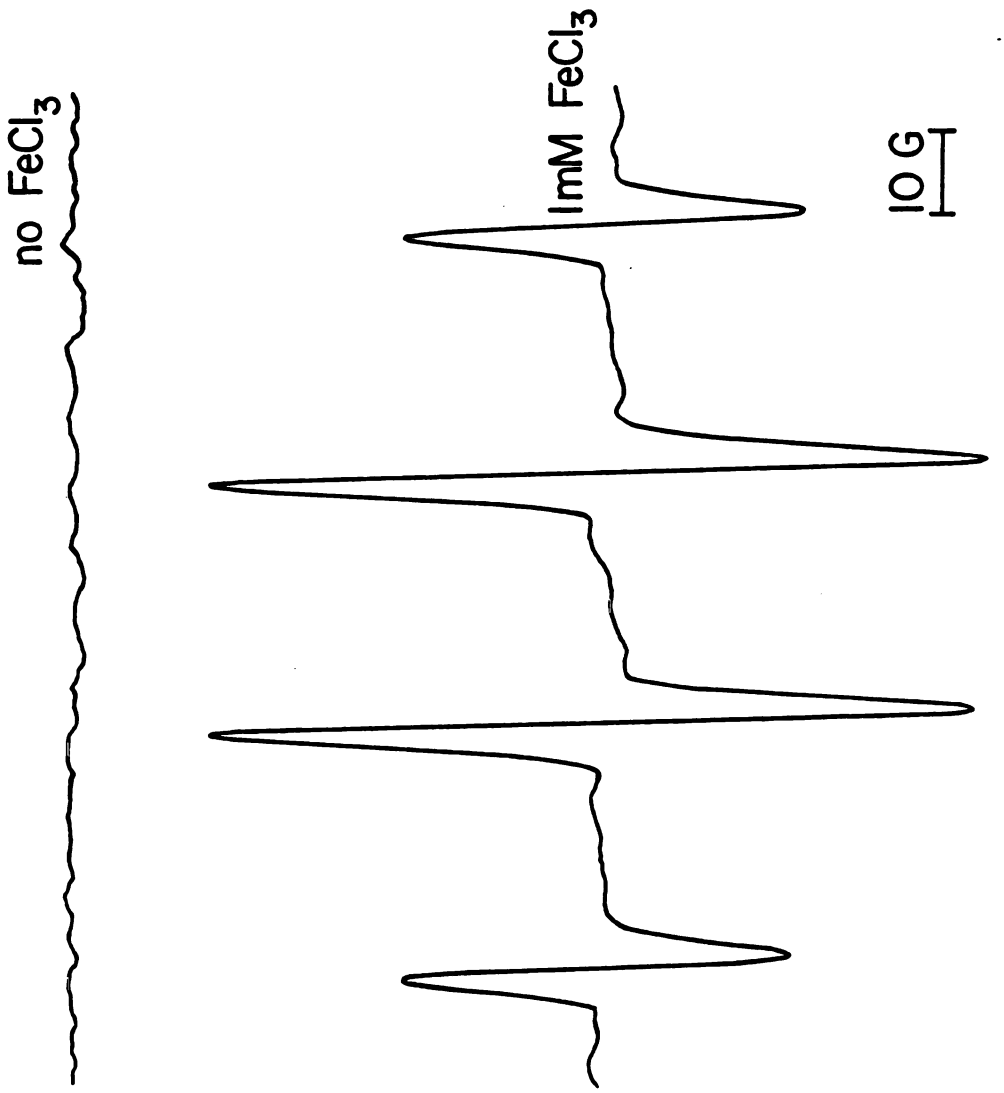
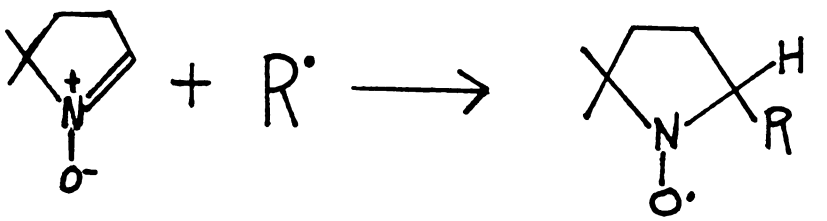


Figure 27.



