# INVESTIGATIONS ON THE MECHANISM OF

# RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE

By

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ABSTRACT

INVESTIGATIONS ON THE MECHANISM OF
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Several investigations concerning the active site and properites of the purified ribulose-1,5-bisphosphate (ribulose-$P_2$) carboxylase/oxygenase (E.C. 4.1.1.39), a chloroplast protein, are discussed in this thesis. This enzyme catalyzes the addition of $CO_2$ or $O_2$ to ribulose-$P_2$ to yield either two molecules of phosphoglycerate or one molecule of phosphoglycerate and one of phosphoglycolate.

When a 50% saturated ammonium sulfate slurry of this enzyme was stored by freezing into tiny beads in liquid nitrogen, the activity could be restored to 90% of the freshly prepared enzyme. Enzyme frozen in this manner was stored in a -80° freezer for up to six months without appreciable loss in activity. Similar results have been obtained for enzyme frozen in the absence of ammonium sulfate.

When the substrate for this enzyme, ribulose-$P_2$, was treated with base, pH 9, or heated, it was found to degrade to inhibitory dicarbonyl compounds, and to epimerize to xylulose-$P_2$, a potent inhibitor of this enzyme. This instability of the substrate may be responsible for the substrate inhibition which has been claimed for this enzyme.

Both xylulose-$P_2$ and xylitol-$P_2$ are potent inhibitors of ribulose-$P_2$ carboxylase/oxygenase. The enzyme was 50% inhibited at a

xylulose-$P_2$ concentration of .56 μM at an active site concentration of .112 μM, but 100% inhibition could not be obtained with this compound. The inhibition due to xylitol-$P_2$ and xylulose-$P_2$ is competitive with respect to ribulose-$P_2$ when these compounds are added to the enzyme with the substrate. If the enzyme is preincubated with either of these compounds, the inhibition appears noncompetitive because of extremely tight binding.

Incubation of this enzyme with both $CO_2$ and $Mg^{++}$ prior to initiation of the assay is required for the maximum rate of either activity. Attempts to stabilize activator $CO_2$ on the enzyme with diazomethane were not successful. While other compounds, such as 2,3-diphosphoglycerate, could not replace the requirement for $CO_2$, there was some stimulation observed at suboptimal $CO_2$ concentrations. O-Methylisourea did not activate the enzyme.

Evidence for a free radical associated with the enzyme has been found through examination of the enzyme during catalysis with EPR spectroscopy at 13° K. The radical was present in all samples of the enzyme even in the absence of substrate. The origin of the radical has not been determined. All samples examined showed the presence of 2 mol of iron/mol of enzyme. Removal of 60% of the iron by dithiothreitol treatment did not alter either activity. A copper signal was observed in only one sample of enzyme and it had been prepared from spinach obtained from Florida. Rapid reaction kinetics performed in conjunction with this study demonstrated a 1 s lag in the carboxylase activity as measured by $^{14}CO_2$ fixation.

Modification by tyrosyl residues with tetranitromethane resulted in

complete inhibition of both activities. This inhibition could be partially prevented through preincubation of the enzyme with ribulose-$P_2$ before addition of the tetranitromethane. Efforts to demonstrate a histidyl residue in the active site through modification with dibromoacetophenone were unsuccessful in that no inhibition was observed.

Claims that the carboxylase and oxygenase activities could be separated through chromatography on Sepharose 6B were not supported. Similarly, no support was found for the claims that differential regulation of the two activities could be achieved through modification with iodoacetamide in the presence of ribulose-$P_2$ or through treatment with hydroxylamine. No support was found for the notion that $O_2$ is required for the activation of both activities. The addition of superoxide dismutase to enzyme assays was found to have no effect on either the carboxylase or the oxygenase activities.

ACKNOWLEDGEMENTS

I must express my gratitude to Dr. N.E. Tolbert who, in addition to supporting me financially, has given a great deal of support, advice, and encouragement.

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I must thank my friend Bridget for her patience and assistance, especially during the preparation of the thesis.

Finally, I must thank my parents whose encouragement and support have enabled me to endure the past few years.

The flowers fall, for all our yearning;

Grasses grow, regardless of our dislike

Sengtsan

from the Hsinhsinming
6th Century A.D.

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| | |
|---|---|
| Arabinitol-$P_2$ | arabinitol-1,5-bisphosphate |
| Bicine | N,N-bis(2-hydroxyethyl) glycine |
| Carboxyarabinitol-$P_2$ | 2-carboxyarabinitol-1,5-bisphosphate |
| Carboxyribitol-$P_2$ | 2-carboxyribitol-1,5-bisphosphate |
| P-CMB | P-choromercuribenzoate |
| DEAE | diethylaminoethyl |
| O-dianisidine | 3,5-dimethyl benzidine |
| DTT | dithiothreitol |
| EDTA | ethylenediamine tetracetate |
| Fructose-$P_2$ | fructose-1,6-bisphosphate |
| P-gluconate | 6-phosphogluconate |
| 2,3-glycerate | 2,3-diphosphoglycerate |
| P-glycerate | 3-phosphoglycerate |
| P-glycolate | 2-phosphoglycolate |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| Pydridoxal-P | pyridoxal-5'-phosphate |
| Ribulose-$P_2$ | ribulose-1,5-bisphosphate |
| SDS | sodium dodecyl sulfate |
| SOD | superoxide dismutase |
| Sugar-P | sugar phosphate |
| Xylitol-$P_2$ | xylitol-1,5-bisphosphate |
| Xylulose-$P_2$ | xylulose-1,5-bisphosphate |

# INTRODUCTION

Ribulose-1,5-bisphosphate (ribulose-$P_2$) carboxylase/oxygenase (E.C. 4.1.1.39) catalyzes the addition of $CO_2$ at $C_2$ of ribulose-$P_2$ to form two molecules of 3-phosphoglycerate (P-glycerate), as well as the addition of $O_2$ at $C_2$ of ribulose-$P_2$ to form one molecule of phosphoglycolate (P-glycolate) and one of P-glycerate. This enzyme accounts for up to 50% of the total leaf protein in some plants. It has been more than thirty years since Wildman and Bonner (1) described this protein and termed it "Fraction-I" protein. At the time of its discovery, its function was unknown, and ribulose-$P_2$ was unknown. It was not until the path of carbon in photosynthesis was elucidated through work in the laboratories of Calvin, Horecker, Ochoa, Racker, and others, that the carboxylase function of this enzyme was discovered (2,3,4,5).

Otto Warburg observed in 1920 (6) that oxygen inhibited $CO_2$ fixation in Chlorella, and further work on Chlorella led Tamiya and Huzisige (7) to conclude that ..."$O_2$ competes, in some way or other, with $CO_2$ (or its derivative $RHCO_2$) for the enzyme." It was more than twenty years before the in vitro oxygenase activity of ribulose-$P_2$ carboxylase was demonstrated in the laboratories of Ogren and Tolbert (8,9,10,11). This oxygenase activity was immediately linked with the "Warburg Effect" and with photorespiration, the light-dependent release of $CO_2$ and concomitant uptake of $O_2$.

Because of the fact that some plants have substantially avoided the loss of $CO_2$ from photorespiration, the so called $C_4$ plants, and because this process apparently wastes energy gained in the photosynthetic light reactions, many authors have suggested that photorespiration serves no useful purpose and should be eliminated. These suggestions have stimulated a great deal of work on photorespiration in general, and on the regulation of ribulose-$P_2$ carboxylase/oxygenase in particular. The approach of enzymologists has been to attempt to elucidate the reaction mechanism of each activity, and then determine whether they can be regulated.

The research reported in this thesis has been mostly directed towards further understanding of the reaction mechanisms of this enzyme. In addition, efforts to repeat claims of differential regulation of the enzyme are reported in Chapter VIII. Chapter III contains research concerning the instability of the substrate ribulose-$P_2$.

## LITERATURE RIEVIEW

### Structure and Properties of Ribulose-$P_2$ Carboxylase/Oxygenase

The general properities of this enzyme are summarized in Table 1. In higher plants and most algae, ribulose-$P_2$ carboxylase is a very large enzyme with a molecular weight of 560,000 (12,13,14,15). In such plants, it is composed of 16 subunits ($A_8B_8$), 8 large (A) subunits of 56,000 molecular weight, and 8 small (B) subunits of 12,000 molecular weight (16,17,18). From calculations based on the size and amount of this enzyme, the concentration in the chloroplast is estimated at 0.4-0.5 mM or 3-4 mM in ribulose-$P_2$ binding sites (19). This *in vito* concentration of up to 250 mg protein/ml has not been obtained *in vito*.

Ribulose-$P_2$ carboxylase is present in all known photosynthetic organisms. In most cases it is an $A_8B_8$ enzyme, but in *Rhodospirillum rubrum* the structure is $A_2$ (14,20), in *Chlorobium thiosulfatophilum* it is $A_6$ (21), and in *Thiobacillus intermedius* (22) and *Anabaena cylindrica* (23) the structure is $A_8$. It has been suggested that small subunits could be lost during purification because of their size (24). Large subunits from a variety of sources have a great deal in common. Antibodies to large subunits will cross-react to large subunits from quite different plants (25, 26) and their amino acid compositions are quite similar (12,24,27). Differences in the small subunit, however, are quite pronounced (25). Different members of the genus *Nicotiana* have been studied extensively in this regard (27,28,29,30).

Table 1

Properties of Ribulose-$P_2$ Carboxylase/Oxygenase from Spinach

| | | |
|---|---|---|
| Molecular weight: | | 560,000 Daltons |
| Subunit Composition: | 8 large (A) | 56,000 Daltons |
| | 8 small (B) | 12,000-14,000 Daltons |

Substrates: D-ribulose-1,5-bisphosphate (ribulose-$P_2$), $CO_2$, $O_2$

Essential cofactor: $Mg^{2+}$

Reaction catalyzed and assay procedure:

(a) carboxylase: Ribulose-$P_2$ + $CO_2$ $\xrightarrow{Mg^{2+}}$ 2,3-phosphoglycerate

(1) determination of $^{14}C$-3-phosphoglycerate with $NaH^{14}CO_3$ as substrate

(2) coupled enzyme assay

(b) oxygenase: Ribulose-$P_2$ + $O_2$ $\xrightarrow{Mg^{2+}}$ 3-phosphoglycerate + phosphoglycolate

(1) oxygen uptake by oxygen electrode or respirometer

(2) phosphoglycolate determination

Activation: $CO_2$ and $Mg^{2+}$, required for both enzyme activites and selected sugar phosphates under suboptimal $CO_2$ conditions

pH-Optimum: 8.2 - 8.6

$K_m$-Values

(a) carboxylase: $K_m(CO_2)$ 10 - 15 µM; $K_m$(Ribulose-$P_2$) 20 - 25 µM

(b) oxygenase: $K_m(O_2)$ ca. 0.4 mM; $K_m$(Ribulose-$P_2$) 25 - 35 µM

Inhibitors: xylulose-$P_2$, xylitol-$P_2$, ribose-5-phosphate, 2-carboxyarabinitol-$P_2$, 2-carboxyribitol-$P_2$, and 6-phosphogluconate

Fuctional groups: sulfhydryl, lysyl, and arginyl residues

It has been claimed by Akazawa's group (3,32,33,34) that ribulose-$P_2$ carboxylase from spinach could be separated into subunits, and that the isolated large subunit maintained its catalytic properties, albeit at a low rate. This separation was accomplished with p-chloromercuribenzoate (p-CMB). Several other groups (Tolbert, Lorimer, Chollet, personal communication) have tried to repeat these experiments without success. Recently Chollet (35) has reported that p-CMB separation of subunits could be accomplished, but upon addition of $Mg^{++}$ (essential for activation and catalysis) the purified large subunits precipitated. Since the enzyme from R. rubrum is an $A_2$ enzyme but still retains all of the control mechanisms of the $A_8B_8$ enzyme, the function of the small subunit remains unclear (36). From the evidence accumulated in Wildman's group (37) the primary structure of the small subunit determines whether the enzyme from tobacco can be easily crystallized.

In the systems which have been considered, studies concerning the three dimensional structure support the $A_8A_8$ model. The electron microscopic evidence of McFadden and coworkers (38) suggest a cubic or pseudorhombic dodecahedron with a 4:2:2 symmetry. This has been supported by the X-ray crystallographic evidence of Baker and coworkers (39). This topic has been recently reviewed by Eisenberg (40). Serological evidence suggest that the small subunits are prevented from reacting with antibodies by the large subunits in the native enzyme (41).

Chemical crosslinking agents such as tetranitromethane, dimethyl suberimidate, dimethyl adipimidate, and methyl-4-mercapto butryimidate have been used with this enzyme (42,43). Mild crosslinking treatment results in a substantial number of AB pairs, and pairs and triples of

B (42). Pretreatment of the enzyme with $HCO_3$ produced no effect on the crosslinking pattern, but pretreatment with ribulose-$P_2$ resulted in a substantial decrease in the amount of crosslinking. Preincubation with both $Mg^{++}$ and $HCO_3^-$ resulted in a substantial increase in the amount of crosslinking (42). Grebanier and coworkers (42) also found a shift in the CD spectrum upon preincubation with $Mg^{++}$ and $HCO_3^-$, which are the conditions for activation of the enzyme (discussed later).

Recently it has been suggested that the carboxylase and oxygenase activities of this enzyme could be separated through chromatography on Sepharose 6B (44). Vigorous efforts by us to confirm this failed (45). Several other groups also could not confirm this report (Randall, Bahr, Chollet, personal communication). While the idea of these two activities being separable is not new, Branden (44) published the first data in support of this notion. Many lines of evidence, from many laboratories provide a proponderance of data that these two activities are inseparable, and that one active site catalyzes both activities (46).

## Biosynthesis of Ribulose-$P_2$ Carboxylase/Oxygenase

Considering the complexity of the structure of this enzyme, the complexity of its biosynthesis is to be expected. The large subunit is coded for by chloroplast DNA and is synthesized on chloroplast ribosomes (28,47,48,49,50,51). The small subunit is coded for by nuclear DNA and is synthesized on cytoplasmic ribosomes (48,50,52). The small subunit is made as a longer precursor protein and is cleaved upon insertion into the chloroplast (53,54). Formation of the large subunit is not required for synthesis of the small subunit (55). The portion of chloroplast DNA which codes for the large subunit has been identified and cloned (49,56).

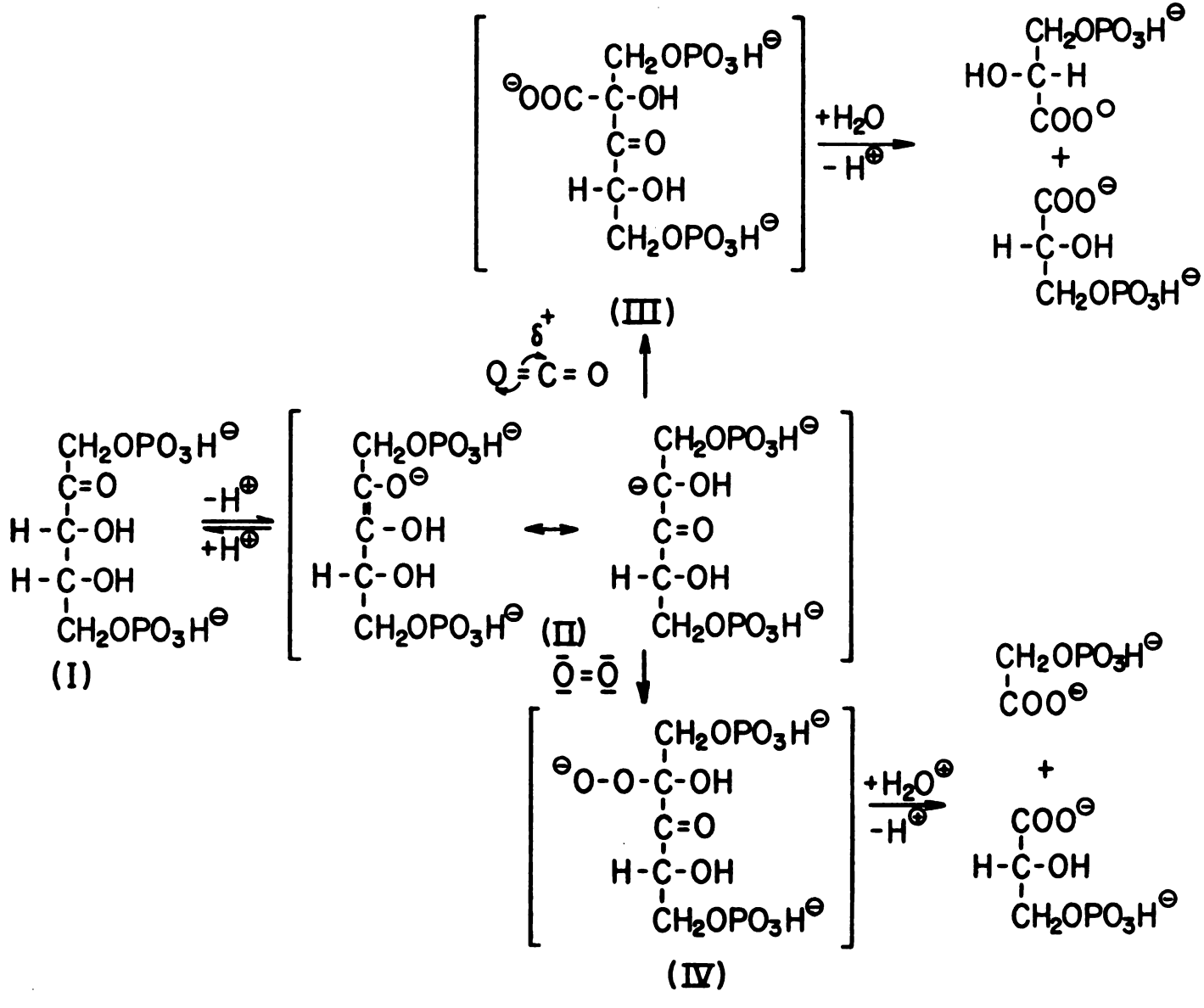
Since the large subunit has thus far proven too difficult to sequence, it is hoped that the sequence of the DNA will soon be determined.

Mechanism of Action

The most widely accepted of the proposed carboxylase mechanisms is shown in Figure 1. This is essentially the mechanism suggested by Calvin (57). A divalent metal, preferably $Mg^{++}$ is required for assay (3,4). The standard free energies for the forward reaction have been estimated at -8.4 to -12.4 KCal/Mol (58,59) and there are no reports of successful efforts to reverse the reaction. The pH optimum for both activities (of the fully activated enzyme) is approximately 8 (60,61). Considering the proton release (Figure 1), the pH undoubtedly aids in the irreversible nature of the reaction. $CO_2$ and not $HCO_3^-$ is the substrate for the reaction (62), it is also the species required for activation (60). While most carboxylating enzymes use $HCO_3^-$, others are known which use $CO_2$ (63).

If the mechanism shown in Figure 1 is correct, ribulose-$P_2$ probably binds to the enzyme before $CO_2$. The postulated oxygenase mechanism is also shown in Figure 1 (11). A comparison of these two mechanisms (Figure 1) has led to the suggestion that the oxygenase reaction is simply an "inevitable consequence" of the carboxylase mechanism (64). Experiments designed to determine the order of substrate binding are very difficult to perform for several reasons: a) $CO_2$ has a dual role as a substrate and as an activator (60,65), so it must always be added first for maximal activity. b) In the carboxylase reaction, both of the products are identical (P-glycerate), yet only one arises from the region of the newly fixed $CO_2$. c) Since $CO_2$ is required for activation of the

Figure 1. Postulated reaction mechanisms of ribulose-$P_2$ carboxylase and oxygenase (11,57).



oxygenase, it is not possible to run the assay with no $CO_2$. d) The binding constants of P-glycerate and P-glycolate are high.

When an enzyme mechanism involves a step such as proton abstraction (first step Figure 1), it is often possible to isolate this part of the reaction and demonstrate proton exchange. This has been attempted for ribulose-$P_2$ carboxylase by Rose's group (66). Ribulose-$P_2$, labeled at C-3 with $^3H$, was incubated with enzyme in the absence of $CO_2$. The results showed no $^3H$ exchange with the medium. Three possible explanations were offered: a) the mechanism does not proceed as suggested; b) the abstracted proton is never free in solution, i.e. it stays attached to the protein; c) $CO_2$ must bind first. This research was performed before the $CO_2$ requirement for activation was established. It probably will not be possible to decide among these three possibilities until some method to activate the enzyme in the absence of $CO_2$ is discovered.