(Eq. 4)

When DMPO was included in the ribulose-P_2 and cyanide reaction mixture, only a trace of a signal (A_N = 14.9 G, A_H^B = 14.8 G) could be observed (Fig. 27). However, if the reagents were not treated with a chelating resin or if 1 mM $FeCl_3$ was included, the ribulose-P_2 and cyanide reaction mixture yielded a strong signal (A_N = 14.9 G, A_H^B = 14.8 G). When the reaction mixture contained ribulose-P_2, the intensity of this signal was enhanced by about 50 fold by the addition of 1 mM $FeCl_3$. With dihydroxyacetone-P, the signal enhancement was even greater (Fig. 27). The hyperfine splitting constants indicated that the hydroxyl radical (·OH) adduct of DMPO had been formed. An identical EPR spectrum was obtained when hydroxyl radical was generated by the Fenton reaction (Eq. 6) (Fenton, 1894).

The presence of the hydroxyl radical adduct of DMPO may arise through the rearrangement of the superoxide adduct of DMPO or from the reaction of DMPO with hydroxyl radical (Finkelstein et al., 1979). In order to differentiate between these possibilities, the experiment was repeated in the presence of 0.1 M ethanol. Hydroxyl radicals will react with ethanol to produce α-hydroxyethyl radicals (Finkelstein et al., 1980). The inclusion of ethanol decreased the intensity of the hydroxyl radical spectrum, and a new signal was observed with the characteristic hyperfine splitting constants of the α-hydroxyethyl adduct of DMPO (A_N = 14.8 G, A_H^B = 22.8 G). Therefore the hydroxyl radical adduct of DMPO was derived from hydroxyl radical in

solution, and was not an artifact of rearrangement of some other DMPO adduct.

The origin of the hydroxyl radical can be explained by the Haber-Weiss reactions (Haber and Weiss, 1934).

$$Fe^{+3} + O_2\text{-} \longrightarrow Fe^{+2} + O_2 \qquad \text{(Eq. 5)}$$

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + {}^{-}OH + \cdot OH \qquad \text{(Eq. 6)}$$

A similar mechanism for the production of hydroxyl radical from superoxide has been proposed by other investigators (Weinstein and Bielski, 1979; Halliwell, 1978; Buettner et al., 1978b; Buettner and Oberley, 1978a). Thus, the trapping of hydroxyl radical in the presence of Fe^{+3} implicates that both O_2- and H_2O_2 had been formed in the oxidation of ribulose-P_2 and cyanide by O_2.

No O_2- adduct of DMPO was observed with the sugar-P and cyanide reaction mixture. Superoxide is not very reactive with DMPO and the superoxide adduct of DMPO is unstable under alkaline conditions (half-life = 35S at pH 8.0) (Finkelstein et al., 1980; Buettner and Oberley, 1978a). The ability to observe the O_2- adduct of DMPO in the sugar-P and cyanide reaction mixture was checked by including NADH and riboflavin, which generated O_2-. No O_2- adduct of DMPO could be observed at pH 8.5. The O_2- adduct of DMPO was observed at pH 7.6 and was dependent on NADH and riboflavin.

These results appeared convincing that ribulose-P_2 oxidation did occur in this system, however, one outstanding observation could not be explained. Why was the O_2 consumed only a fraction of the sugar-P included in the reaction? Robertson et al. (1981) claimed that the

oxidation of the carbohydrate did go to completion as measured by cytochrome c reduction.