An analogue of the 3-keto branched chain intermediate (III in Figure 1) would be expected to inhibit the enzyme if the postulated reaction mechanism is correct. Lane's group discovered that the carboxylation reaction was inhibited by HCN (67). This was due to reaction of HCN with ribulose-$P_2$. Lane's group then went on to make 2-carboxyribitol bisphosphate from the HCN and ribulose-$P_2$ adduct (68). This compound was a potent inhibitor of both ribulose-$P_2$ carboxylase (68,69) and oxygenase (70). Their product was actually an isomeric mixture of 2-carboxyribitol-bisphosphate and 2-carboxyarabinitol-bisphosphate, but it has consistently been referred to as 2-carboxyribitol-bisphosphate. The $K_D$ for the mixture is $10^{-8}$ M (69), and divalent metal is required for

maximal inhibition (69). Ribulose-$P_2$ (69,71) and carboxyribitol-$P_2$ (71) have similar effects on UV absorption spectrum of the carboxylase. Mildvan's group demonstrated that the addition of carboxyribitol-$P_2$ to enzyme which had been incubated with $H^{13}CO_3^-$ and $Mn^{++}$ abolished the effect of divalent metal on the relaxation rate of water protons, or of $H^{13}CO_3^-$ (72). This would suggest that either the $H^{13}CO_3^-$ was forced from the region of the $Mn^{++}$ or its rapid dissociation was blocked. It has been demonstrated that carboxylase treated with $H^{14}CO_3^-$ and carboxyribitol-$P_2$ binds $^{14}CO_2$ stoichiometrically, and the enzyme-$^{14}CO_2$-carboxyribitol-$P_2$ complex is stable to passage over Sephadex G-75 (73). Since this experiment was done under conditions required for activation of the enzyme, and because of the similarity of carboxyribitol-$P_2$ to the postulated intermediate III (Figure 1), this work has been used as evidence that the activator and catalytic $CO_2$ sites are distinct.

Recently, the mixture has been resolved into its two isomers, carboxyribitol-$P_2$ and carboxyarabinitol-$P_2$ (74,75). Of the two compounds, it is the carboxyarabinitol-$P_2$ which is the better inhibitor. The $K_{off}$ of carboxyribitol-$P_2$ is less than $10^{-11}$ M. If one assumes that the better inhibitor most closely resembles the reaction intermediate III (Figure 1), then the intermediate probably has the arabino- rather than the ribo-configuration (75). This raises a potential problem with the stereochemistry of the P-glycerate which arises from the top half of the ribulose-$P_2$. It is known that the configuration is D, but if the intermediate is in the arabino- form, the P-glycerate formed with $CO_2$ would appear to be L. This problem is under investigation by John Pierce in Tolbert's laboratory.

The postulated intermediate III has been synthesized (76). The authors stated that the compound, 2-carboxy-3-ketoribitol-1,5-bisphosphate, rapidly decomposed in the absence of carboxylase either through loss of $CO_2$, or to a mixture of D and L P-glycerate. The intermediate was also a substrate for the enzyme, but the stereochemistry of the products was not determined (76). It is not clear whether the synthesized intermediate was really the ribo- form or whether it could have been the arabino- form.

Rabin and Trown (77,78,79) have suggested a somewhat different mechanism (Figure 2) for the carboxylase reaction. In their model there is a covalent attachment of the substrate, ribulose-$P_2$, at C-2 to a sulfhydryl group on the protein. This mechanism would involve loss of the oxygen at C-2. Two separate laboratory groups have recently demonstrated that the oxygen atoms at both C-2 and C-3 are retained during the reaction (80,81). These reports would suggest that either no covalent attachment exists at these carbons, or that the enzyme returns to the product the same oxygen that it removes from the substrate.

As far as the oxygenase mechanism is concerned (Figure 1) it has been shown conclusively that P-glycolate arises from the top two carbons of ribulose-$P_2$ (82). Studies with $^{18}O$ have shown that the oxygen fixed in the oxygenase reaction is incorporated into the carbonyl carbon of P-glycolate (11).

The first step in the postulated mechansim of Figure 1 is the abstraction of a proton from C-3 of ribulose-$P_2$ by a base on the protein. Sugar bisphosphates which do not have the ribo- configuration at C-2, C-3, have been found to be potent inhibitors of the enzyme. These are

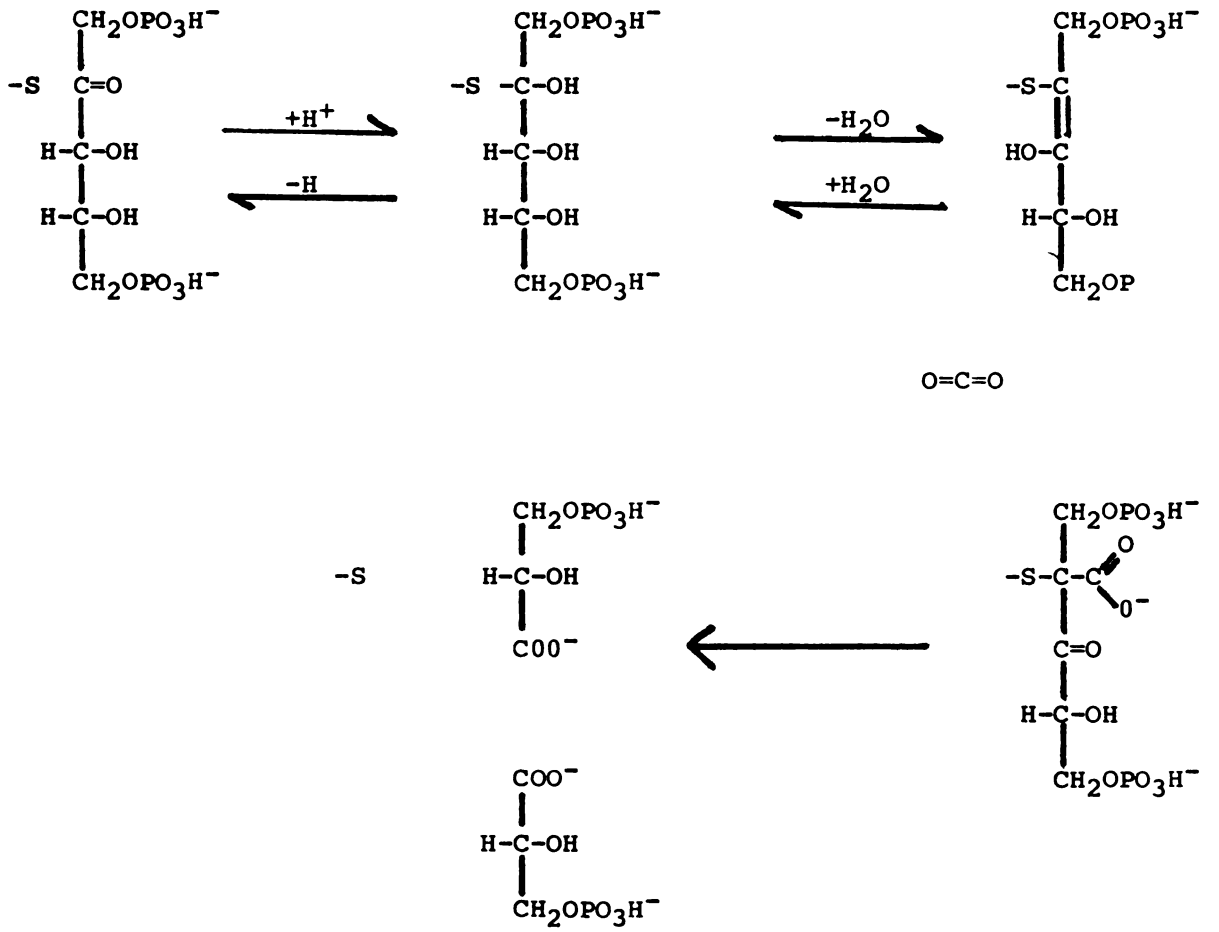



Figure 2. Postulated reaction mechanism of ribulose-$P_2$ carboxylase involving a covalent intermediate (Rabin and Trown 77,78,79).

xylitol-1,5-bisphosphate (xylitol-$P_2$) (83), xylulose-1,5-bisphosphate xylulose-$P_2$) (84), and arabinitol-1,5-bisphosphate (arabinitol-$P_2$) (John Pierce, personal communication). Besides their obvious similarities to ribulose-$P_2$ in size and bisphosphate nature, these compounds all posess a hydroxyl at C-3 which could allow hydrogen bonding in the region where proton abstraction occurs. The importance of the stereochemistry at C-4 is not known.

The oxygenase mechanism shown in Figure 1 was proposed (11) by analogy to the carboxylase mechanism of Calvin (57). There is a major problem with the mechanism as written; $O_2$ is in the triplet state, i.e. it has two unpaired electrons, while the intermediate II (Figure 1) is singlet. Such a reaction is termed "spin-forbidden" (85). The energy level of triplet $O_2$ is 22 kcal/mol below the lowest energy singlet state (85). To perform this transition, the oxygen must be reduced to the level of $H_2O$. This could be done one electron at a time generating $O_2^-$ as the first product, two electrons at a time proceeding through $H_2O_2$, or four electrons at a time breaking the O=O bond. Two known ways of solving this problem are: complexing the $O_2$ with a transition metal to unpair the electrons, or forming a resonance-stabilized free radical. Such a free radical would need to be stabilized by a cofactor such as a flavin, or some similar prosthetic group on the protein (85,86). Hamilton states this very strongly (85); "Thus, <u>every</u> <u>oxygenase</u> has one or both of the following characteristics: (a) the enzyme requires a transition metal ion (usually iron or copper) for activity; (b) the enzyme has a cofactor or substrate, the reduced state of which gives a highly resonance stabilized free radical by the loss of an electron or the equivalent of a hydrogen atom." (emphasis in original).

Wildner (87) suggested that the form of oxygen involved in the oxygenase reaction was $O_2^-$, however he offered no data to support this. The only data published to support this claim is from Bhagwat and Sane (88). Their data were that superoxide dismutase (SOD), purified by them from bovine erythrocytes, inhibited ribulose-$P_2$ oxygenase, they offer no data concerning the carboxylase function. We tried to repeat their conditions, using SOD from Sigma and found no effect on either activity (11, Paech and McCurry unpublished observations, Chapter VIII, this thesis). Lorimer (personal communication) also has attempted this experiment using copper penicillamine, a small molecular weight SOD, and found no effect. Wildner (personal communication) claims to have confirmed Bhagwat and Sane (88). If $O_2^-$ is the real substrate, then it must be made by the enzyme from $O_2$, because its concentration in free solution is much too low to account for the observed rates. If it were being made by the enzyme then it could simply be unavailable for reaction with SOD. Data will be presented later in this thesis (Chapter VI) concerning EPR spectroscopy performed during catalysis. These spectra fail to show any $O_2^-$ signal. $Mg^{++}$ is not capable of performing the role of a transition metal in regards to the required spin-inversion. The potential involvement of $O_2^-$ remains to be resolved.

Activation

There are two ways in which $CO_2$ is involved in the mechansim of this enzyme. It is a substrate, and it is essential for maximal activation of either carboxylase or oxygenase activity. The requirement of $CO_2$ for activation was discovered by Pon and coworkers (65,89) in 1963. This early work described $CO_2$ as a homotropic effector for

ribulose-$P_2$ carboxylase, and stated that prior to initiation of the reaction, the enzyme required incubation with $CO_2$ amd $Mg^{++}$ for maximal activity. It was not suggested that $CO_2$ was required at more than one site. This report was relatively ignored by others in the field. Akazawa's group confirmed the effect (90), but it still did not become routine to activate the enzyme before each assay. It was through the work of Bahr and Jensen (91,92) on enzyme prepared from freshly ruptured chloroplasts that the problem of activation became fully recognized. They observed that carboxylase prepared from freshly ruptured chloroplasts had a lower $K_m$ for $CO_2$ than purified enzyme did. This increase in $K_m$ was traced to loss of $CO_2$ from the enzyme. The requirements for full activation of both activities was then worked out by Lorimer and coworkers (60,61). One of the most fundamental features of this discovery was that the pH optimum for fully activated ribulose-$P_2$ oxygenase was found to be 8.0-8.2 and 9.2-9.4 (61). Fully activated enzyme did not show any differential regulation with respect to sugar phosphates (93), as had been claimed for the unactivated enzyme (70).

Several compounds have been shown to stimulate the enzyme to varying degrees, these include 6-P-gluconate, fructose-$P_2$, NADPH, P-glycerate (94,95,96,97) and pyridoxal-P (98). All of these compounds activate only under certain conditions: a) they must be present only in small amounts, b) they must be incubated with the enzyme prior to the addition of ribulose-$P_2$, and c) they must be used at suboptimal concentrations of $CO_2$ or the effect will not be observed. In no case is the activation from these compounds as great as the activation from saturating amounts of $CO_2$. When these compounds are present in the assay in high concentrations, they are inhibitory (96,97). It has been suggested that

a separate sugar-P site exists for the activator role of these compounds, with their inhibitory role being at the ribulose-$P_2$ catalytic site (95,97,98). Similarly, it has been argued that ribulose-$P_2$, under conditions of low $CO_2$ will bind to this sugar-P site and inhibit the enzyme (94,98,99).

A model for control of this enzyme, involving five distinct sites for various compounds has been proposed (97). This model involves the catalytic site for the binding of ribulose-$P_2$, $CO_2$, and $O_2$, an allosteric site for the binding of ribulose-$P_2$, a second allosteric site for the binding of $Mg^{++}$ and $HCO_3^-$, a third allosteric site for the binding of 6-P-gluconate and fructose-$P_2$ and a fourth allosteric site for NADPH, with some lack of specificity of the sites. This model seems unnecessarily complicated. A simpler model has been proposed by Tolbert's group (100) and is shown in Figure 3. In this model, there is one active site for ribulose-$P_2$ and $CO_2$, and one activator site for $Mg^{++}$ and $CO_2$. The sugar-P effectors exert their effect at the catalytic site, and the positioning of $O_2$ is uncertain. It has been known for some time that incubation of the carboxylase with ribulose-$P_2$ in the absence of $CO_2$ results in inhibition of the enzyme activity (97) and thus the proposed allosteric site for ribulose-$P_2$. Recently we have discovered that the substrate ribulose-$P_2$ is quite labile with respect to base (101). Under mild base treatment, including storage at pH 8.0 and -20°, ribulose-$P_2$ can epimerize to xylulose-$P_2$, as well lose the phosphate group from C-1 (101). That these products may account for ribulose-$P_2$ inhibition is discussed in more detail in Chapter III.

The necessity of a divalent metal (preferably $Mg^{++}$) for the activity

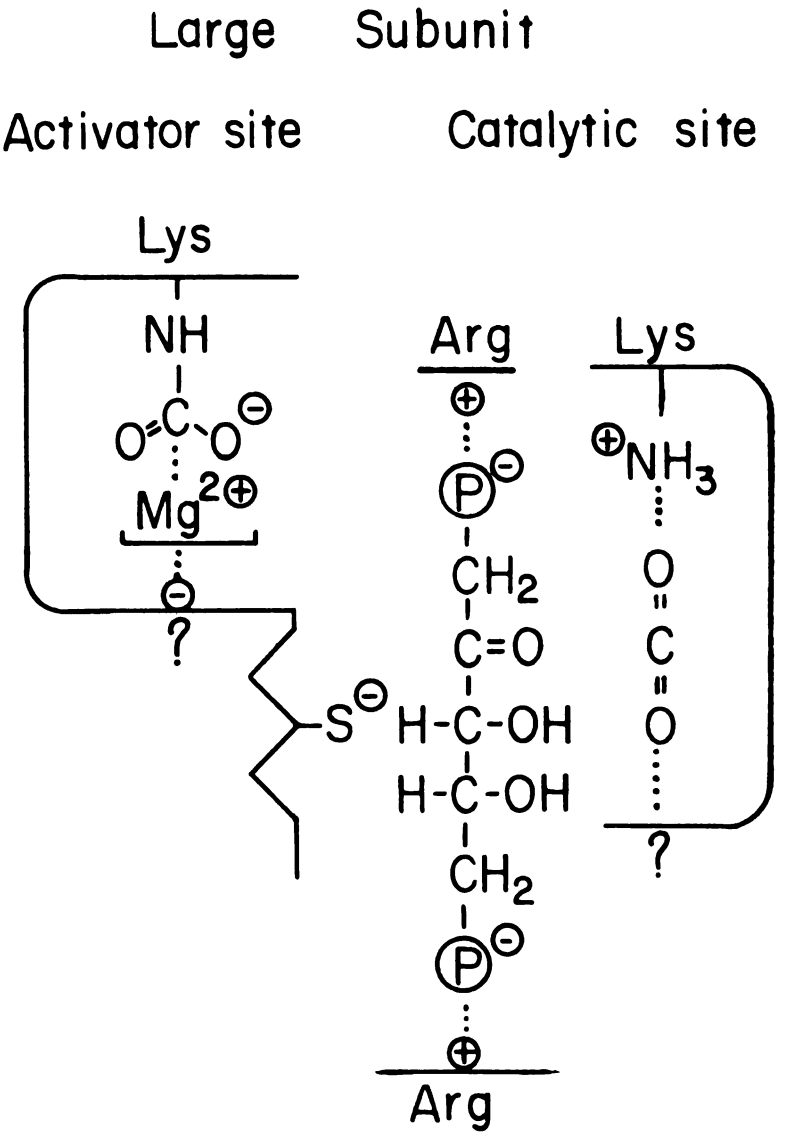



Figure 3. Model for the catalytic and activator sites of ribulose-$P_2$ carboxylase (100).

of this enzyme has been known since the identification of the function of this protein (3,4). Pon observed that $Mg^{++}$ was required for the homotropic effect of $CO_2$ (65). Two groups have considered the role of metal through the use of $Mn^{++}$ and EPR spectroscopy (72,102). Chu and coworkers (102) found a stoichiometry of 3 $Mn^{++}$/mol of enzyme, a rather surprising ratio for an enzyme with 8 active sites. The explanation may lie in the fact that these workers failed to supply any $CO_2$ to their enzyme-$Mn^{++}$ incubation mix. If it had not been for the presence of endogenous $CO_2$ in their samples the ratio would have been smaller. Miziorko and Mildvan (72) were aware of the effect of $CO_2$ and found that when the carboxylase was incubated with sufficient $CO_2$ for complete activation, a stoichiometry of 8 mol of $Mn^{++}$/mol of enzyme was found. They were able to determine the distance from $Mn^{++}$ to $H^{13}CO_3^-$ to be 5.4 A. This was determined by measuring the effect of $Mn^{++}$ on the longitudinal relaxation rate of the $H^{13}CO_3^-$ with $^{13}C$-NMR. It is clear from their data that the $^{13}C$ signal is not a carbamate. Addition of carboxyribitol-$P_2$ (probably the isomeric mixture) abolished the effect of $Mn^{++}$. My intrepretation of this data is that the $CO_2$ in the enzyme-$CO_2$-Mn complex exchanged so slowly that no signal for this $CO_2$ was observed (discussions with John Pierce helped clarify this point). This data also implies that carboxyribitiol-$P_2$ binds very close to the Mn. It is clear that carboxyribitol-$P_2$ requires $M^{++}$ for maximal binding (69).

Miziorko (73) has recently demonstrated that enzyme activated with $Mg^{++}$ and $H^{14}CO_3^-$ and then mixed with carboxyribitol-$P_2$ (the isomeric mixture) incorporates $^{14}C$ in stoichiometric amounts. If carboxyribitol-$P_2$ is really an analogue of the reaction intermediate (III Figure 1) then it should exclude $CO_2$ from the catalytic site. Using this

reasoning then the bound $CO_2$ represents activator $CO_2$.