To test the dependence of O_2 consumption on ribulose-P_2, ribulose-P_2 was treated with activated ribulose-P_2 carboxylase under catalytic conditions or a control solution which lacked enzyme. Carbon fixation was determined, and 100% of the ribulose-P_2 was converted to product in the presence of enzyme. The addition of cyanide to either solution still gave identical rates and stoichiometries of O_2 consumption. Neither glycerate-3-P and glycolate-2-P, products of the carboxylase and oxygenase reactions, exhibited cyanide dependent O_2 consumption. Ribulose-P_2 was treated with alkali to eliminate phosphate and to generate a diketo monophosphate product (Paech et al., 1978) and this preparation was no more reactive with cyanide in O_2 consumption then ribulose-P_2. Therefore, O_2 consumption dependent on the addition of cyanide to ribulose-P_2 solutions was not due to ribulose-P_2, but was presumably due to a contaminant present in the ribulose-P_2 preparation.

Discussion

The substrate present in ribulose-P_2 and other solutions of sugar-P which reacts with cyanide and O_2 is unknown. The results show that O_2 may be reduced by one electron to O_2^-. This was indicated by the inhibition of O_2 reduction by MnSOD, NBT^{2+} and cytochrome c. Hydrogen peroxide was also a product of the reaction, presumably via the dismutation of O_2^-, as indicated by the peroxidase coupled reaction and the release of O_2 after the addition

of catalase to the reaction mixture. The addition of ferric iron to the reaction generated hydroxyl radical, as predicted by the Haber-Weiss reactions if both O_2^- and H_2O_2 were present.

Although the nature of the substrate for this reaction is unknown, the addition of cyanide generates a reductant capable of reducing O_2 to O_2^-. The redox potential for the reduction of O_2 to O_2^- is approximately -0.3 V (Fee and Valentine, 1977). The contaminant was present in each of several ribulose-P_2 preparations. Two preparations of dihydroxyacetone-P from Sigma also showed reactivity with O_2 in the presence of cyanide.

Cyanide reacts with carbonyls to form nitriles. The cyano substituent is strongly electron-withdrawing and the pK of an α proton will be decreased. The increased acidity of the cyano adduct may generate carbanions, which in principle could react as proposed by Robertson et al. (1981) in Scheme 5. Although this scenario represents a potential mechanism, the explanation for the non-stoichiometric reaction with O_2 remains obscure.

CHAPTER 7

THE EFFECT OF SOME POTENTIALLY REGULATORY COMPOUNDS ON RIBULOSE-P_2 CARBOXYLASE/OXYGENASE.

Ribulose-P_2 carboxylase/oxygenase catalyzes the first committed reaction for each of two important metabolic pathways, photosynthesis and photorespiration. The regulation of the carboxylase and oxygenase activities and the ratio of these activities are important parameters which dictate photosynthetic performance. As summarized in the literature review, the only known factors effecting the ratio of carboxylase to oxygenase are the tensions of CO_2 and O_2, temperature, and the metal ion used in activation. Only the two former parameters are physiologically important. Nevertheless, any compound or condition which might regulate the activities or the ratio of ribulose-P_2 carboxylase/oxygenase would be of mechanistic interest to basic scientists and agronomic interest to applied scientists. This chapter summarizes the effect of several compounds on ribulose-P_2 carboxylase/oxygenase which have potential as regulators of the enzyme activity.

Materials and Methods

Ribulose-P_2 carboxylase/oxygenase was purified, stored, activated and assayed as described in Chapter 1. Ribulose-P_2 was synthesized and purified as described in Chapter 1.

Fructose 2,6-P_2 was a gift from Dr. Simon J. Pilkis, Department of Physiology at the Vanderbilt University, Nashville, Tennesee. Trans-2-hexenal was obtained from Aldrich. Ribose-5-P was obtained from Sigma.

Results and Discussion

The Effect of Fructose-2,6-P_2 on Ribulose-P_2 Carboxylase/Oxygenase. Fructose-2,6-P_2 is a potent activator of ATP-dependent phosphofructokinase from several sources (Hers and Van Schaftingen, 1982), a potent activator of the PP_i dependent phosphofructokinase from mung beans (Sabularse and Anderson, 1981), and a potent inhibitor of fructose bisphosphatase from several sources (Hers and Van Schaftingen, 1982). The possibility that this compound also exerts some regulatory effect on ribulose-P_2 carboxylase was tested.

Activated ribulose-P_2 carboxylase was weakly inhibited by fructose-2,6-P_2 under the standard assay conditions (Table 4). Inhibition was greater when ribulose-P_2 was decreased to twice K_m (0.05 mM). This response suggests that fructose-2,6-P_2 was a competitive inhibitor with respect to ribulose-P_2, but a more thorough kinetic analysis was not performed. Ribulose-P_2 oxygenase was apparently not effected by fructose-2,6-P_2, but the standard deviations were high in these measurements.

The effect of fructose-2,6-P_2 was also tested on activation of ribulose-P_2 carboxylase (Fig. 28). Effectors of the enzyme activation state are typically weak competitive inhibitors with respect to ribulose-P_2 and modify the activation state only under suboptimal activation conditions (eg. 1 mM HCO_3^-) (McCurry et al., 1981). Fructose-2,6-P_2 behaved in a manner typical of an effector with 39% stimulation of carboxylase activity observed at 0.3 mM fructose-2,6-P_2. This response is weaker than that observed with gluconate-6-P which resulted in 110% stimulation of carboxylase activity at 0.5 mM. Fructose-2,6-P_2 appears not to have any unusual effects on ribulose-P_2 carboxylase/oxygenase and is probably not involved in the regulation of this enzyme in vivo.

Table 4. The Effect of Fructose-2,6-P_2 on Ribulose-P_2 Carboxylase/Oxygenase.

	[Ribulose-P_2] μM	[Fructose-2,6-P_2] μM	Rate (μmol·min^{-1}· mg^{-1}protein)	% of control
A.Carboxylase				
	500	0	1.87 ± 0.08	100
	500	100	1.67 ± 0.05	89
	50	0	1.13 ± 0.06	100
	50	100	0.65 ± 0.05	45
B.Oxygenase				
	500	0	0.112 ± 0.008	100
	500	100	0.112 ± 0.012	100

Figure 28. The Effect of Fructose-2,6-P_2 on Ribulose-P_2 Carboxylase Activation.

Ribulose-P_2 carboxylase was incubated under standard activation conditions except that $NaHCO_3$ was 1.0 mM and fructose-2,6-P_2 was included. Aliquots of enzyme (40 µg) were assayed under standard conditions (10 mM $NaHCO_3$) for 15 s to minimize activation during the assay.