One likely way for $CO_2$ to be bound for activation would be through a carbamate to the $\varepsilon$-amino group of a lysyl residue (Figure 3). Such a carbamate could be stabilized by $M^{++}$, perhaps through some additional anion at the active site. Using the enzyme from R. rubrum, O'Leary and coworkers (103) have demonstrated carbamate formation with $^{13}CO_2$ using $^{13}C$-NMR. While they did not rule out the possibility of a Tris-$CO_2$ carbamate in their paper, they have since run this control and ruled it out as a possible source of the signal (F. Hartman, personal communication). Since catalytic $CO_2$ would not be expected to be bound as a carbamate, this data can be used as further evidence of two distinct $CO_2$ sites.

If there are two sites for $CO_2$ and the activator $CO_2$ does not participate in catalysis, then the activator species might be identifiable by means of an isotope trapping experiment. Lorimer (104) has performed such an experiment. The enzyme was fully activated using $Mg^{++}$ and high specific activity $H^{14}CO_3^-$. The activated enzyme was injected into a large excess of $H^{12}CO_3^-$ and ribulose-$P_2$ and allowed to catalyze for a short period. Subsequently, the enzyme was rapidly separated from the smaller molecular weight species by gel filtration in the presence of a large excess of $Mg^{++}$. The resulting enzyme fraction contained a 40 fold higher specific activity (of the $^{14}C$) than would have been expected if the activator $CO_2$ were used in catalysis. This is evidence for a distinct activator site for $CO_2$, and argues against the suggestion that the bound activator $CO_2$ is subsequently fixed (65,89).

In an effort to irreversibly activate the carboxylase, two groups

have used the isoelectronic analogue of $CO_2$, cyanate (105, 106). In each case it was found to be a potent inhibitor, although the mode of inhibition was not agreed upon (105,106).

Active Site Characterization

Armed with some knowledge of the mechanism of an enzyme, it is often possible to make some assumptions about the nature of the catalytic site. With much less data available to them than is available today, Rabin and Trown (77,79) made some speculations about the active site of ribulose-$P_2$ carboxylase. They proposed a sulfhydryl group adjacent to C-2 of ribulose-$P_2$ for covalent attachment during catalysis. There may be such a sulfhydryl, but it doesn't function as suggested (80,81). They also suggested that some sort of base was involved in the binding of $CO_2$. This has been shown to be the case for $CO_2$ activation and is likely to be true for the catalytic $CO_2$ as well. Lastly, they suggested the presence of a base to function in the proton abstraction at C-3 of ribulose-$P_2$. They offered some data on the presence of SH groups. Using 5,5'-dithiobis-(2-nitrobenzoic acid) Ellman's reagent (107), they found 4 fast-reacting SH groups/mol enzyme. Two of these could be protected by preincubation with ribulose-$P_2$. No explanation for this stoichiomentry was offered.

Pyridoxal-5'-P has been found to be a potent inhibitor of ribulose-$P_2$ carboxylase/oxygenase (108,109). This compound is known to react with primary amino groups, such as the ε-amino groups of a lysyl residue, through formation of a Schiff base. Paech and Tolbert (110) demonstrated that at pyridoxal-P concentrations sufficient to cause 100% inhibition of both activities of the enzyme purified from spinach, only

the ε-animo group of lysyl groups were modified by reduction of Schiff base with $NaBH_4$. The inhibition by pyridoxal-P is competitive with respect to ribulose-$P_2$ and $CO_2$ (108,110). There is clearly one class of rapidly reacting lysyl groups amounting to 8 mol per mol of enzyme (110). The enzyme from spinach requires the binding of 16 mol of pyridoxal-P per mol of enzyme for complete inhibition (110), however proteolysis of carboxylase modified by pyridoxal-P followed by reduction with $NaB^3H_4$, shows only one significant peptide and several minor ones (111,112). Data obtained from R. rubrum, an $A_2$ enzyme, suggest that modification of only one lysyl group per enzyme molecule results in complete inhibition (113,114). Pyridoxal-P is an especially good probe for the active site since it can be used either as a covalent modifier, or as a non-covalent competitive inhibitor for kinetic studies.

There has been research to design chemical compounds which resemble the substrate ribulose-$P_2$, but form stable complexes with the enzyme. These affinity labels have been made and used with success by Hartman's group (115). The best results have been with compounds such as 3-bromo-1,4-dihydroxy-2-butanone-1,4-bisphosphate (116,117,118) and N-bromoacetyl ethanolamine phosphate (119). These reagents modify both cysteine and lysine residues, and activation causes a shift towards modification of lysyl residues. The modification of a sulfhydryl lends credence to earlier reports of SH groups in the active site (77, 120).

Incubation of the enzyme with 3-bromo-1,4-dihydroxy-2-butanone-1,4-bisphosphate resulted in the modification of two different lysyl residues (121). Tryptic peptides containing these modified residues have been sequenced (121). Activation of the enzyme before modification results in

an increase in the amount of one of these peptides, termed "peptide I" by the authors. Recently, a tryptic peptide has been obtained and sequenced from enzyme modified with pyridoxal-P and $NaBH_4$ (111,112). Both the pyridoxal-P and the butanone derivative modify the same lysyl residue on "peptide I". Lorimer has had some success in attaching $^{14}CO_2$ to the enzyme with diazomethane (personal communication). It is attached to a lysyl residue, and the sequence is being determined.

Essentially all of the compounds which compete with ribulose-$P_2$ for the catalytic site are phosphate esters. Several groups have designed experiments to determine the nature of the residues responsible for binding the phosphates. In other proteins arginyl residues perform this function (122). The reagents of choice for arginyl residues are diketo compounds, such as cyclohexanedione and glyoxal (123). Results with butanedione (124,125) indicate the presence of 2 to 3 reactive arginyl residues per large subunit, which can be protected by preincubation with ribulose-$P_2$. This work was done with R. rubrum (124) and Pseudomonas oxalaticus (125). Paech and Spellman (Tolbert's group, unpublished) have found similar results with spinach, and Chollet (126) has repeated the experiments on tobacco. To date, no one has reported any data concerning arginyl residues at a distinct activating site.

# CHAPTER I

# BASIC TECHNIQUES

## Introduction

The work discussed in this thesis concerns aspects of research on ribulose-$P_2$ carboxylase/oxygenase. While the specialized methods are discussed with each set of experiments, the basic techniques common to all sections are described in this chapter.

## Purification of ribulose-$P_2$ carboxylase/oxygenase

Glycylglycine, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), Bicine (NN-bis-(2-hydroxyethyl)-glycine), β-mercaptoethanol, and polyvinylpolypyrrolidone were from Sigma Chemical Company. Ultra-pure ammonium sulfate was from Schwarz-Mann. The spinach used came from a variety of sources: grown in our growth chambers, green house, or field plots; purchased locally; flown in from Oklahoma, California, Florida, or Georgia. No differences were found in the activity of different preparations of enzyme which could be unequivocally attributed to the source of the spinach.

Ribulose-$P_2$ carboxylase/oxygenase was pruified from spinach leaves in the following manner: 500-1000 g of washed, deveined leaves were ground with a two fold excess (v/w) of grinding buffer. The buffer at pH 7.8 to 8.2 was 25mM Bicine (HEPES or glycylglycine were used occasionally) with 1 mM EDTA, 10-50 mM β-mercaptoethanol, and 0-2%

polyvinylpolypyrrolidone (w/v). The grinding and subsequent steps were performed at 4°. The leaves were ground in a 4 l Waring blender at slow speed for 40-60 sec. The resulting homogenate was allowed to stand for 10-15 min to facilitate decanting by allowing the cellular debris to rise to the top of the homogenate. The homogenate was filtered through 2-8 layers of cheesecloth and 1-4 layers of miracloth. The excluded debris was discarded.

The homogenate was centrifuged at 10,000-11,000 x g for 30-45 min. The resulting supernatant was poured through either miracloth or glass wool for removal of dark green lipid material. The supernatant was made 37% saturated with a solution of saturated ultrapure $(NH_4)_2SO_4$. The saturated solution had been previously prepared, stored at 4°, and the pH adjusted to 7-8 with $NH_4OH$. The enzyme solution was then stirred for 5-20 min and allowed to stand for an additional 10-40 min before centrifugation as before. The supernatant from the second centrifugation was made 50% saturated in $(NH_4)_2SO_4$ to precipitate the carboxylase and allowed to stand and then centrifuged as before.

The precipitate from the 50% $(NH_4)_2SO_4$ centrifugation was resuspended in 25 mM buffer (Bicine, HEPES, etc.), 1 mM EDTA, 10 mM β-mercaptoethanol, pH 7.8-8.2. The resuspension volume was kept as low as possible; from 500 g of leaves, the volume was 25-30 ml. Great care was taken in resuspending the precipiate employing gentle stirring with a glass rod. The resuspended precipitate was centrifuged at 27,000 x g for 10-15 min for clarification. The supernatant from this spin was either loaded onto a Sepharose 4B column (2.5 x90 cm) and chromatographed overnight, or stored overnight for zonal centrifugation. In the latter

case a 10-30% (w/w) linear sucrose gradient was used in a 750 ml zonal rotor and spun at 30,000 rpm for 4 hr. The buffer in either case was the resuspension buffer.

In either case fractions were colected and monitored by their absorbance at 280 nm. A sample Sepharose 4B profile is shown in Figure 4. The fractions containing carboxylase (the first peak after the void volume) were pooled. The pool was loaded onto DEAE cellulose (Whatman DE 52) in a 6 x 40 cm column. The column had been previously equilibrated with the resuspension buffer. After application of the protein sample, the column was washed with 500-1000 ml of resuspension buffer. The sample was eluted with a 0-0.4 M $NaHCO_3$ 2 l gradient. The fractions were monitored as before. A sample profile is shown in Figure 5. The peak fractions were pooled and usually stored as 50% ammonium sulfate slurry at 4°. Under these conditions the specific activity remained relatively constant for 3-4 weeks. The yield of purified carboxylase from 500 g of spinach leaves was 0.7-1.0 g.

To prepare enzyme for assay, the desired amount of slurry was centrifuged at 15,000-20,000 x g for 10-20 min. The precipitate was usually resuspended in a buffer at pH 8.0 to 8.2 of 100 mM Bicine, 1 mM DTT, and 0.4 EDTA. The resuspension volume was adjusted so that the resulting protein concentration was 20-40 mg/ml. The sample was placed in dialysis tubing and dialyzed overnight against the resuspension buffer. Improved storage methods are discussed in Chapter II.

Figure 4. Elution profile of ribulose-$P_2$ carboxylase from Sepharose 4B column. The fractions were diluted 20 fold with the column buffer for determination of their absorbance at 280 nm. Fractions 22-34 were pooled for the DEAE column. Fraction size was 11.5 ml.

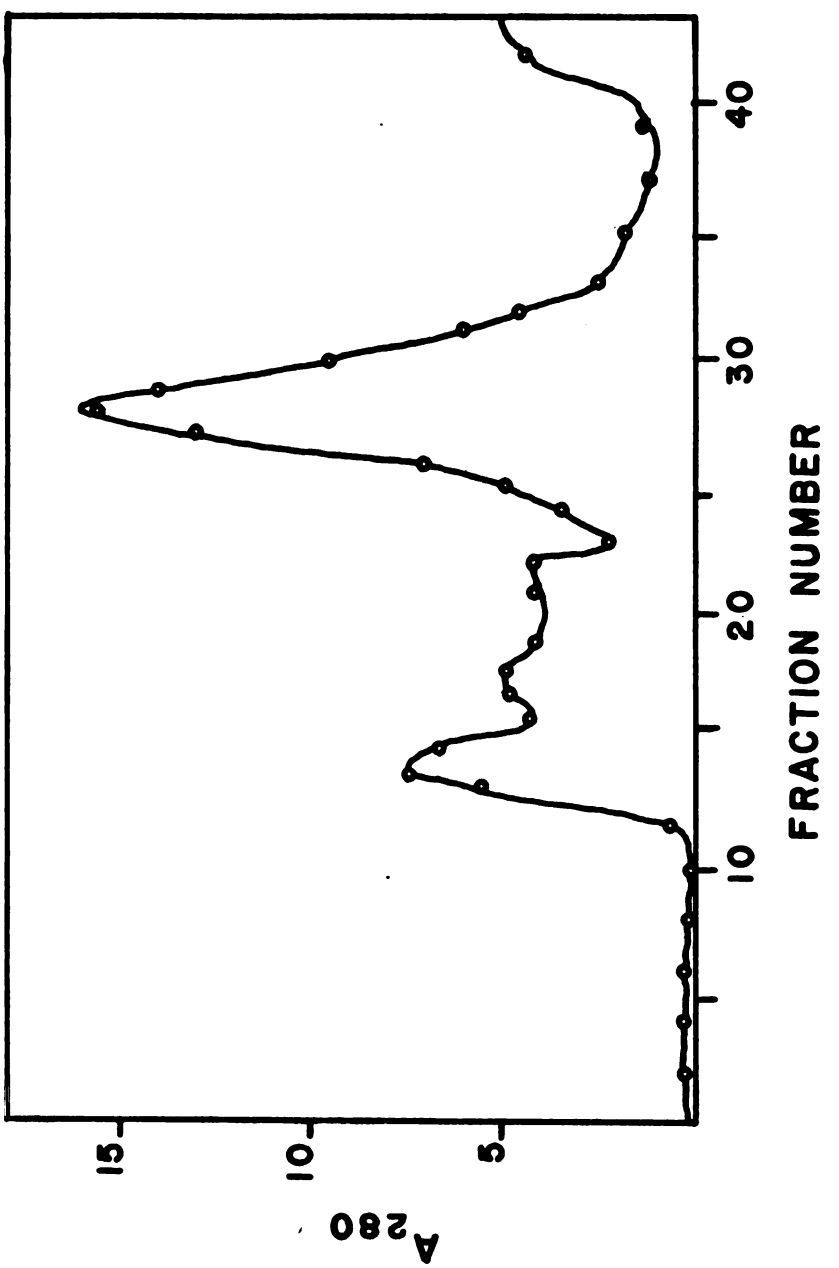
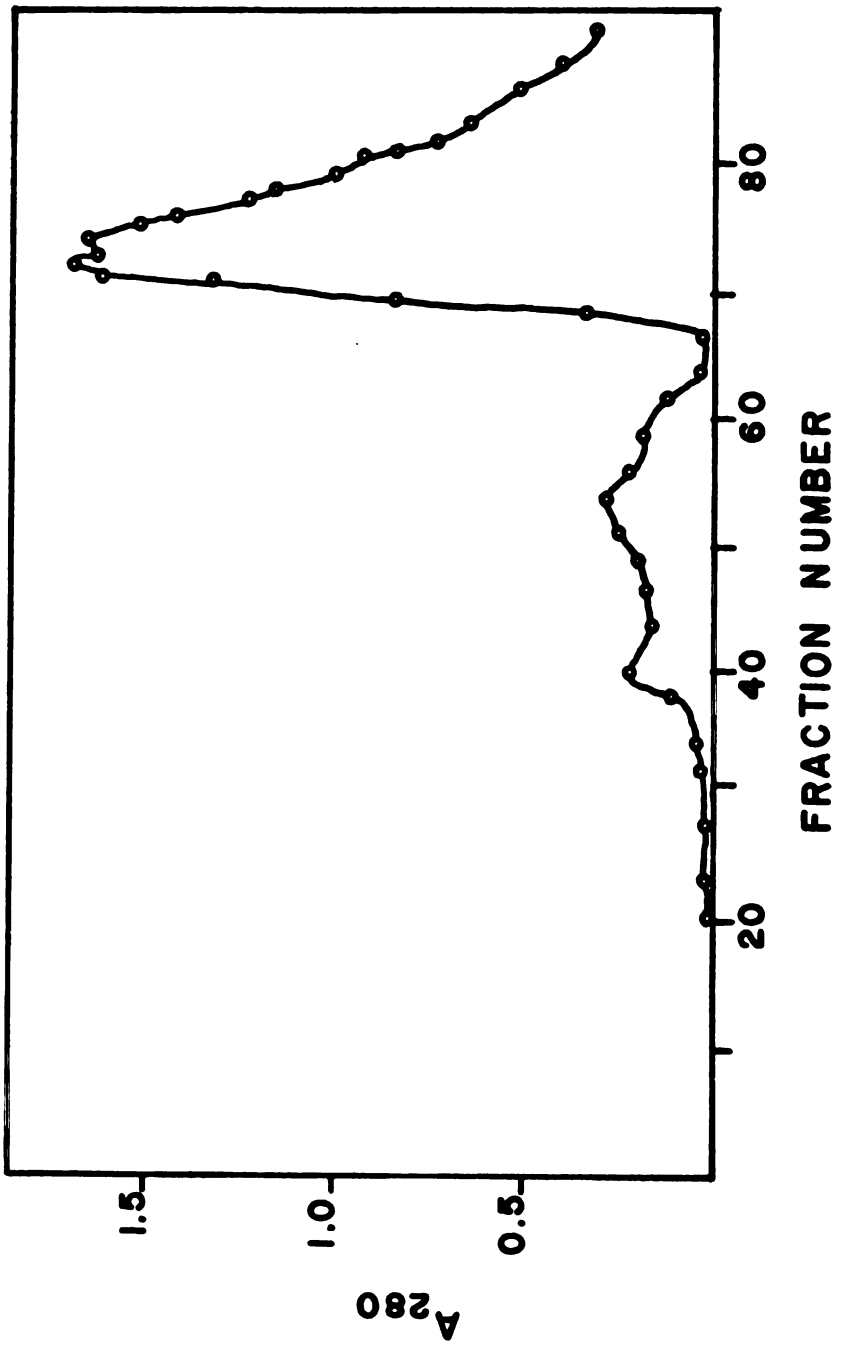

15
10
5
$A_{280}$
10
20
30
40
FRACTION NUMBER

Figure 5. Elution profile of ribulose-$P_2$ carboxylase from DEAE cellulose column. The column conditions are described in the text. The fractions were diluted 10 fold with the column buffer (No $NaHCO_3$) for determination of their absorbance at 280 nm. Fractions 69-85 were pooled and made 50% saturated in ammonium sulfate for storage. The fraction size was 20 ml.


1.5
1.0
0.5
$A_{280}$
20
40
60
80
FRACTION NUMBER

Synthesis of Ribulose-$P_2$

While ribulose-$P_2$ can be purchased from the Sigma Chemical Company, it is quite expensive, so the ribulose-$P_2$ used in the experiments described in this thesis was synthesized in the laboratory. The procedure was that of Horecker's group (127) with some modifications. The ribose-5-P, ATP, phosphoribosisomerase, and phosphoribulokinase were all purchased from the Sigma Chemical Company.

The reaction mixture contained the following: 1 mMol ribose-5-P, 1 mMol ATP, 1 mMol $MgCl_2$, 0.1 mMol DTT, and 0.1 mMol $Na_2EDTA$ in a reaction volume of 100-200 ml. The DTT, EDTA, and $MgCl_2$ were dissolved in 50-100 ml of water and the pH was adjusted to 7.9. The ATP was added and the pH was readjusted to 7.9. Next, 100 U each of phosphoriboisomerase and phosphoribulokinase were added and the pH readjusted (when necessary). The ribose-5-P was dissolved in up to 50 ml of water, the pH adjusted to 7.9, this solution was used to initiate the synthesis. The reaction was monitored on a pH meter and the pH was kept between 7.5-7.9 through frequent addition of small amounts of 0.1 M KOH. When the pH was constant for 10 min the reaction was considered to be complete. This took up to 90 min. When the reaction was completed the enzymes were usually precipitated by the addition of 10-15 ml of 50% trichloroacetic acid at 4°. When this step was used, the solution was made 0.2 M with $CH_3CO_2Na$ and the nucleotides extracted with activated charcoal. The high salt concentration was necessary to prevent the ribulose-$P_2$ from binding to the charcoal. The charcoal was added in 20 g amounts, stirred and filtered. this step was repeated until the ribulose-$P_2$ solution had less than 1.0 O.D. at 260 nm. The concentration of the ribulose-$P_2$ solution

was measured at this time, by the orcinol reaction (128). The preparation was loaded onto a Dowex-1 column (1.5 x 20 cm) in the formate form. The column was washed with water and the effluent monitored with the orcinol reaction. If orcinol positive material was found at this stage, the column was either overloaded, loaded too fast, or inadequately washed before application of the sample. When no more orcinol positive material was removed with the water wash, the column was washed with 2 N formic acid to remove monophosphates (e.g. ribose-5-P). The ribulose-$P_2$ was eluted from the column with a 2 N HCOOH to 2 N HCOONa linear gradient, 200 ml total volume. Running orcinol reactions through the fractions collected from the gradient revealed a single ribulose-$P_2$ peak. This purification has been recently simplified through suggestions from J. Pierce. Addition of myokinase to the reaction mix (suggested by A. Serianni) results in the removal of ADP and in the formation of AMP and ATP. This solution can then be introduced directly on to a Dowex-1 C1 column. The column is then eluted in a stepwise manner for separation of the mono-, di-, and triphosphates. This method works well for the preparation of xylulose-$P_2$ (84). With this method the coupling enzymes are not precipitated before the Dowex column thus enabling the salt concentration to be kept low.