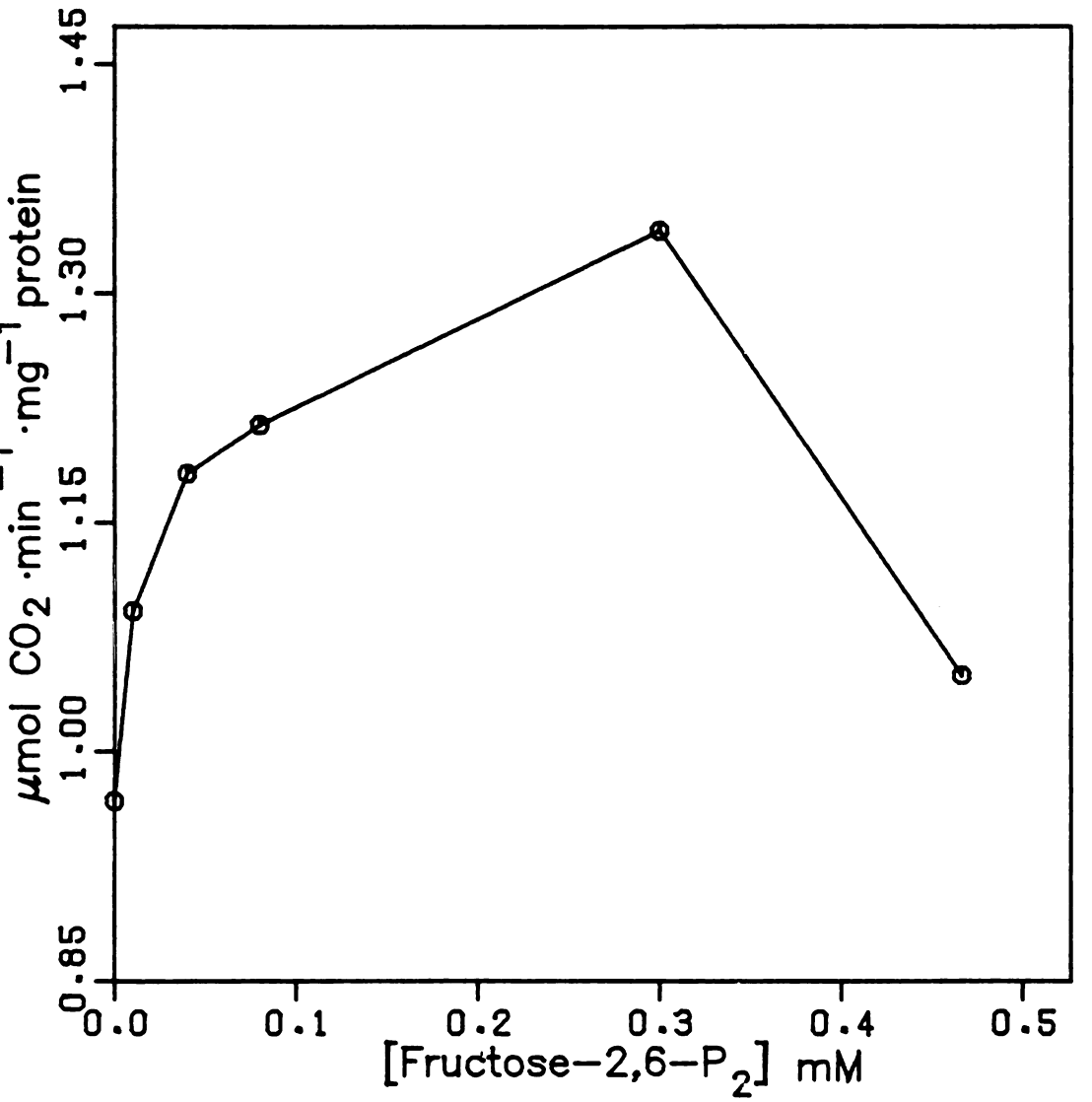

μmol CO2 ·min−1 ·mg−1 protein
0.85
1.00
1.15
1.30
1.45
0.0
0.1
0.2
0.3
0.4
0.5
[Fructose−2,6−P2] mM

Figure 28.

The Effect of Trans-2-hexenal on Ribulose-P_2 Carboxylase/Oxygenase. Trans-2-hexenal was tested on ribulose-P_2 carboxylase/oxygenase for two reasons. First trans-2-hexenal is present in millimolar concentrations in crude plant extracts and is responsible for the distinctive odor of freshly cut grass. This could represent a physiologically important compound. However further literature search on this topic indicated that it was not actually present in plants but rather was generated as a metabolite in crude leaf extracts due to lipoxygenase activity (Spoehr and Nye, 1943). Secondly, an abstract at the 1982 Federation of American Societies for Experimental Biology (Martin and Tabita, 1982) reported that activated enzyme incubated in the presence of aldehydes showed an increased carboxylase to oxygenase ratio. Glyoxylate has been demonstrated not to selectively inhibit carboxylase or oxygenase activity (Chapter 3). Trans-2-hexenal was added to activated enzyme at 1.25 mM and activity was followed over one hour (Fig. 29). The inhibition followed psuedo-first order kinetics with a rate constant for inhibition of 0.021 min^{-1} for each activity. Therefore trans-2-hexenal demonstrated no selectivity for inhibition of ribulose-P_2 carboxylase or oxygenase. No physiological role for trans-2-hexenal may be postulated, since this compound is produced in crude plant extracts and is not present in

Figure 29. The Effect of Trans-2-hexenal on Ribulose-P_2 Carboxylase and Oxygenase Activities.