After isolation and identification of the ribulose-$P_2$ peak, by either of the above metnods, the pH of the pool was raised carefully to 5 through the addition of $NH_4OH$. A 1.2 fold molar excess of Ba $(CH_3COO)_2$ was added and the solution was mixed with equal volumes of ethanol. The sample was cooled to 4°, centrifuged, and the precipitated products washed several times with cold 50% ethanol. The Ba salt of ribulose-$P_2$ was dried and stored dessicated at -20°.

To prepare a ribulose-$P_2$ solution for use, the Ba-ribulose-$P_2$ salt was mixed with carefully washed Dowex-50 ($H^+$ form) in the approximate ratio of 120 mg of the Ba-salt to 1 ml of Dowex. The Dowex mix was vortexted and let stand for 5 min, filtered, and washed to a volume of 1 ml. The pH was carefully raised with KOH to 6.5-7.0. Subjecting the substrate to high pH, even briefly, results in epimerization to xylulose-$P_2$ and degredation products are formed (see Chapter III).

Assay of Ribulose-$P_2$ Carboxylase

The basic features of this assay have been reported previously (84, 129), and are included here for continuity and convenient reference. The rationale behind the assay is to mix the enzyme with $NaH^{14}CO_3$ of known specific activity in the presence of ribulose-$P_2$ for a defined amount of time. Determination of radioactivity incorporated into acid stable products allows calculation of the amount of $CO_2$ fixed. The assay volume was either 0.25 or 0.50 ml. The assay buffer was 100 mM Bicine, 0.4 mM EDTA, 20 mM $MgCl_2$, and 10-20 mM $NaH^{14}CO_3$ (200-300 cpm/nMol) at pH 8.0-8.2. The protein concentration in the assay was usually 80 µg/ml. The ribulose-$P_2$ concentration was 0.5 mM.

Before assay, the enzyme was activated by incubation with 10 mM $NaHCO_3$, 20 mM $MgCl_2$, and 1 mM DTT in Bicine buffer at pH 8.0-8.2, for at least 10 min at 30°. The activation was performed in a sealed container with a minimum volume of gas space over the sample. The protein concentration was typically 2 mg/ml. The reaction was initiated by the addition of activated enzyme to the assay mix containing ribulose-$P_2$. The reactions were run for 1 min or less and terminated by the addition of 0.2 ml of 2 N HCl. All assays were run in scintillation vials. After

addition of the HCl the samples were dried slowly (to minimize charring) in a 90° oven. The combination of the acid and the heat resulted in the removal of the unreacted $^{14}CO_2$.

The dried samples were resuspended in 1 ml of water, and 9 ml of scintillation cocktail was added. The cocktail was composed of 2 l of toluene, 1 l of Triton-X-100, 12 g of 2,5-diphenyloxazole (PPO), and 0.15 g of 1,4-bis(2(5-phenyloxazoly)) benzene (POPOP). The vial was shaken until the water and cocktail were thoroughly mixed. The samples were counted in a Packard Tri-Carb Liquid Scintillation Counter.

Assay of Ribulose-$P_2$ Oxygenase

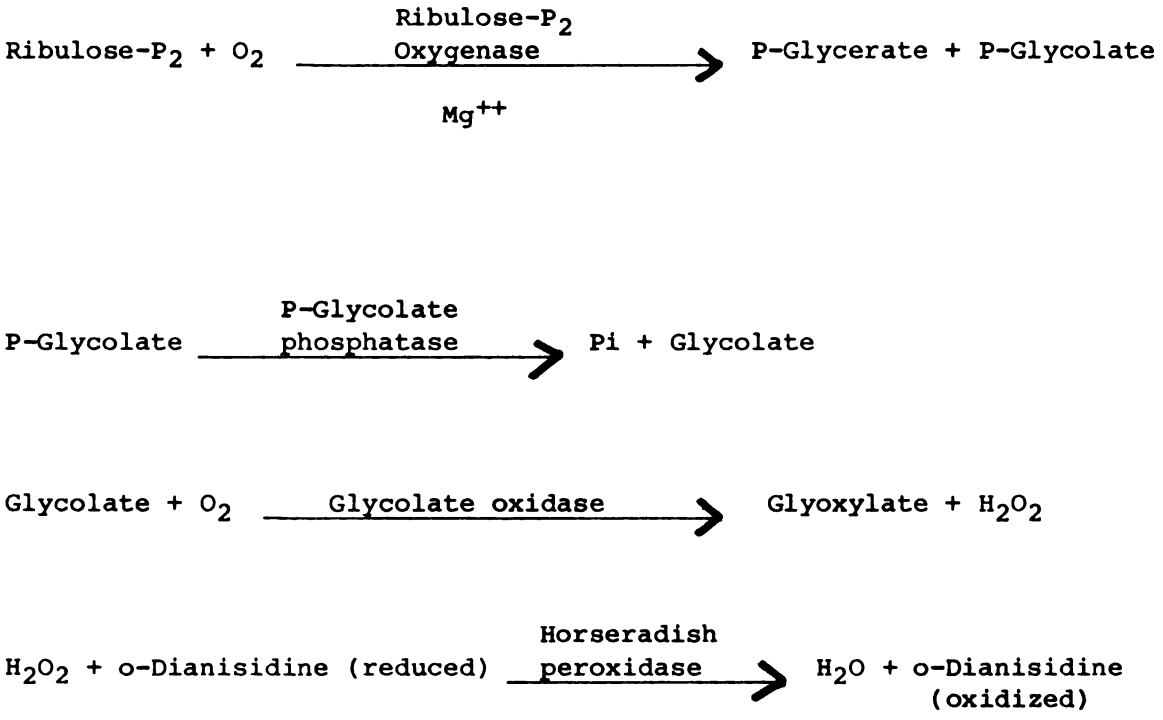
The most commonly used assay for ribulose-$P_2$ oxygenase is the monitoring of $O_2$ uptake with an oxygen electrode. This technique was used for the experiments described in this thesis. The electrode, from Rank Brothers (Bottisham, Cambridge, England), had a water jacket and the temperature was maintained at 30° with a Haake water circulator. The attached recorder was calibrated between atmospheric oxygen (21%) and zero oxygen (achieved by the addition of dithionite to the assay buffer in the electrode chamber). The assay buffer consisted of 100 mM Bicine, 20 mM $MgCl_2$, 0.4 mM EDTA, and up to 1 mM DTT, in a final volume of 0.5-1.0 ml. The ribulose-$P_2$ concentration was 0.5 mM. The assay was initiated by the addition of enzyme activated in the same manner as for the carboxylase (previous section, this Chapter). The protein concentration was 4-8 mg/ml for activation and 160-320 µg/ml in the assay. Under these conditions the reaction had a 5-10 s lag and the rate was linear for 1-2 min.

Occasionally, requirements for a particular experiment make a spectrophotometric assay desirable. While there is a very good spectrophotometric assay for the carboxylase (4,130), no such assay exists for the oxygenase. I have worked on developing such an assay in collaboration with Christian Paech and others in the laboratory. This assay couples ribulose-$P_2$ oxygenase through P-glycolate phosphatase, glycolate oxidase, and horseradish peroxidase to the chromaphore 3,3'-dimethyl benzidine (o-dianisidine). The reaction sequence is given in Table 2. The disadvantages of this particular spectrophotometric assay include the availability and required purity of the coupling enzymes, as well as the toxicity of the o-dianisidine. The advantages include increased sensitivity, a shorter lag than with the oxygen electrode, and the ability to monitor product formation rather than $O_2$ uptake (especially in a crude extract). When the coupling enzymes are available in the required specific activites this can be used as a rate assay; it can also be used as an end point assay.

In a coupled assay, the conditions must be such that the first enzyme in the series be rate limiting. Under these conditions there will be a finite lag, the duration of which is determined by the kinetic properties of the coupling enzymes (131). This time lag is the time required for the system to achieve steady state. The lag time for any one enzyme of the series is given by the ratio of its Km and maximum velocity, while the lag for the entire process is given by the simple sum of the individual lags (131). These features have been discussed in detail for a system with two coupling enzymes (132), and the general case has been described as well (131). Knowledge of the specific activity and Km of each of the coupling enzymes allows one to detrmine whether the

Table 2

Reaction Sequence of the
Coupled Oxygenase Assay



coupled system will work without running assays.

Because of the requirement that ribulose-$P_2$ oxygenase be activated with $CO_2$, and because this $CO_2$ rapidly dissociates from the enzyme under the low $CO_2$ conditions necessary for the oxygenase assay, the oxygenase only fixes $O_2$ at a linear rate for 1-2 min. This requires that the lag period be kept as short as possible. Considering the amount of time required for mixing, making the lag shorter than 5 s would be unnecessary. The final enzyme in the series, horseradish peroxidase has a rate constant for $H_2O_2$ of $10^8$ ($M^{-1} \times s^{-1}$) (133). As a result its Km is essentially zero. According to the catalogue from Boehringer (Biochemica Information) the peroxidase couples to o-dianisidine with a rate 8-10 times higher than other electron donors. These two facts mean that a few units of the peroxidase in an assay makes the lag time of this step essentially zero. Therefore a lag of 2.5 s could be allowed for P-glycolate phosphatase and glycolate oxidase. The Km's of these two enzymes, in this system have been determined to be 160 μM and 400 μM respectively (Christian Paech, unpublished). The required amount of each of these two enzymes is given by U=Km/T, where U is the number of units of activity, and T is the acceptable lag in min. For P-glycolate phosphatase, 2.5 sec = .042 min, and Km = 160 μM so U = 3.8 Mol/min.ml. For glycolate oxidase, the required number of units is 9.6. We have been able to prepare P-glycolate phosphatase of high specific activity. George Santora in Tolbert's lab has prepared this enzyme from tobacco with a specific activity of 70 U/ml; this is clearly enough for the coupling assay purposes described here. Glycolate oxidase has been more difficult. Initially it seemed that the method of Harris and Stern (134) would be the way to purify the enzyme. They reported a specific

activity of 3092.5 μMol/min/mg protein. We were unable to reproduce this work. Harris (personal communication) belatedly informed us that they had made a 1000 fold error in reporting their data and that the correct activity was 3.0925 μMol/min/mg protein. As of this writing, we have not been successful in obtaining glycolate oxidase of adequately high specific activity to make this coupled assay a reality. Nicola Selph in Tolbert's lab is working on this problem and hopefully the enzyme will be available in the near future.

# CHAPTER II

## STABILITY AND STORAGE OF RIBULOSE-$P_2$ CARBOXYLASE/OXYGENASE

### Introduction

This enzyme is generally stored as a 50% $(NH_4)_2SO_4$ slurry at 4°. Under these conditions it is relatively stable for 3-4 weeks. After this period of time it rapidly loses activity down to 5-10% of the activity of the freshly purified enzyme. This loss does not seem to be due to the cold lability of this enzyme. The nitrogenase protein is subject to similar losses in activity but it has been stored with success by slowly dripping it into liquid nitrogen and freezing it as small "peas" followed by storage at -80° (W.H. Orme-Johnson, personal communication). this chapter contains the results of storage under a variety of conditions including the liquid nitrogen method, and a discussion of a method for restoration of lost activity through treatment of the enzyme with high concentrations of DTT. Early results of this work have been reported (135).

### Materials

See Chapter I.

### Methods

The enzyme was purified and assayed as described in Chapter I. The studies with glycerol, sucrose, and $(NH_4)_2SO_4$ were done by mixing the reagents being tested with the enzyme and storing it in the manner

indicated. For the liquid nitrogen studies, the enzyme was frozen by dripping it slowly into an erlenmeyer flask containing liquid nitrogen. The flask was positioned in an ice bucket at the base of a ring stand under a separatory funnel containing the enzyme. Care was taken to freeze the enzyme into individual beads ("peas") rather than into chains. This required frequent additions of liquid nitrogen to the flask to replace that which had boiled off.

When high DTT concentrations were used, the enzyme was made 50-100 mM DTT for several hours and then dialyzed against 100 mM Bicine pH 8.0. Because of the absorbance of oxidized DTT at 280 mM, it must be removed before determination of the protein concentration by 280 nM absorbtion. For routine determinations of the protein concentration, the method of Paulsen and Lane (136) was used: $A_{280}$/ml x 0.61 = the protein concentration in mg/ml.

## Results and Discussion

Storage of the carboxylase for six weeks under a variety of conditions is shown in Table 3. The sample frozen in 50% $(NH_4)_2SO_4$ retained its activity the best, but it was still lower than the activity of the freshly prepared enzyme by 40%. In this experiment, the samples were dialyzed overnight, activated with 2 mM DTT, 20 mM $MgCl_2$, and 10 mM $NaHCO_3$, and held at 30° for 1 hour before assay.

Recently, Nigel Hall in Tolbert's lab has discovered that incubation of aged enzyme of reduced specific activity could be restored to a specific activity approximating that of the freshly prepared enzyme, by incubation with 50-100 mM DTT for a period of hours (135,137). Neither

Table 3

Comparison of Various Storage Methods for Purified Ribulose-$P_2$ Carboxylase

| | Additions for storage | | | |
|---|---|---|---|---|
| Storage temperatures | No additions | 30% sucrose | 20% glycerol | 50% saturated ammonium sulfate |
| -20° | 3.8 ± 9% | 4.1 ± 11% | 4.1 ± 13% | 4.8 ± 10% |
| 4° | 3.3 ± 6% | 4.1 ± 2% | 3.3 ± 11% | 4.0 ± 2% |
| 22° | 3.3 ± 6% | 1.9 ± 3% | 3.0 ± 7% | 3.5 ± 4% |

Enzyme from spinach leaves was stored under the conditions indicated for 6 weeks, dialyzed overnight, and activated before assay. Conditions are described in the text. The assay volume was 0.25 ml and the specific activity of the $NaH^{14}CO_3$ was 220 cpm/nMol. Values shown are cpm x $10^{-3}$ and are the average of 3 assays. The original activity before storage was about 8,000 cpm.

β-mercaptoethanol nor Na-ascorbate worked as well as DTT. This means that enzyme can be conviently stored as a 50% $(NH_4)_2SO_4$ slurry at 4° for 2-3 months without substantial loss in restorable activity.

The possibility of storing the enzyme by freezing it in small beads in liquid nitrogen was investigated. Enzyme frozen in this manner was subsequently stored in a -80° freezer. Two storage conditons were investigated. In one case, a 50% $(NH_4)_2SO_4$ slurry was frozen, in the other case, a dialyzed protein solution of 30 mg/ml was frozen. In the first case, enzyme stored for 6 months retained 70% of its original activity, addition of 50 mM DTT restored the activity to 85 to 95% of its original activity. In the second case, enzyme stored for seven weeks retained 35% of its original activity and addition of 50 mM DTT restored the enzyme to 105% of its original activity. It is expected that when the optimum conditions for the freezing are determined, it will be possible to store the enzyme in this manner for months to years with no adverse effects on the enzyme activity.

# CHAPTER III

# STABILITY AND STORAGE OF RIBULOSE-$P_2$

## Introduction

In the more than 20 years since its discovery, many workers have discussed the substrate inhibition of the carboxylase/oxygenase by ribulose-$P_2$ (3,65,97,136). This phenomenon has been attributed to an allosteric binding site (97), or competitive binding for ribulose-$P_2$ and $CO_2$ (108), and such binding can produce a conformation change (71,138). Since the elucidation of the required conditions for activation (60), it seems clear that in some cases (97) the ribulose-$P_2$ was inhibiting by simply preventing activation. An additional reason for this inhibition is the presence of certain inhibitors in various preparations of ribulose-$P_2$. Because of the discovery that with different ribulose-$P_2$ solutions of apparently identical concentrations, different initial rates of carboxylation were obtained, a study was undertaken to determine the stability of ribulose-$P_2$. These results have been reported at two meetings (Appendix 1 (100),139) and have been published (101). The publication is included in this thesis as Appendix 2, and therefore only the results are summarized in this chapter.

## Results and Discussion

Since the ribulose-$P_2$ had been prepared in our laboratory (Chapter I) chance contamination seemed unlikely and substrate degradation was suspected. One possible source of contamination was tested first

however. Because of a report that at least one batch of ATP from the Sigma Chemical Company had been contaminated with $NaVO_3$ (140) this compound was tested and found to have no effect on carboxylase activity at concentrations up to 10 mM.

When one sample of ribulose-$P_2$ was incubated at pH 11 and 30° for varying amounts of time (Appendix 2, Figure 2) and then tested for inhibition of the carboxylase, it became clear that the degree of inhibition was dependent on the duration of the high pH treatment. The inhibitory features of these treatments were tested in an experiment designed by Christian Paech. The enzyme was mixed with the treated sample of ribulose-$P_2$ and catalysis was allowed to proceed until the substrate concentration was zero. Then untreated ribulose-$P_2$ was added and the enzyme was assayed. This was possible since the product from the first incubation (P-glycerate) is known to have little effect at these concentrations.

After realizing that the inhibitory compounds could be generated through base and or heat treatment, a careful analysis of the ribulose-$P_2$ was carried out. The analysis revealed that xylulose-$P_2$ (a known inhibitor of this enzyme (84)) and a dicarbonyl compound, were present. Other dicarbonyl compounds have been used to demonstrate the presence of arginyl residues in this enzyme, and they are good inhibitors (124,125,126). It was determined that both of these compounds could be formed by exposure of ribulose-$P_2$ to strong base, even for a brief period. The conversion of ribulose-$P_2$ to xylulose-$P_2$ is a reversible reaction simply involving epimerization at C-3. The dicarbonyl compound arises from an irreversible $\beta$-elimination of the phosphate at C-1. This

explained why frequently the amount of inorganic phosphate increased as a ribulose-$P_2$ solution aged. Under the conditons of the assay the phosphate at C-1 is lost at the rate of 1.5% per hour. During the course of this study the ribulose-$P_2$ solution that had the most inhibitory effect was prepared from ribulose-$P_2$ which had been purchased from Sigma. At that time we attributed this to the fact that it was an old solution. At present Sigma claims that their ribulose-$P_2$ is 95% pure by TLC, phosphate and pentose phosphate assays, but then they go on to say that with their assay using the carboxylase, the substrate is only 85-90% pure.

One conclusion from this research was a new hypothesis concerning the condition of the enzyme in the chloroplast. At the pH and temperature in a chloroplast, the instability of ribulose-$P_2$ could pose a problem. In the light, the internal pH can exceed 8 and on a hot day the temperature can be above 40°. However, partial enzyme inactivation does not seem to occur in the chloroplast. Even when the ribulose-$P_2$ is made to accumulate to concentrations higher than the concentration of active sites of the carboxylase, activity is fully restored within 10 min after the additon of bicarbonate (141). It has been suggested that only a portion of the carboxylase in the chloroplasts is functional at any one time (19), and while this has been disputed (D. Walker, personal communication), it leaves open the possibility that under normal conditions, when the amount of enzyme active sites probably exceeds the substrate, ribulose-$P_2$ is not free in solution long enough to be broken down or epimerized. It may be bound immediately to the enzyme.

# CHAPTER IV

# INHIBITOR STUDIES

## Introduction

Frequently, when a new enzyme activity is discovered, several compounds are assayed for their efficacy as substrates to determine the specificity of the enzyme. Ribulose-$P_2$ is the only known substrate for the carboxylase/oxygenase (130). A knowledge of substrate specificity often allows one to make certain assumptions about what parts of the substrate are most important for its binding to the active site of the enzyme, but when only one substrate is known, these assumptions cannot be made. One route for determining the requirements for substrate binding is through the use of competitive inhibitors which closely resemble the substrate. The analogue of the proposed reaction intermediate (III in Figure 1), 2-carboxyribitol-$P_2$, has been known for many years to be a potent inhibitor (68,69). Recently it has been shown that the epimer of carboxyribitol-$P_2$, 2-carboxyarabinitol-$P_2$, is an even better inhibitor (74,75). This probably means that carboxyarabinitol-$P_2$ is more like the intermediate. Xylitol-$P_2$ (83) has been reported to be a good inhibitor and arabinitol-$P_2$ has also been found to be inhibitory (F. Ryan, personal communication, 1975; J. Pierce, personal communication, 1979). Ribitol-$P_2$ has been reported to be not inhibitory (83), but this has not been confirmed with a different preparation of ribitol-$P_2$. The report on xylitol-$P_2$ claimed that the pattern of inhibition was noncompetitive with respect to ribulose-$P_2$ (83). This chapter reports the results of

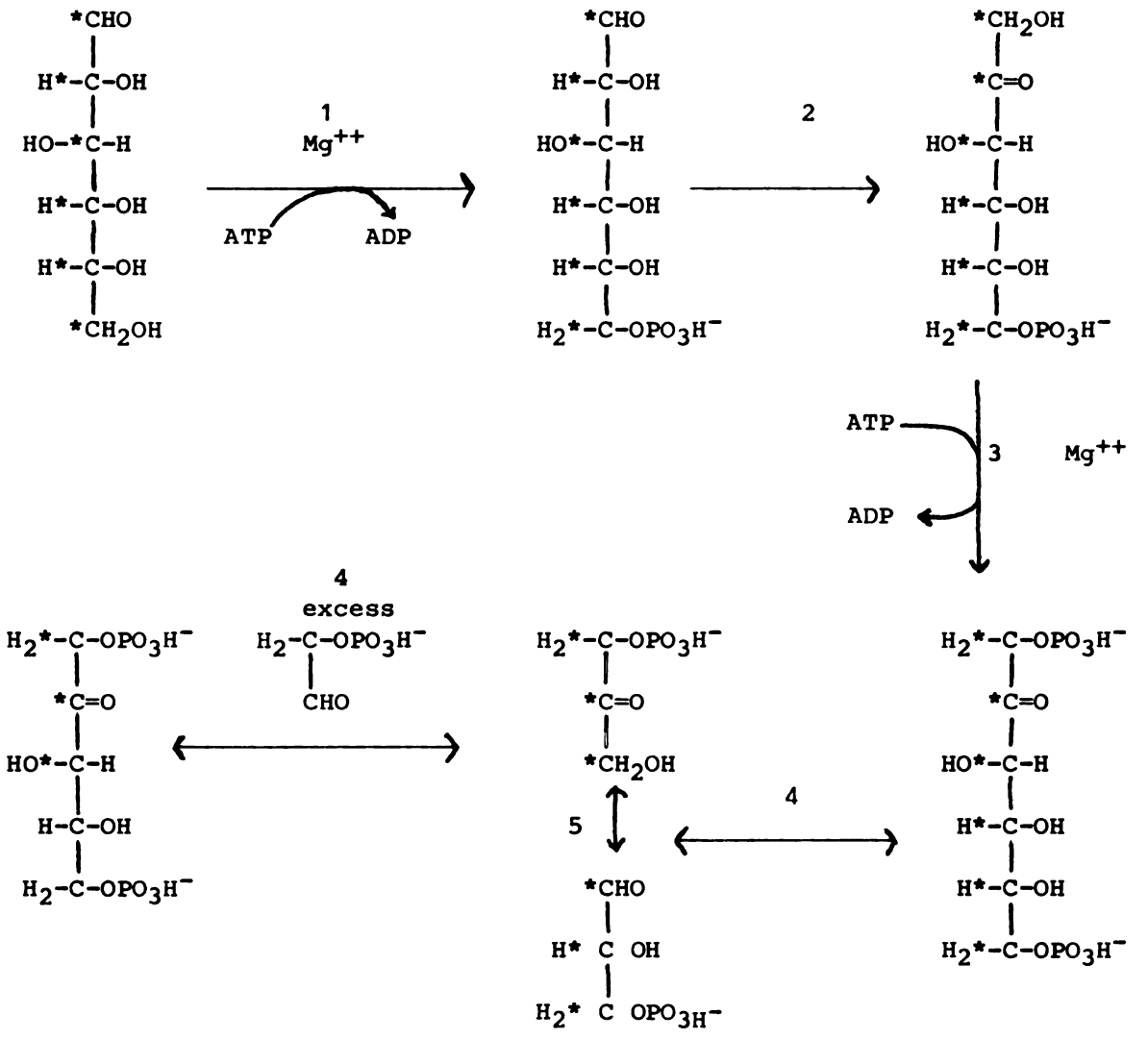
experiments with xylitol-$P_2$ and xylulose-$P_2$ and presents data in support of the hypothesis that both of these compounds are competitive, tight-binding, non-covalent inhibitors of ribulose-$P_2$ carboxylase/ oxygenase.

## Materials

The $^3$H-xylitol-$P_2$ was synthesized for us in the laboratory of R. Barker according to the method of Hartman and Barker (142). The xylulose-$P_2$ was synthesized according to the method of Byrne and Lardy (143), as described previously (84). The enzyme and substrate were prepared in the manner described in Chapter I. The assays were performed in the manner described in Chapter I. Amersham was the source of (U-$^{14}$C) α-D-glucose. Glycolaldehyde phosphate and dihydroxyacetone phosphate were from Calbiochem. The compounds, 2-pyridinecarboxaldehyde, 3-pyridinecarboxaldehyde, 4-pyridinecarboxaldehyde, and carboxymethoxylamine hemihydrochloride were all obtained from Aldrich. Iminodiacetic acid was from Sigma. The 2-phosphate and the 2-sulfate derivatives of ascorbic acid were gifts from B. Tolbert (U. of Colorado).

## Methods

$^{12}$C-Xylulose-$P_2$ was prepared from the condensation of glycolaldehyde-P and dihydroxyacetone phosphate with aldolase (84). Xylulose-$P_2$ was prepared with a $^{14}$C label (at C-1, C-2, and C-3) from U-$^{14}$C-α-D-glucose, according to the scheme in Figure 6. The radioactive products were purified by ion exchange chromatography on a Dowex-1 $Cl^-$ column (2.1 x 50 cm), and eluted with a series of HCl concentrations. The elution profile is shown in Figure 7. In order to get a satisfactory



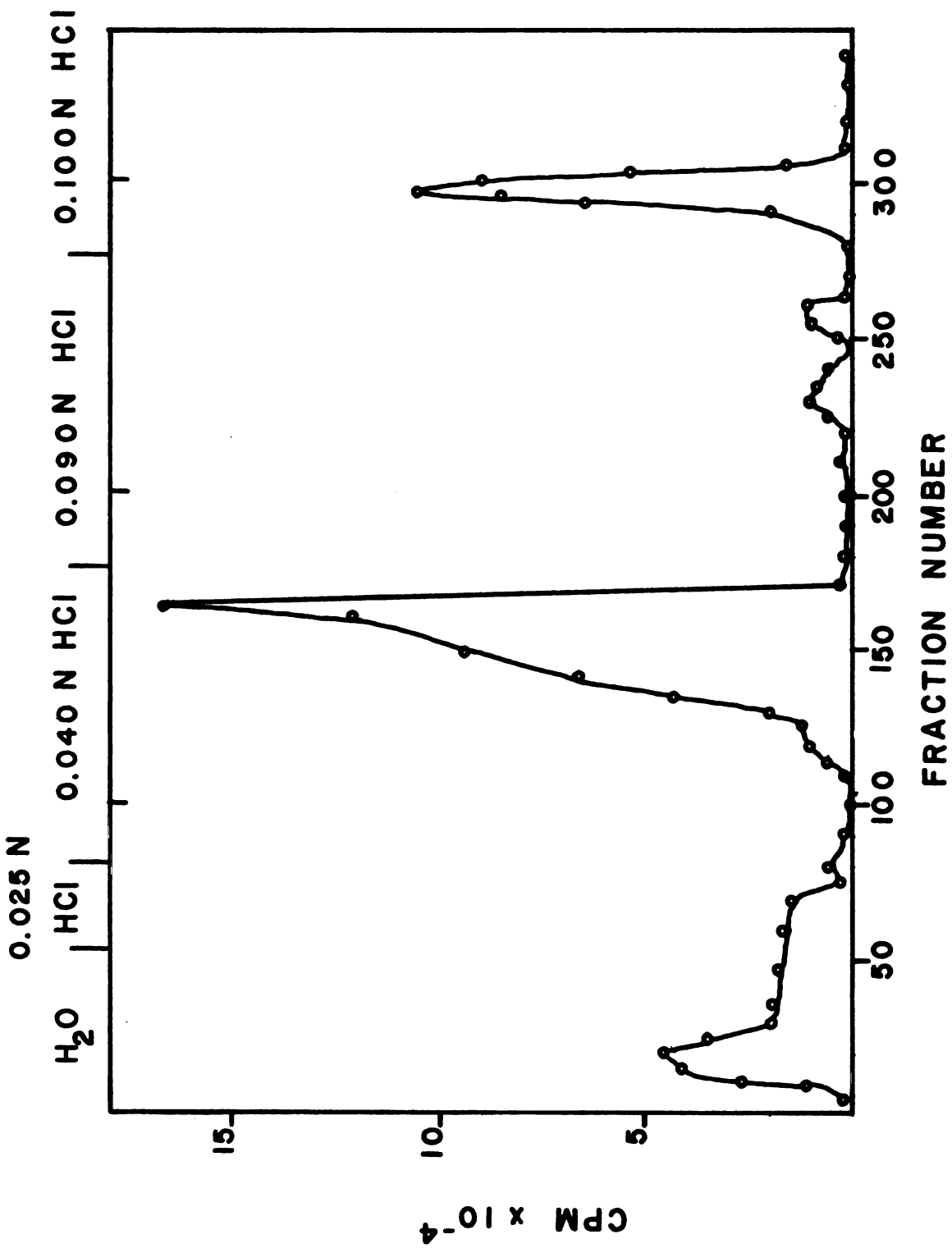

$$ADP + ADP \xrightarrow[Mg^{++}]{6} ATP + AMP$$

Figure 6. Reaction scheme for the synthesis of $^{14}C$-xylulose-$P_2$ from $^{14}C$-U-α-D-glucose. The enzymes were:

1) Hexokinase
2) Phosphoglucoisomerase
3) Phosphofructokinase
4) Aldolase
5) Triose phosphate isomerase
6) Myokinase

*denotes $^{14}C$

Figure 7. Elution profile of $^{14}C$-xylulose-$P_2$ from a Dowex-1 Cl column. Increasing concentrations of HCl were used to elute the labeled compounds as indicated in the figure. Fractions 280-310 were pooled and lyophilized for use in the binding experiments described in the text.

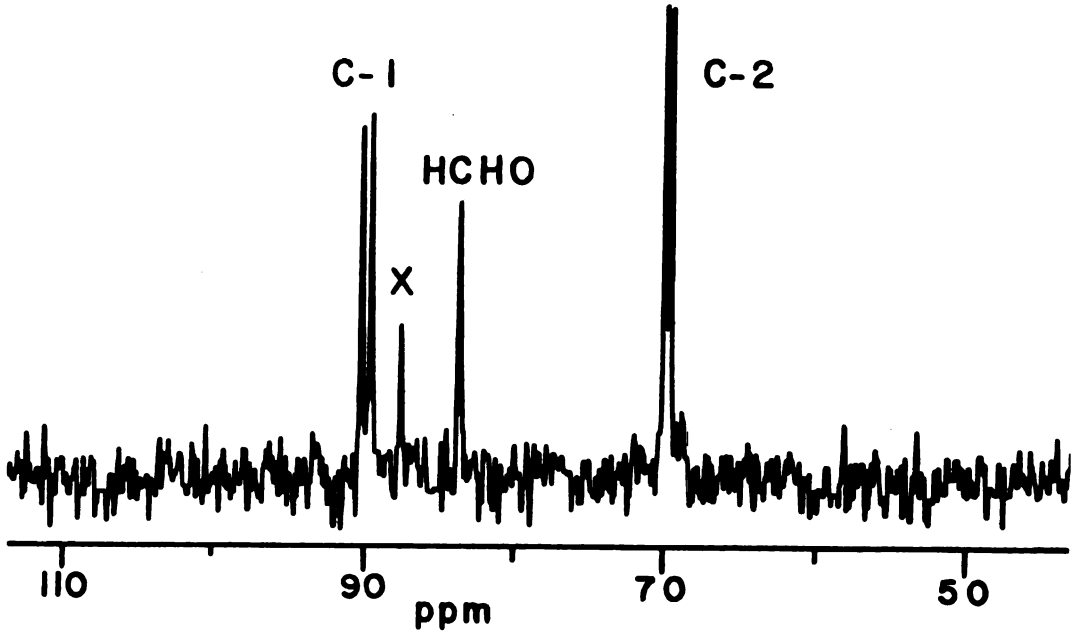

H2O
0.025 N HCl
0.040 N HCl
0.090 N HCl
0.100 N HCl
CPM x 10^-4
15
10
5
50
100
150
200
250
300
FRACTION NUMBER

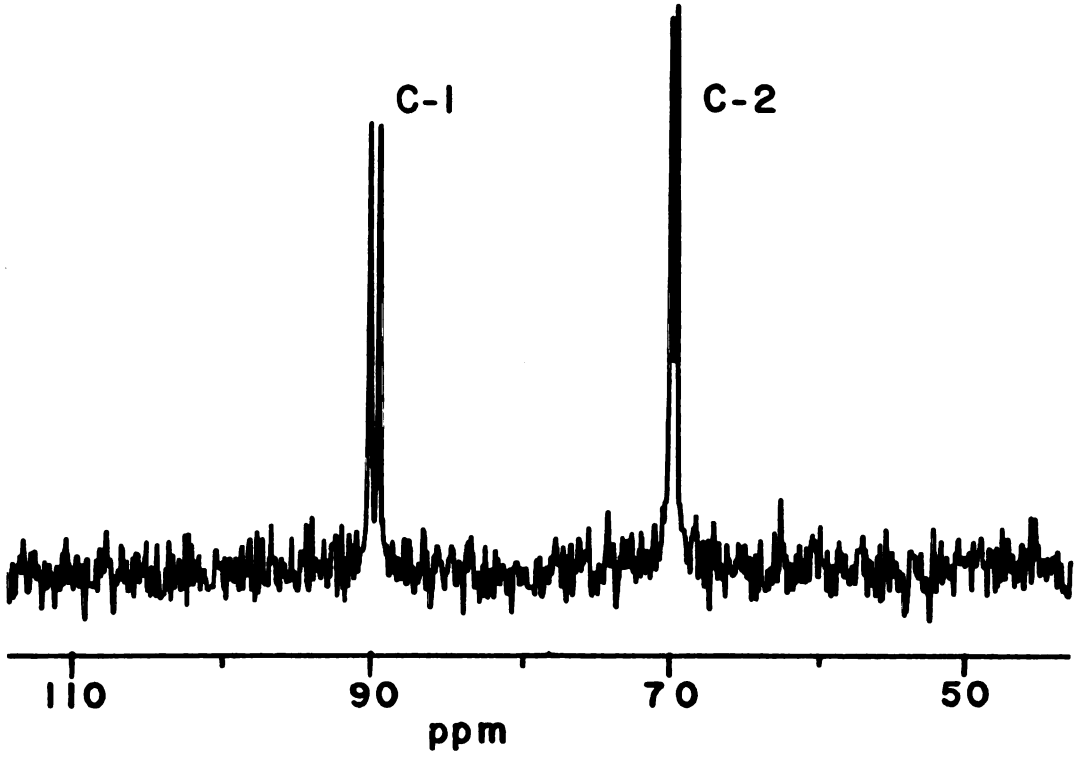
yield of $^{14}$C-xylulose-$P_2$, it was necessary to drive the reaction with a large excess of glycolaldehyde-P. Because of this, the synthesis was performed with no dilution of the initial specific activity. While this created the problem of having a very small amount of material to purify (about 1 μMol) it could not be avoided. This synthesis resulted in about 200 nMol of xylulose-$P_2$ with a specific activity of $3.73 \times 10^5$ dpm/nMol. This was diluted with unlabeled xylulose-$P_2$ to a specific activity of $2 \times 10^3$ dpm/nMol before use.

Because of the large amount of glycolaldehyde-P needed for this synthesis, and its cost if purchased, the glycolaldehyde-P was synthesized. The method of synthesis was a cleavage of α-glycerophosphate (DL mix) with a 1.2 fold excess of $NaIO_4$. This reaction was run at pH 5 and followed on a pH meter. The sample was cooled and the $NaIO_3$ was filtered off. A small amount of $Ba(CH_3COO)_2$ was added to scavenge any remaining iodate. This solution was filtered and the filtrate extracted overnight with ether in a continuous liquid-liquid extraction apparatus at 4°. The sample was concentrated and the $^{13}$C-NMR spectrum determined. The spectrum revealed glycolaldehyde-P with two contaminants, HCHO and an unknown compound (Figure 8a). These contaminants were removed with a DEAE-acetate (Whatman DE-52) column (2 x 50 cm) with a linear gradient of 0.05-0.80 M $NaCH_3COO$ at pH 4.5. The $^{13}$C-NMR spectrum of the product was determined, and is shown in Figure 8b. These techniques were modified from the procedure of Serianni and coworkers (144) with the advice of J. Pierce and A. Serianni. Glycolaldehyde-P from this preparation was used for synthesis of $^{12}$C-xylulose-$P_2$. Storage of the concentrated glycolaldehyde-P at pH 6 resulted in a yellow color which increased with freezing and thawing.

Figure 8. a) The proton decoupled 15.08 MHz $^{13}$C-NMR spectrum of glycolaldehyde-P revealing contamination by formaldehyde and an unknown compound (x). The spectrum wa determined at acidic pH.

b) $^{13}$C-NMR spectrum of glycolaldehyde-P after further purification by chromatography on DEAE-acetate. The conditions were the same as in a).


C-1
C-2
HCHO
X
110
90
70
50
ppm


C-1
C-2
110
90
70
50
ppm

Additional glycolaldehyde-P used in this work was supplied by J. Pierce.

The binding studies were done with a 0.7 x 60 cm column of Sephadex G-25 (fine). The column buffer was 100 mM bicine, 1 mM DTT, and 20 mM $MgCl_2$. The columns were run at room temperature.

Xylulose-$P_2$ and Xylitol-$P_2$: Discussion of Results

The conclusion of Ryan and coworkers (83) that the inhibition due to xylitol-$P_2$ was noncompetitive was based on data obtained after the enzyme and inhibitor had been incubated together for a period of time before initiation of the reaction. A very tight-binding inhibitor will frequently appear noncompetitive, even if it binds to the catalytic site (145). This is because such an inhibitor essentially removes enzyme from the reaction mix, i.e. its binding constant to the enzyme is so low that the population of the enzyme to which the substrate can bind is severely reduced. Xylitol-$P_2$ requires 20 min to reach full inhibition (83), and because of this, the substrate cannot reach equilibrium with the enzyme during a 1 min assay. When xylitol-$P_2$ and ribulose-$P_2$ were presented to the enzyme simultaneously the pattern of inhibition was competitive (Figure 9). When the procedure of Ryan et al (83) was repeated, i.e. a 20 min incubation of the enzyme with xylitol-$P_2$ the pattern of inhibition was noncompetitive (Figure 10). I have found similar results with xylulose-$P_2$ (84, Appendix III).

An attempt was made to determine a binding constant for xylitol-$P_2$ using a Paulus equilibrium dialysis device (147). These experiments were not successful due to the long times required for the diphosphate to pass through the membrane. If xylitol-$P_2$ is really

Figure 9. Inhibition pattern of ribulose-$P_2$ carboxylase when xylitol-$P_2$ and ribulose-$P_2$ were added to the enzyme simultaneously. The pattern of inhibition is shown to be competitive. A) 40 μM xylitol-$P_2$; B) 20 μM xylitol-$P_2$; C) 10 μM xylitol-$P_2$; D) 5 μM xylitol-$P_2$; E) no xylitol-$P_2$. V equals nMol $^{14}C$-$CO_2$ fixed/min/assay.