Trans-2-hexenal (100 mM) was prepared in absolute ethanol and added to enzyme at 1.25 mM. The final concentration of ethanol was 0.8% (v/v). Aliquots were taken and assayed for carboxylase and oxygenase activities by the standard assays. Carboxylase assays were performed at each time point in duplicate. Oxygenase assays are single determinations.

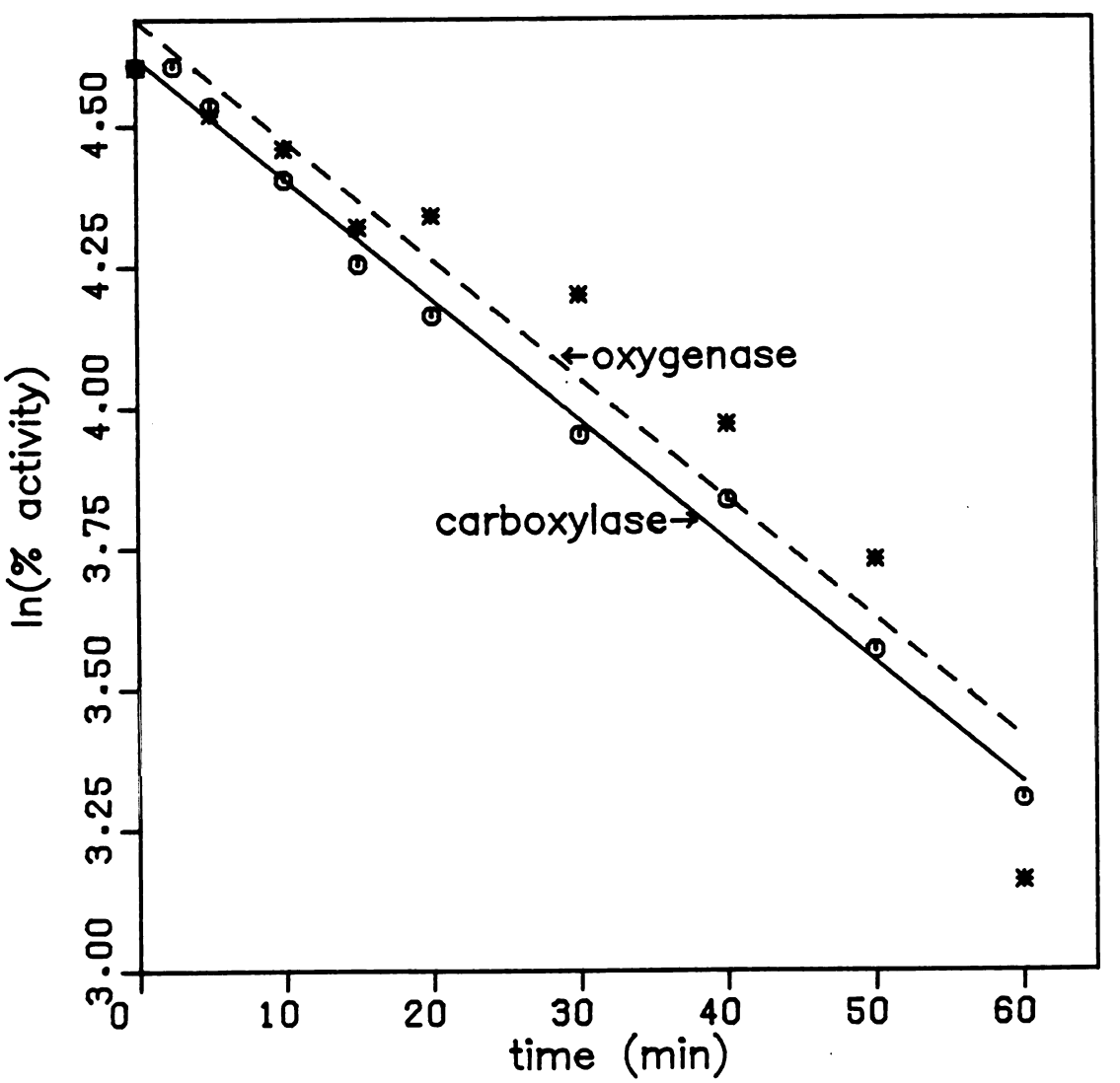

ln(% activity)
4.50
4.25
4.00
3.75
3.50
3.25
3.00
0
10
20
30
40
50
60
time (min)
←oxygenase
carboxylase→

Figure 29.

plants per se.