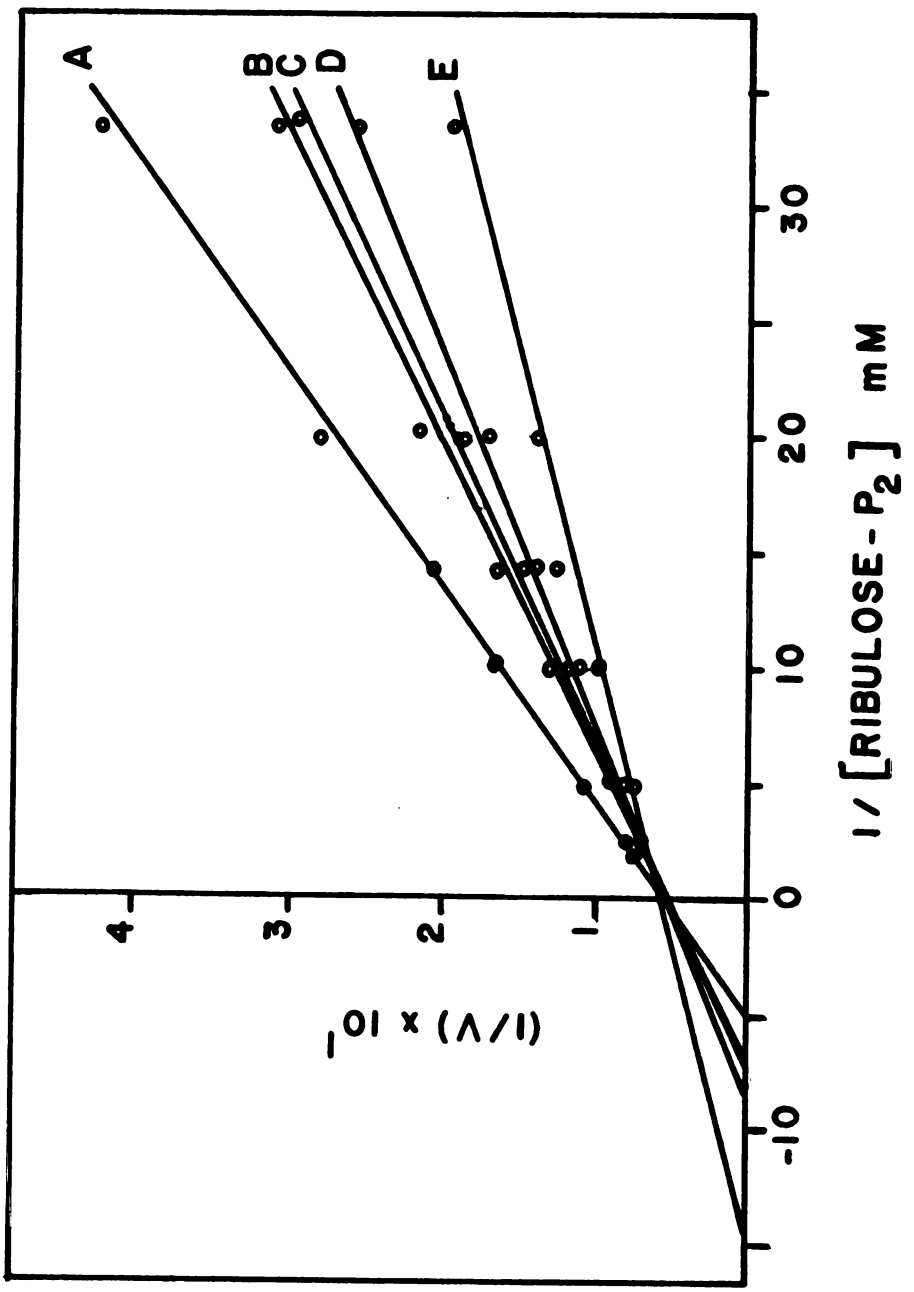
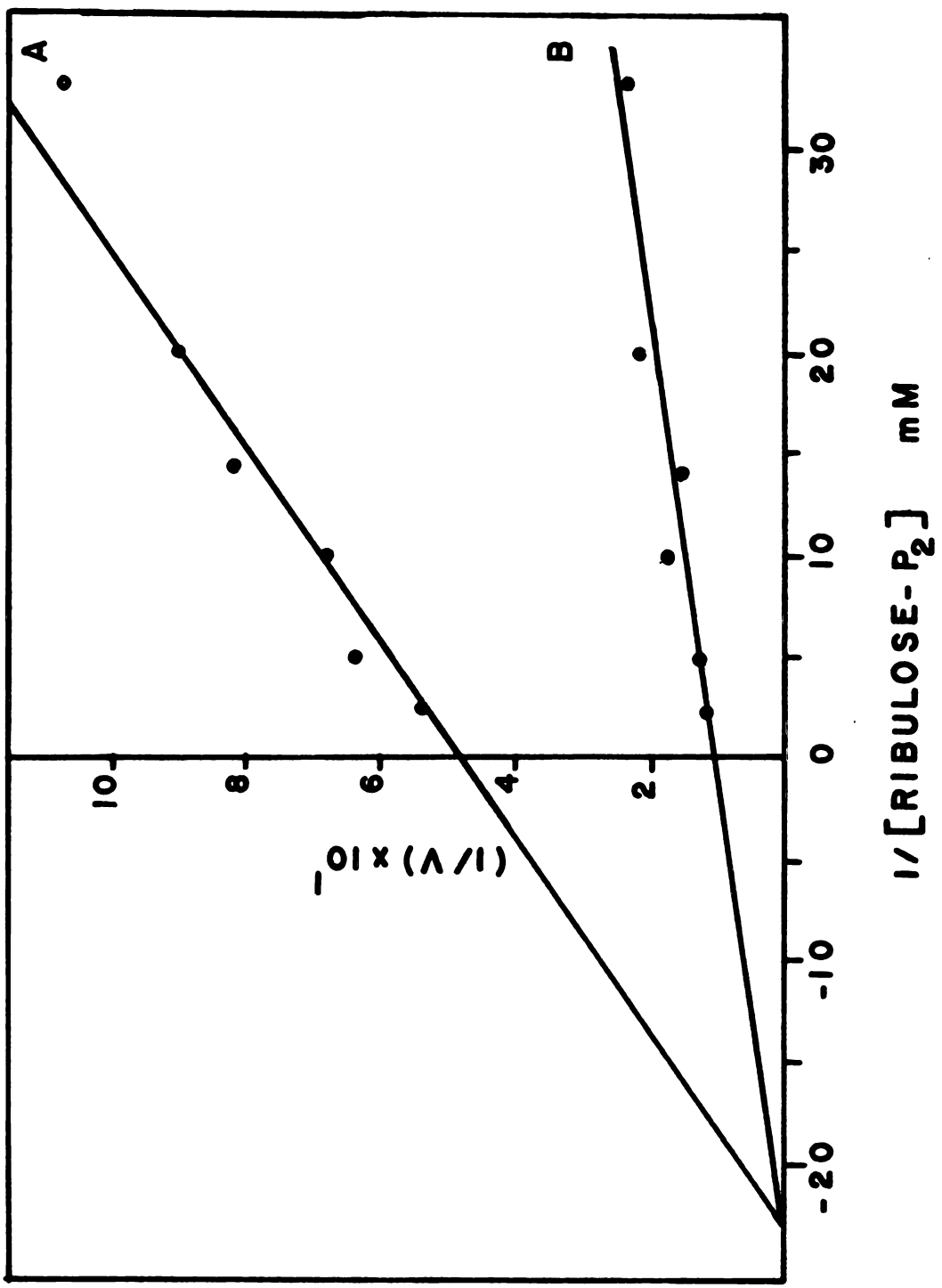

A
B
C
D
E
4
3
2
1
0
-10
10
20
30
(1/V) x 10
1/[RIBULOSE-P2] mM

Figure 10. Inhibition pattern of ribulose-$P_2$ carboxylase when the enzyme and xylitol-$P_2$ were incubated together for 20 min prior to initiation of the reaction with ribulose-$P_2$. A) 10 µM xylitol-$P_2$; B) no xylitol-$P_2$. V equals nMol $^{14}C$-$CO_2$ fixed/min/assay.

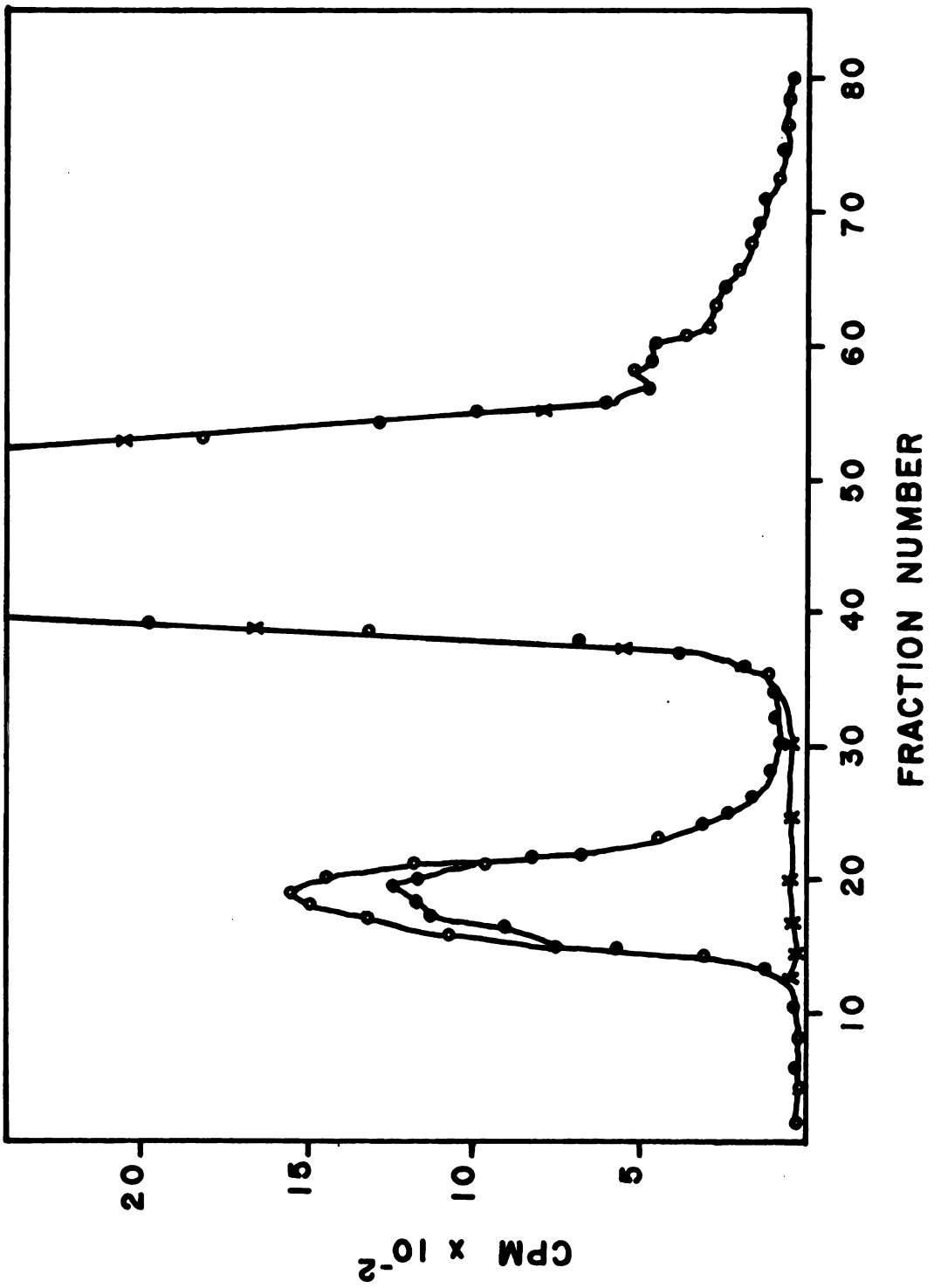

A
B
10
8
6
4
2
0
-10
-20
10
20
30
(1/V) x 10
1/[RIBULOSE-P2] mM

competitive, then preincubation of the enzyme with ribulose-$P_2$ should prevent xylitol-$P_2$ binding, at least partially. To test this hypothesis, ribulose-$P_2$ was incubated with carboxylase, in the absence of $Mg^{++}$, for 20 min and then incubated with $^3H$-xylitol-$P_2$ for an additional 20 min. This sample was chromatographed through Sephadex G-25 (Figure 11) to separate bound from unbound xylitol-$P_2$. The difference between the sample with and without ribulose-$P_2$ was quite small, suggesting that the binding constant for xylitol-$P_2$ is somewhat lower than the 1 μM suggested for ribulose-$P_2$ (148). Incubation of the enzyme with iodoacetamide (IAA) completely prevented the binding of xylitol-$P_2$ (Figure 11). Treatment of the enzyme with IAA also prevents the binding of pyridoxal-P (110).

Both xylulose-$P_2$ and xylitol-$P_2$ are potent inhibitors of the carboxylase. When the xylulose-$P_2$ concentration was 0.56 μM and the enzyme was 0.15 μM (1.12 μM in sites) the enzyme was 50% inhibited (84). The carboxylase specific activity was 0.5 μMol/min/mg protein. While most investigators are working with a pure protein, there is a wide variety in the specific activities. The highest specific activity ever seen in this laboratory was 2.8 μMol/min/mg protein for the enzyme purified from spinach leaves, this was an isolated case, and the average is 1.0-2.0 μMol/min/mg protein. Lorimer (personal communication) has found a specific activity of 2.9 in one isolated case, also from spinach. The specific activity values for the enzyme prepared from other organisms are even more variable. The relationship between the specific activity of the enzyme and its ability to bind either xylitol-$P_2$ or xylulose-$P_2$ remains to be elucidated.

Since both xylitol-$P_2$ and xylulose-$P_2$ bind so tightly to the enzyme,

Figure 11. Column profile from Sephadex G-25 (fine) showing complete separation of bound and unbound $^{3}H$-xylitol-$P_2$. The protein, as determined either by absorbance at 280 nm or by assay, coincided with the first peak (bound $^{3}H$-xylitol-$P_2$). The enzyme at 3 mg/ml was incubated with $^{3}H$-xylitol-$P_2$ (400 μM) for 20 min. The specific activity of the $^{3}H$-xylitol-$P_2$ was 575 cpm/nm. The enzyme and xylitol-$P_2$ were incubated together for 20 min and then chromatographed (open circles). In a second experiment the enzyme was incubated with ribulose-$P_2$ for 20 min before the introduction of the xylitol-$P_2$ (closed circles). In a third experiment the enzyme was incubated with iodoacetamide for 20 min prior to the addition of xylitol-$P_2$ (X-X). In each case 2 ml was loaded on the column and the fraction size was 0.3 ml, and 12 ml of void volume was collected before the fraction collector was started.


CPM x 10⁻²
20
15
10
5
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20
30
40
50
60
70
80
FRACTION NUMBER

the possibility that they were attached covalently was considered. To test this hypothesis, an experiment was run to determine whether sodium dodecyl sulfate (SDS) would release the inhibitor from the enzyme. In one case the carboxylase was incubated with $^{3}H$-xylitol-$P_2$ and the bound and unbound portions were separated with the Sephadex G-25 column as before. The fractions with the protein-xylitol-$P_2$ complex were pooled and made 1% SDS, dipped into boiling water for 2-3 min and rerun on the Sephadex G-25 column (with 1% SDS added to the buffer). The results are shown in Figure 12. Since the SDS treatment resulted in the complete removal of $^{3}H$ from the protein fraction it was concluded that xylitol-$P_2$ binding was not covalent. A similar experiment was conducted with xylulose-$P_2$. In this case $^{14}C$-xylulose-$P_2$ was incubated with three different samples of activated enzyme. The control sample was chromatographed as before. Both of the remaining samples were made 1% SDS, one sample was heated at 65° for 2 hr, and the remaining sample was placed in a boiling water bath for 2 min. Since the label did not remain bound to the protein that had been boiled (Figure 13), it was concluded that the interaction of xylulose-$P_2$ with the enzyme was not covalent. The fact that the sample which had been heated to 65° in 1% SDS for 2 hr still bound significant xylulose-$P_2$ attests to the remarkable heat stability of this enzyme. It is not known whether this bound portion represents $A_8B_8$ enzyme or individual large subunits. It should be possible to isolate large subunits and determine whether they will bind either of these bisphosphates. While the isolated large subunit is insoluble in the presence of $Mg^{++}$ (35), $Mg^{++}$ is not required for xylitol-$P_2$ binding (McCurry, unpublished), and therefore is probably not required for the binding of xylulose-$P_2$.

Figure 12. Column profile from Sephadex G-25 (fine) showing separation of bound and unbound $^{3}$H-xylitol-$P_2$ with and without SDS. Enzyme at 10 mg/ml was incubated with 2 mM $^{3}$Hxylitol-$P_2$ for 20 min and added to a column (open circles). The $^{3}$H-xylitol-$P_2$ specific activity and column conditions were the same as in Figure 11. Fractions 18-25 were pooled, made 1% SDS, and boiled for 2 min. One half of the boiled pool was added to the column and run again (closed circles). The column buffer in the second case was made 1% SDS before it was run.