The Effect of Ribose-5-P on Ribulose-P_2 Carboxylase/Oxygenase. Ribose-5-P is a negative effector of ribulose-P_2 carboxylase/oxygenase (McCurry et al., 1981). Apparently it binds more tightly to the unactivated form of the enzyme and draws the equilibrium away from activation. Glycolate-2-P phosphatase is also inhibited by ribose-5-P in a manner indicative of partial competitive inhibition with respect to glycolate-2-P (H. David Husic, personal communication). A plot of apparent Km (glycolate-2-P) versus ribose-5-P concentration showed a nonlinear trend at high inhibitor concentration.

The inhibition of ribulose-P_2 carboxylase/oxygenase by ribose-5-P was examined (Fig. 30). The inhibition by ribose-5-P was essentially competitive with a K_I of 5.4 mM. Some deviation from competitive inhibition is noted at the highest concentration of inhibitor (10 mM); V_{max} was diminished by 18% under these conditions. This may be the result of ribose-5-P acting as a negative effector and decreasing the activation state of the enzyme and consequently V_{max}. The plot of apparent K_m (ribulose-P_2) versus inhibitor is relatively linear over the range of inhibitor concentration tested (Fig. 30).

The response of ribulose-P_2 carboxylase to ribose-5-P may be explained as a combination of competitive inhibition and deactivation due to the negative effector phenomenon.

Figure 30. The Inhibition of Ribulose-P_2 Carboxylase by Ribose-5-P.

Enzyme (4 μg) was activated and assayed under standard conditions with variable ribulose-P_2 concentration (58-690 μM) and variable ribose-5-P concentration (0 to 10 mM). Assays were performed in triplicate and kinetic parameters were analyzed by the method of Wilkinson (1969) with the aid of a computer program. These data indicate a K_I of 5.4 mM for ribose-5-P.

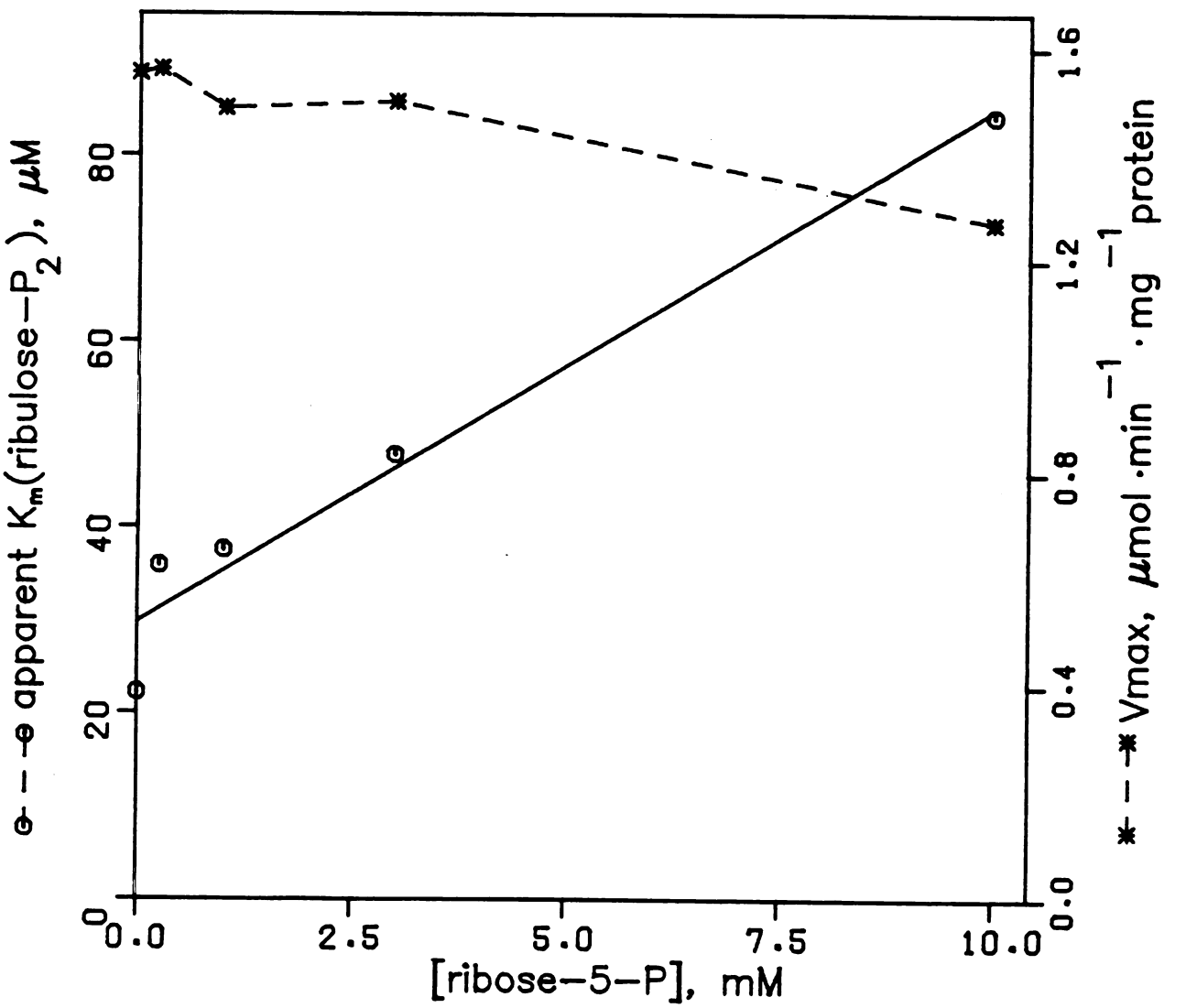

⊖ — ⊖ apparent K_m(ribulose–P_2), μM
✱ — ✱ Vmax, μmol·min^{-1}·mg^{-1} protein
[ribose–5–P], mM
0
20
40
60
80
0.0
0.4
0.8
1.2
1.6
0.0
2.5
5.0
7.5
10.0

Figure 30.

Ribose-5-P is a poor inhibitor of ribulose-P_2 carboxylase and probably plays no physiological role for regulating this enzyme.

List of Publications

Cook, C.M., R.M. Mulligan and N.E. Tolbert. The Stimulation and Inhibition of Spinach Ribulose Bisphosphate Carboxylase/Oxygenase by Glyoxylate. Manuscript in Preparation.

Mulligan, R.M. and N.E. Tolbert. The lability of an Intermediate from the Ribulose Bisphosphate Carboxylase Reaction. Manuscript accepted by Archives of Biochemistry and Biophysics.

Mulligan, R.M., B. Wilson and N.E. Tolbert (1983). The Effects of Glyoxylate on Photosynthesis by Intact Chloroplasts. Plant Physiology (In Press)

Pierce, J.W., S.D. McCurry, R.M. Mulligan and N.E. Tolbert (1982). Activation and Assay of Ribulose Bisphosphate Carboxylase/Oxygenase. Methods in Enzymology 89:47-55.

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Mitchell, M.J., R.M. Mulligan, R. Hartenstein, and E.F. Neuhauser (1977). Conversion of Sludges into "Topsoils" by Earthworms. Compost Science 18:28-32.

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Mulligan, R.M. and N.E. Tolbert (1981). Enzymatic Mechanism of Ribulose Bisphosphate Carboxylase/Oxygenase. Plant Physiology 67s:189.

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Mulligan, R.M. and N.E. Tolbert (1979). Localization and Properties of 3-Phosphoglycerate Phosphatase in Spinach Chloroplasts. Plant Physiology 63s:64.

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