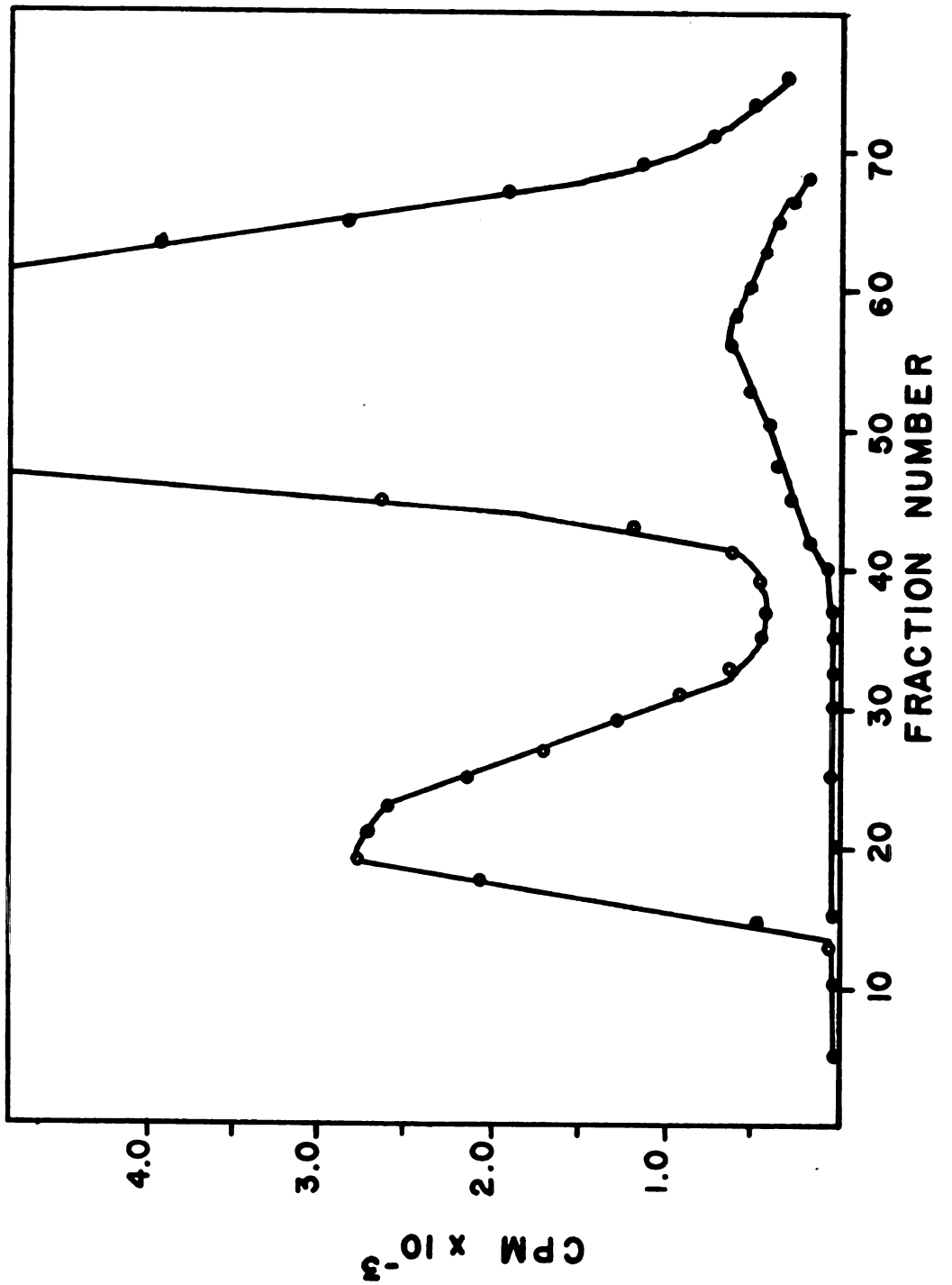
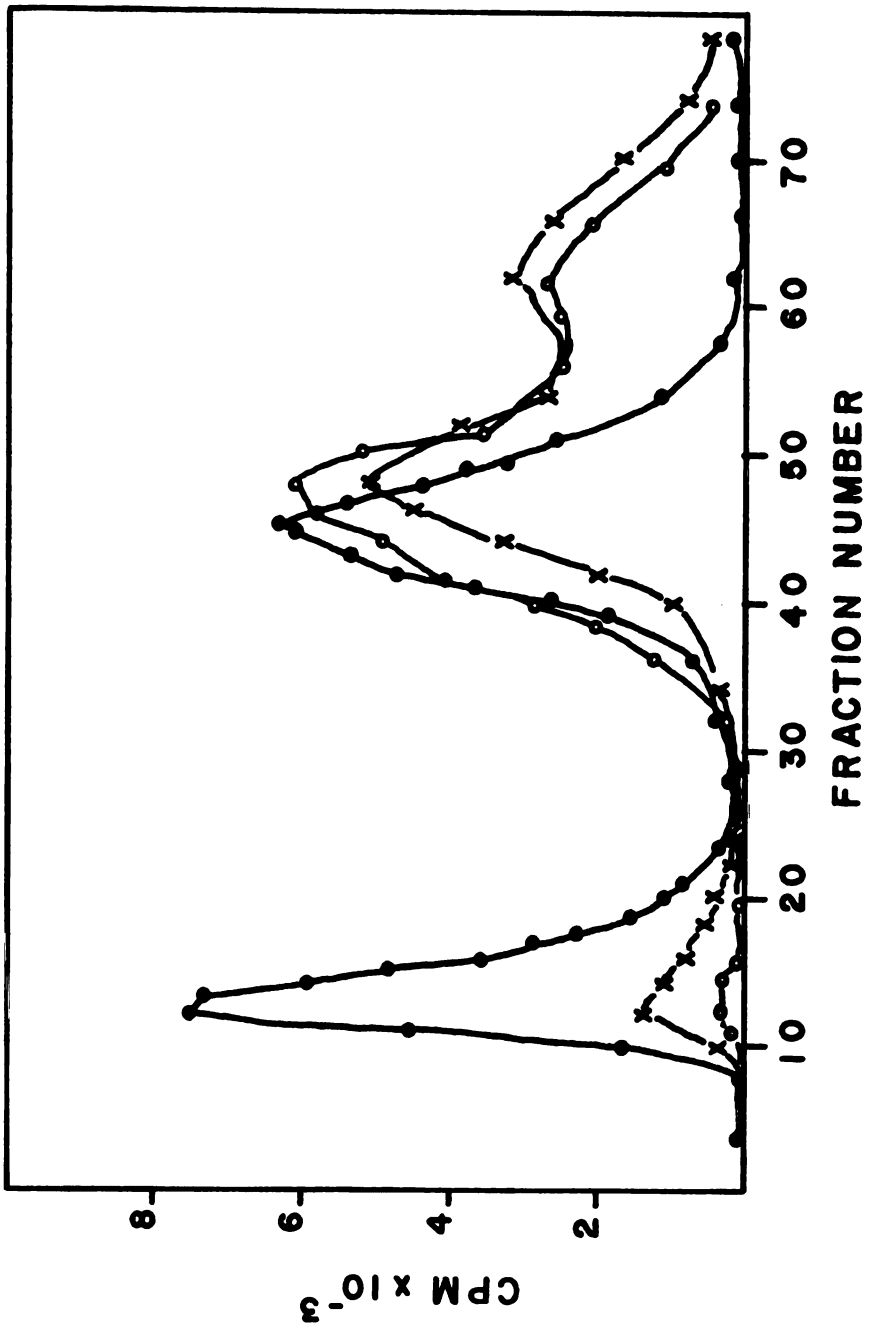

CPM x 10^-3
4.0
3.0
2.0
1.0
10
20
30
40
50
60
70
FRACTION NUMBER

Figure 13. Column profile from Sephadex G-25 (fine) showing separation of bound and unbound $^{14}$C-xylulose-$P_2$ to the carboxylase in the presence and absence of SDS. $^{14}$C-xylulose-$P_2$ was incubated with activated enzyme and chromatographed on Sephadex G-25 (closed circles). Another sample of this mixture was made 1% SDS and either incubated at 65° for 2 hr (X-X) or boiled for 2 min (open circles) before chromatography. The incubation conditions were 3 mg protein/ml and 50 µM xylulose-$P_2$. The specific activity of the $^{14}$C-xylulose-$P_2$ was 3900 cpm/nM. One ml of sample was applied, otherwise the column conditions were as in Figure 11.


CPM x 10^-3
2
4
6
8
10
20
30
40
50
60
70
FRACTION NUMBER

While xylitol-$P_2$ was expected to be an inhibitor, it was surprising that the degree of inhibition was so large. Ryan and coworkers (83) postulated that the reason for the inhibition, besides the size and charge, was due to the configuration at C-3. They pointed out in this regard that xylitol-$P_2$ is structurally similar to 6-P-gluconate. The even greater inhibition by xylulose-$P_2$ follows this hypothesis since the keto- group at C-2 makes it more similar to the substrate, ribulose-$P_2$. We have suggested that the effect of the stereochemistry at C-3 could be hydrogen bond formation with a basic group in the active site (84,100), but this suggestion has not yet been proven.

Discussion of Other Inhibitors

Because of the known inhibitory effect of isonicotinyl hydrazide and α-hydroxy-2-pyridine methane sulfonic acid (148,149) on photorespiration, pyridine derivatives, substituted variously at C-2, C-3, and C-4 with formaldehyde, were tried as inhibitors of ribulose-$P_2$ carboxylase. The concentration employed was 1 mM. When the reaction was initiated with activated enzyme (untreated by inhibitor), the 2 substitued pyridine inhibited the carboxylase 11%, and the 3 and 4 derivatives were not effective. Preincubation of the enzyme with the compounds for 11 min before initiation of the reaction was slightly more effective in that the 4 derivative give 25% inibition, the 3 derivative gave 11%, and the 2 derivative gave 19%. Since these compounds were such poor inhibitors at the high (1mM) concentration, their inhibition was not investigated further. Similarly, carboxymethoxylamine hemihydrochloride ((aminoxy) acetic acid) produced no significant inhibition of ribulose-$P_2$ carboxylase/oxygenase.

During the washing of a Dowex or Chelex resin, compounds will often leach out of the resin. These compounds may be colored, or they may be colorless. Inhibition of enzymes have been reported for some of these compounds (150). In two cases during the work of this thesis an unplanned inhibition of the enzyme occured which could be traced to either Dowex or Chelex. In the Dowex case the resin had been improperly washed and the resulting inhibitory mixture was highly colored (orange). In the case with Chelex, a bicine buffer was passed over the resin for removal of trace metals. The buffer was allowed to stay in the column overnight before the column was run the next day. The effluent was colorless and the pH was the same as the buffer flowing onto the column. This effluent was quite inhibitory and had a slight odor. Iminodiacteic acid is a constituent of Chelex resins, and some amount of it leaches out (151) during washing. So iminodiacetic acid was tried as an inhibitor of the carboxylase. Concentrations up to 1 mM proved to have no effect on the carboxylase activity. Since these inhibitory effects could be avoided by a more careful laboratory procedure, iminodiacetic acid and other effluent products were not investigated further.

Due to its size similarity to ribulose-$P_2$, ascorbic acid was tried as an inhibitor of both carboxylase and oxygenase activities. Very high concentrations of ascorbic acid (20 mM) resulted in up to 40% inhibition of both activities with no differential regulation. The C-2 phosphate and C-2 sulfate derivatives of ascorbic acid were given to us by B. Tolbert. At concentrations of 1 mM the sulfate derivative inhibited the carboxylase 18% and the phosphate derivative inhibited 41%. In these experiments the inhibitor was preincubated with the enzyme for 20 min before initiation of the reaction. Essentially the same results were obtained for the oxygenase.

# CHAPTER V

# ACTIVATION STUDIES

## Introduction

Normally ribulose-$P_2$ carboxylase/oxygenase is activated by incubation with 10-20 mM $NaHCO_3$ and 20 mM $MgCl_2$ for at least 10 min at 30°. For most purposes, this is an acceptable method. However, the consistant required presence of $CO_2$ with the enzyme means that some experiments are difficult or impossible to perform. Similarly it has not been possible to distinguish between the effect of $CO_2$ binding at the activator site from the effect of $CO_2$ binding at the catalytic site. Because of the requirement of $Mg^{++}$ for activation the role of $Mg^{++}$ in catalysis cannot be determined. As a result of these problems, attempts were made to activate the enzyme irreversibly. This chapter reports the unsuccessful efforts to activate the carboxylase by unconventional means (other than with $CO_2$).

## Materials

Diazald, diallyltartardiimide, $NdCl_3$, O-methylisourea hydrogen sulfide, and the diazomethane generating kit were purchased from Aldrich. Sigma supplied the 2,3-diphosphoglycerate.

## Methods

Diazomethane ($CH_2N_2$) was prepared according to the manner explained in the instructions from Aldrich which came with their $CH_2N_2$ generating

kit (product no. Z10,025-0). $CH_2N_2$ can be generated from several different starting compounds. Diazald was used for this work since it is relatively non-toxic and stable. The procedure involved dissolving the Diazald in ether (all ether used in this synthesis was first made peroxide-free by washing with an acidic $FeSO_4$ solution) and slowly dripped into a distilling flask containing a basic alcohol solution heated to 65°. The flask was connected to a condenser with two receiving flasks in series kept below 0°. The distillation was carried out in an efficient hood, behind an explosion shield. Safety goggles and heavy rubber gloves were worn for all work around the distillation apparatus and while handling the $CH_2N_2$. In each experiment, the $CH_2N_2$ was generated immediately before use. Since $CH_2N_2$ is very volatile and it explodes when it crystallizes, the glassware was checked for scratches before each use and under no circumstances was ground glass used for any of the containers. Excess $CH_2N_2$ was disposed of by reaction with acetic acid and the product was discarded.

Gel electrophoresis was performed in glass tubes (0.5 x 13 cm) with a Tris/glycine buffer, pH 8.7 in 8 M urea with 7.5% acrylamide, essentially according to Ornstein (152) and Davis (153). SDS electrophoresis was performed at 0.1% SDS and 7.5% acrylamide essentially according to Weber and Osborn (154). In certain experiments the N,N'-methylenebisacrylamide was repalced with N,N'-diallyltartardiimide so that the gel could be solubilized (155). A gel slicing apparatus was fashioned from razor blades and 10 cm bolts, this produced slices of uniform thickness (approximately 3 mm).

## Discussion of Results with Diazomethane

Before the realization that two molecules of $CO_2$ were probably involved in the mechanism of the carboxylase, data was reported suggesting that $CH_2N_2$ could be used to covalently attach $^{14}CO_2$ to the active site of this enzyme (156). I began this project with the idea of obtaining a peptide with $^{14}CO_2$ attached covalently to the active site. The initial experiment was to incubate the enzyme with $NaH^{14}CO_3$ for 30 min in the presence of $Mg^{++}$ and then add an excess of $CH_2N_2$. The result was to be run on 8 M urea gels to determine whether there was any specificity to the $^{14}CO_2$ binding. Unfortunately treatment of this enzyme with $CH_2N_2$ in ether resulted in total denaturation of the protein. This protein could not be dissolved in 8 M urea. Working on the assumption that some of the protein did go into solution, the urea gels were run, but no protein was detectable on them and the $^{14}C$, as determined by liquid scintillation counting, was in the range of background. In the next experiment, the initial set up was the same, but the sample was resuspended in 1% SDS and boiled for 10 min. This sample was then run on 0.1% SDS gels. The gels were sliced, each slice was minced with forceps and counted. The scintillation cocktail described in Chapter I was modified by the addition of 5% Cabosil to aid in suspending the gel particles. In later experiments, diallytartardiimide was substituted for methylene bisacrylamide in the gels, to give gels which were soluble in 2% periodic acid. The $^{14}C$ label was incorporated into both the large and small subunits. Frequently a significant amount of label was incorporated into a high molecular weight species which barely moved into the gels. This species was assumed to be denatured protein which had not been resolubilized by the SDS treatment. The amount and

position of incorporated label was very unpredictable. Both carboxyribitol-$P_2$ (a gift from M.D. Lane) and xylitol-$P_2$ were used in effort to demonstrate exclusion of $^{14}CO_2$ using the same procedure. The enzyme was incubated with the inhibitors before the addition of the $NaH^{14}CO_3$. From the data, it appeared that carboxyribitol-$P_2$ prevented some $CO_2$ binding, but the results were very irreproducable.

Due to the irreproducibility of the $CH_2N_2$ experiments they were dropped. It was felt that if the diazomethane could be made and mixed with the enzyme in a solvent other than ether, i.e. one which did not cause such a degree of disorder in the protein, this line of research might be made to work. Recently, Lorimer working in conjunction with Miziorko, has succeeded in such an experiment (Lorimer, personal communication). Their procedure was to incubate enzyme, which had been activated with $Mg^{++}$ and $NaH^{14}CO_3$, with carboxyribitol-$P_2$ to lock $^{14}CO_2$ into the activating site. The enzyme was then separated from the unbound $^{14}CO_2$ on a Sephadex column in the method of Miziorko (73). This sample was then treated with $CH_2N_2$ which had been prepared with Methyl Cellosolve (2-methoxyethanol) as the solvent. Methyl Cellosolve is miscible with water. After protease treatment of the labeled enzyme they isolated a single labeled peptide with a label on a lysyl residue. The sequence of the peptide will be determined for comparison with the peptides from Hartman's group (121), and the peptide of Spellman and coworkers (111,112). It is anticipated that this will be direct evidence for the involvement of a lysine group in the activation process.

Diazomethane is much too severe a treatment for covalent attachment of $CO_2$ with the retention of activity. Perhaps a better alkylating agent will be found.

Results with O-Methylisourea

Sheen (157) reported that treatment of ribulose-$P_2$ carboxylase/oxygenase from tobacco with 10 mM O-methylisourea resulted in only 10% inhibition of the enzyme activity. This compound reacts with lysyl residues and forms homoarginine (123). Because of the known inhibition of the enzyme when lysyl residues were modified with pyridoxal-P (108,110,113, 114) it was surprising that the inhibition from the O-methylisourea treatment was not greater. This suggested two different possibilities: a) the conversion of critical lysines to homoarginines was not toxic for activity; or b) inadequate modification occurred. If modification occurred, but was not toxic, then perhaps the enzyme had been irreversibly activated. Since it was not clear from Sheen's report (157) how the experiments were done, several conditions were attempted. The reaction is slow and modification is usually done at high pH (10-11) and at a low temperature (123). Initially, modification of ribulose-$P_2$ carboxylase was attempted at pH 8.5, 9.5, and 10.5, for varying times at 30° or 0°. The results from such an experiment are shown in Table 4. A high concentration of O-methylisourea (0.4m) was used because the buffer system was bicine-ammediol (2-amino-2methyl-1,3-propanodiol) which was expected to react with the excess O-methylisourea. The results of this experiment indicate that while both the elevated pH and the O-mehylisourea inhibited the enzyme to some extent, the treated enzyme still had the same requirements for activation as the untreated control enzyme. In addition, this compound did not remove the effect of 6-P-gluconate as an activator.

Since incubation at pH 10.5, even without the modifying agent,

Table 4

Modification of Ribulose-$P_2$ Carboxylase with O-Methylisourea at Different pH's

| Incubation pH | Activation Conditions | | | | | |
|---|---|---|---|---|---|---|
| | A | | | | B | |
| | 20 mM $MgCl_2$ | ——— | $MgCl_2$ | ——— | 1 mM P-gluconate | ——— |
| | 10 mM $NaHCO_3$ | 10 mM $NaCHO_3$ | ——— | ——— | 20 mM $MgCl_2$ 1 mM $NaHCO_3$ | 20 mM $MgCl_2$ 1 mM $NaHCO_3$ |
| | cpm x $10^{-3}$ | | | | cpm x $10^{-3}$ | |
| 8.5 + | 7.0 ± 6% | 0.7 ± 0% | 2.4 ± 1% | 0.5 ± 2% | 107.6 ± 5% | 42.2 ± 3% |
| 8.5 - | 7.3 ± 9% | 0.8 ± 0% | 2.5 ± 8% | 0.6 ± 0% | 127.8 ± 3% | 42.2 ± 3% |
| 9.5 + | 5.2 ± 0% | 0.6 ± 1% | 2.0 ± 2% | 0.3 ± 3% | 96.0 ± 2% | 35.6 ± 4% |
| 9.5 - | 5.5 ± 1% | 0.7 ±12% | 2.0 ± 2% | 0.5 ± 4% | 114.5 ± 1% | 32.3 ± 1% |
| 0.5 + | 1.1 ± 0% | 0.2 ± 5% | 0.6 ± 8% | 0.2 ± 3% | 18.8 ± 3% | 8.6 ± 0% |
| 0.6 - | 0.8 ± 3% | 0.1 ± 9% | 0.4 ± 5% | 0.1 ±11% | 16.6 ± 2% | 4.9 ± 4% |

The enzyme samples were incubated with or without 0.4 M O-methylisourea at the indicated pH for 1 hour at 30°, followed by dialysis against three changes of 100 mM Bicine at pH 8.0 and then activated for 30 min as indicated. The values shown are means of 3 assays.

The assay conditions were 20 mM $MgCl_2$ and 10 mM $^{14}C$-$NaHCO_3$ (at a specific activity of 230 cpm/nMol) in A, and 20 nM $MgCl_2$ and 1 Mm $^{14}C$-$NaHCO_3$ (at a specific activity of 10,000 cpm/nMol) in B.

resulted in substantial inhibition, a long term modification was tried at pH 9.5. Enzyme was incubated in the presence and absence of O-methylisourea, and with and without substrate, ribulose-$P_2$, at pH 9.5 and 30° overnight (Table 5). From these data, it appears that incubation with ribulose-$P_2$ protected the enzyme from inactivation at the high pH, but ribulose-$P_2$ may have slightly increased the inhibition from the O-methylisourea. This may be due to exposure of more lysyl residues through a conformational change caused by the binding of the ribulose-$P_2$ at the catalytic site. Experiments with cross-linking reagents suggest that the binding of ribulose-$P_2$ causes the protein to "loosen" resulting in less cross-linking (42). Since the exploratory experiments indicated that the potential for o-methylisourea as an irreversible activator was small, this project was not pursued.

Results with Neodymium

While the requirement of the carboxylase for $Mg^{++}$ has been known for some time, a recent report demonstrated that $Mn^{++}$ could replace $Mg^{++}$ in the oxygenase assay, but not in the carboxylase assay (158). This result has been confirmed by N. Hall in Tolbert's lab (159). Hall found that the effect of the metal appeared to be in activation. It seems likely that $Mn^{++}$ would stabilize a carbamate as well or better than $Mg^{++}$. This data could be used to support the hypothesis that $Mg^{++}$ is required in the carboxylation reaction as well as for activation, but not in the oxygenase reaction, and that $Mn^{++}$ fails in this catalytic role. Because of the variation in the effects of various metals it was suggested (J.F. Morrison, personal communication) that an element of the lanthanide series might have an interesting effect on activation. Neodymium, in the

Table 5

Modification of Ribulose-$P_2$ Carboxylase with O-Methylisourea in the Presence of Ribulose-$P_2$

| | o-Methylisourea Treated | | | Untreated | |
|---|---|---|---|---|---|
| | + Ribulose-$P_2$ | ——— | cpm x $10^{-3}$ | + Ribulose-$P_2$ | ——— |
| A | 1.9 ± 9% | 5.6 ± 20% | | 12.3 ± 8% | 9.7 ± 13% |
| B | 0.5 ± 9% | 1.5 ± 7% | | 3.9 ± 9% | 3.2 ± 3% |

The treated samples were made 50 mM in O-methylisourea in 25 mM Bicine buffer and incubated overnight at pH 9.5 and 30°. The ribulose-$P_2$ concentration for incubation was 1 mM and without added $Mg^{++}$. The samples were dialyed overnight to remove excess O-methylisourea.

The numbers shown are cpm x $10^{-3}$, and are the means of 3 assays.

A was activated and assayed with 20 mM $MgCl_2$ at 10 mM $NaHCO_3$.

B was activated and assayed with 20 mM $MgCl_2$ at 1 mM $NaHCO_3$.

The specific activity of the $^{14}C$-$NaHCO_3$ was 230 cpm/nmol.

form of $NdCl_3$, was tried (Table 6). $Nd^{+3}$ did not replace $Mg^{+2}$ for catalysis or activation. $Nd^{+3}$ was inhibitory, probably by interfering with $Mg^{+2}$ binding. Similar results were obtained for the oxygenase reaction, i.e. $Nd^{+3}$ behaved differently than either $Mg^{++}$ or $Mn^{++}$.

Results with Phosphorylated Compounds

Hemoglobin is another protein which binds $CO_2$ as a carbamate (160). It also binds $O_2$ and sugar phosphates, and the sugar phosphate binding competitively regulates the $CO_2$ binding (160,161). The $CO_2$ and the phosphates bind at the N-terminus of the subunits (160). The best regulator for $CO_2$ binding to hemoglobin is 2,3-diphosphoglycerate. Because of carbamate formation in activation of ribulose-$P_2$ carboxylase (103), the known effect of sugar phosphates on activation of the carboxylase (97), and the high concentration of 2,3-diphosphoglycerate in soybean leaves (162), this compound was investigated for its effect on the activation of the carboxylase.

A representative sample of the data from the 2,3-diphosphoglycerate experiments is shown in Table 7. There is some small amount of stimulation at sub-optimal $CO_2$ concentrations and even the hint of stimulation at 10 mM $NaHCO_3$. While these results were reproducible, the standard deviations of replicate experiments were large enough to account for the difference. This compound is not an activator in the absence of added $CO_2$. Based on these data it is unlikely that 2,3-diphosphoglycerate plays a role in the in vivo activation of this enzyme.

Because of the fact that a variety of phosphorylated compounds,

Table 6

The Effect of Neodymium Chloride on Activation and Assay of Ribulose-$P_2$ Carboxylase

| Assay | Activation | | | |
|---|---|---|---|---|
| | $NdCl_3$ | $MgCl_2$ | * $NdCl_3$ + $MgCl_2$ | * $MgCl_2$ + $NdCl_3$ |
| | cpm x $10^{-3}$ | | | |
| $NdCl_3$ | 0.0 | ____ | _____ | _____ |
| $MgCl_2$ | 0.8 ± 2% | 24.1 ± 1% | 14.4 ± 3% | 22.6 ± 1% |

The concentrations of $MgCl_2$ and $NdCl_3$ were both 20 mM.

Each assay contained 360 µg/ml protein. The specific activity of the $NaHCO_3$ was 230 cpm/nmol. Values shown are the means of 3 assays.

Addition of $NdCl_3$ to the oxygenase assay gave similar results, and there was no activity in the absence of $Mg^{++}$.

*The compound listed first was incubated with the enzyme for 10 min in the presence of 10 mM $NaHCO_3$, followed by 10 min with the compound listed second.

Table 7

The Effect of 2,3-Diphosphoglycerate on Activation of Ribulose-$P_2$ Carboxylase

| | Activation Conditions | Results cpm x $10^{-3}$ |
|---|---|---|
| 1. | Enzyme alone | 0.3 ± 4% |
| *2. | Enzyme + $MgCl_2$ + $NaHCO_3$ | 6.8 ± 9% |
| *3. | Same as 2 + 1 μM 2,3-glycerate-$P_2$ | 7.3 ± 9% |
| *4. | Same as 2 + 10 μM 2,3-glycerate-$P_2$ | 7.4 ± 10% |
| *5. | Same as 2 +100 μM 2,3-glycerate-$P_2$ | 8.8 ± 3% |
| *6. | Same as 2 + 1 mM 2,3-glycerate-$P_2$ | 8.4 ± 7% |
| 7. | Enzyme + $MgCl_2$ + $NaHCO_3$ | 9.7 ± 4% |
| 8. | Same as 7 + 1 μM 2,3-glycerate-$P_2$ | 10.1 ± 6% |
| 9. | Same as 7 + 10 μM 2,3-glycerate-$P_2$ | 9.6 ± 1% |
| 10. | Same as 7 +100 μM 2,3-glycerate-$P_2$ | 10.2 ± 3% |
| 11. | Same as 7 + 1 mM 2,3-glycerate-$P_2$ | 10.0 ± 1% |
| 12. | Enzyme + 10 μM 2,3-glycerate-$P_2$ | 1.2 ± 1% |
| 13. | Enzyme + 1 mM 2,3-glycerate-$P_2$ | 1.7 ± 6% |

The samples were activated for 30 min before initiation of the reaction.

The assay time was 30 s, and the results are the means of 3 assays.

In all cases the $MgCl_2$ concentration was 10 mM for activation (if used) and 10 mM for assay. All samples were assayed at 10 mM $NaHCO_3$. The specific activity of the $^{14}C$-$NaHCO_3$ was 230 cpm/nmol.

*In these cases the $NaHCO_3$ concentration for activation was 1 mM, in all other cases it was 10 mM (if used).

including phosphate and pyrophosphate (M. Spellman, personal communication) cause some degree of activation of the carboxylase under sub-optimal $CO_2$ concentrations, a reorientation of our working hypothesis is probably in order concerning the activation of this enzyme. It seems certain from the work of Lorimer and others that one aspect of $CO_2$ activation is the formation of a carbamate which is stabilized by $M^{++}$. The effect of sugar-P's clearly cannot replace this phenomenon. Perhaps the effect of sugar phosphates is a non-specific anion activation at the ribulose-$P_2$ site, and this manifests itself only under sub-optimal $CO_2$ conditions, because this activation is normally performed by $HCO_3^-$. The rationale behind this hypothesis is that the binding energy of effector causes a conformational change in the protein which allows it to bind substrate readily. This idea for activation of an enzyme has been discussed by Koshland (163). If this explanation is representative of the real condition then the inhibition due to sugar-P is due to their relative life time in the active site. One might expect then that ribulose-$P_2$ would activate the carboxylase, but it might be too fast to observe under normal assay conditions. Data will be presented in Chapter VI with rapid reaction kinetics showing an initial lag, as if such a ribulose-$P_2$ activation did occur.

CHAPTER VI

# EPR SPECTROSCOPY, IRON, AND RAPID RECTION KINETICS

## Introduction

The proposed oxygenase mechanism (Figure 1) contains an apparently spin-forbidden step (see Literature Review). Therefore, the possibility that the reaction could proceed through a free radical was explored in a joint research project between Dr. Tolbert's group and Dr. W.H. Orme-Johnson at the University of Wisconsin. Dr. Peach and I made two trips to Dr. Orme-Johnson's laboratory to make use of his expertise and equipment for EPR spectroscopy and rapid-quenched enzyme reactions. This Chapter contains the results of these two research trips.

## Materials

The enzyme was prepared in the manner described in Chapter I, the spinach was obtained locally, flown in from California, or flown in from Florida. Ultrapure: $Mg(NO_3)_2$, KOH, HCl, and $NaHCO_3$ were from the Alfa Division of the Ventron Corporation. Bathophenanthroline, tetranitromethane, and hydroxylamine hydrochloride were from Sigma.

## Methods

The EPR spectroscopy was performed on a Varian V-4500 spectrometer at 13°K. The individual settings for the spectrometer are described in the appropriate Figure legends. The rapid-quench apparatus was from

Update Instruments. The instrument consisted of two syringes: one with substrate, and one with activated enzyme. At time = 0 the enzyme and substrate were forced simultaneously into a mixing chamber and subsequently into either 2N HCl (for the fast reaction enzyme assays) or into isopentane at 77°K (for the EPR studies). The duration of the assay was a function of the length of the tube connecting the mixing chamber and the quenching solution. The samples that were quenched in isopentane were stored in liquid nitrogen until the EPR spectroscopy was performed. Conducting enzyme assays for very short reaction times (e.g. 10 ms) poses serious mixing problems, so the ratio of volumes of enzyme and substrate had to be kept close to 1 to 1. In an effort to get the final sample for the EPR as concentrated as possible, the starting concentration of enzyme was 40-60 mg/ml. The sample volume was about 400 µl so at least 10 mg of enzyme was used in each reaction. The assay times varied from 3 ms to 10 s.

Discussion of the EPR Spectroscopy

The initial approach was to determine the EPR spectrum of the enzyme during catalysis. The rationale behind this was to determine whether a radical signal was present, and if it was, whether the signal changed during the course of the reaction. The rapid-quench apparatus was used to prepare frozen samples from the initial portion of a reaction progress curve, and then to study them in the EPR. Since the activity under study in this case was the oxygenase reaction, care was taken to saturate both the enzyme and the substrate with $O_2$ before beginning the reaction. Even with this precaution it was not possible to optimize the oxygenase reaction. Normally in an oxygenase assay, activated enzyme is diluted 25

fold, so that the carryover of inhibitory $HCO_3^-$ from activation is only 0.4 mM. However in this case, the ratio of volumes required for adequate mixing resulted in a carryover of around 5 mM $HCO_3^-$. As a result, it was impossible to eliminate the carboxylase reaction and the oxygenase was substantially inhibited.

The immediate results were the discovery of a radical signal in the g=2 region of the spectrum and an iron signal in the g=4 region (Figure 14). A radical signal was present in every enzyme sample that was run. control samples were prepared by mixing the activated enzyme with water instead of substrate and waiting a defined time before quenching the "reaction". Figure 15 shows a 3 s assay, a 3 s control, and a difference spectrum created by storing the first two assays in a computer and displaying the difference. The radical has a certain amount of hyperfine splitting, but the experiments to date do not reveal much more. According to Dr. Orme-Johnson, the signal probably does not represent superoxide since it is missing certain hyperfine features present in the spectrum of superoxide.

Because the radical was always present in the enzyme samples, it seemed reasonable to consider possible sources of radicals on the enzyme surface. Two potential sources of radical signals were cysteine and tyrosine. In an effort to determine the source of the radical signal four samples of enzyme with a concentration of 20 mg/ml were prepared. a) A control sample was prepared with no additions, but was otherwise treated exactly like the test samples. b) An enzyme sample was treated with $C(NO_3)_4$ which reacts with tyrosine residues, in a competitive manner with respect to ribulose-$P_2$ (164, and Chapter VII this thesis) and

Figure 14. The EPR spectrum of ribulose-$P_2$ carboxylase at 20 mg/ml. This was performed in a Varian V-4500 spectrometer under the following conditions: microwave frequency 9.0503 GHz, 0.3 s time constant, 10 mW microwave power, modulation amplitude of 10 G, modulation frquency of 100 kHz, and a scan rate of 1000G $min^{-1}$, at 13°K. The g values were calculated from $(h\nu)=g\beta$ (H) where H (the field strength) varied from 0.1-4.1 kG, $\nu$ is the microwave frequency, h is Planck's constant ($6.6251 \times 10^{-27}$ erg sec), $\beta$ is the Bohr magneton ($0.92729 \times 10^{-20}$ erg gauss). The signal in the g=4 region is indicative of Fe (III). There is a radical in the g=2 region.

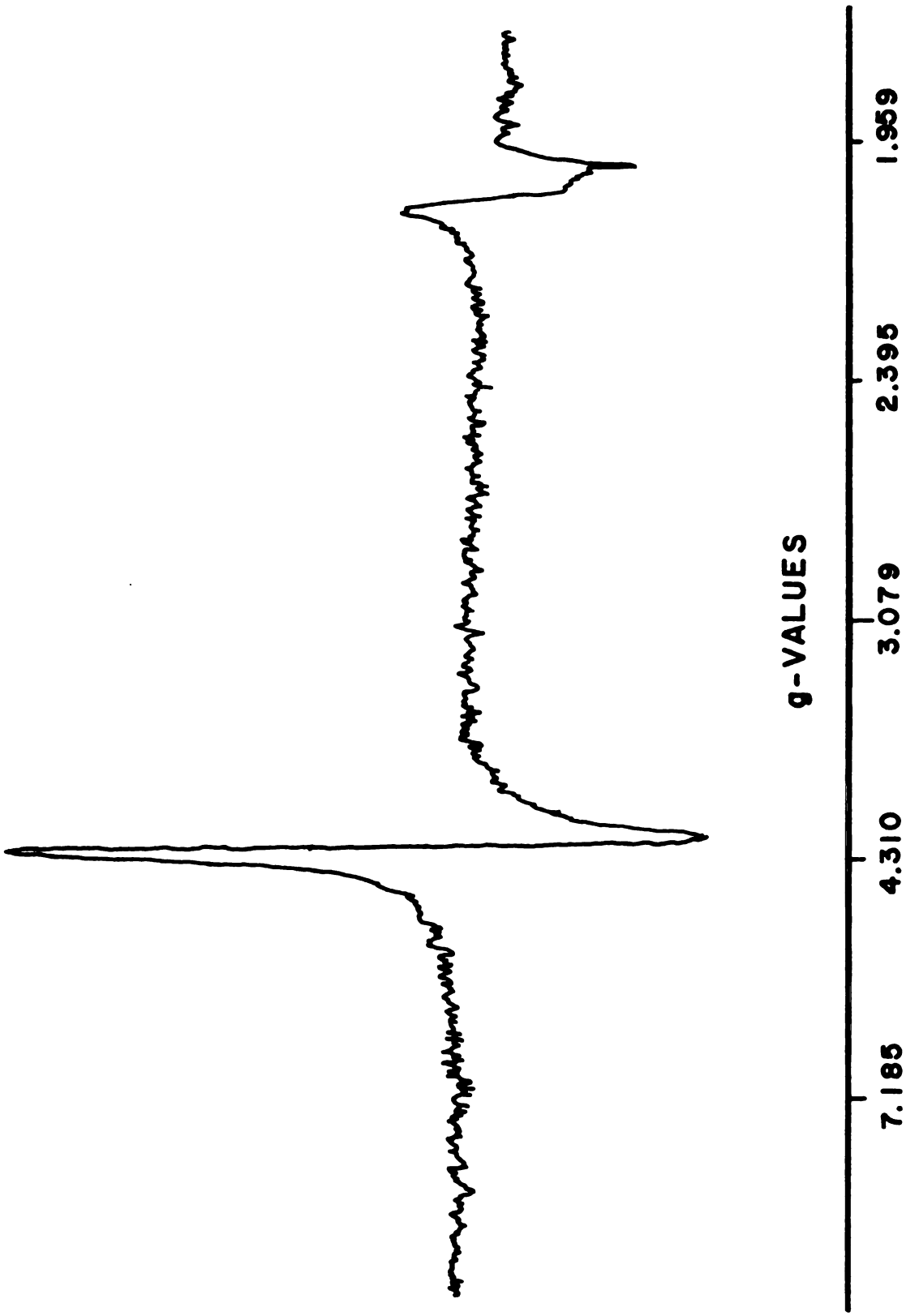
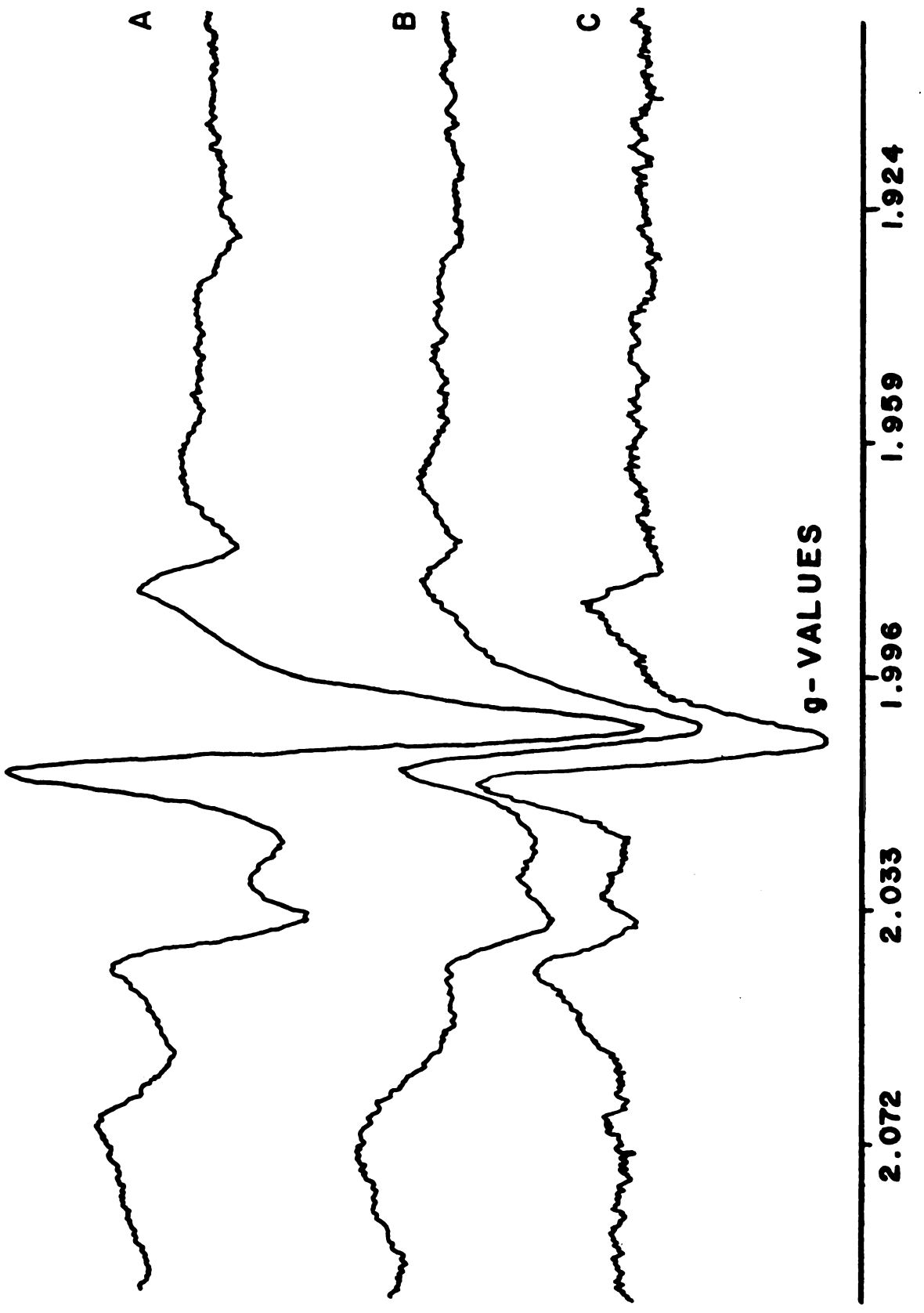

g-VALUES
7.185
4.310
3.079
2.395
1.959

Figure 15. EPR spectra of ribulose-$P_2$ carboxylase under three conditions. A) Water was mixed with the enzyme at time zero and incubated for 3 s at 0° before quenching in isopentane at 77°K. The microwave frequency was 9.0634 GHz, microwave power at 1 mW, time constant of 3 s, and a scan range of 2.845-3.645 kG. All other conditions were the same as Figure 14. B) Ribulose-$P_2$ was mixed with the enzyme at time zero and the reaction was allowed to proceed for 3 s at 0° before quenching as in A. C) Both of the spectra from A and B were stored in a computer and the difference spectrum was determined.

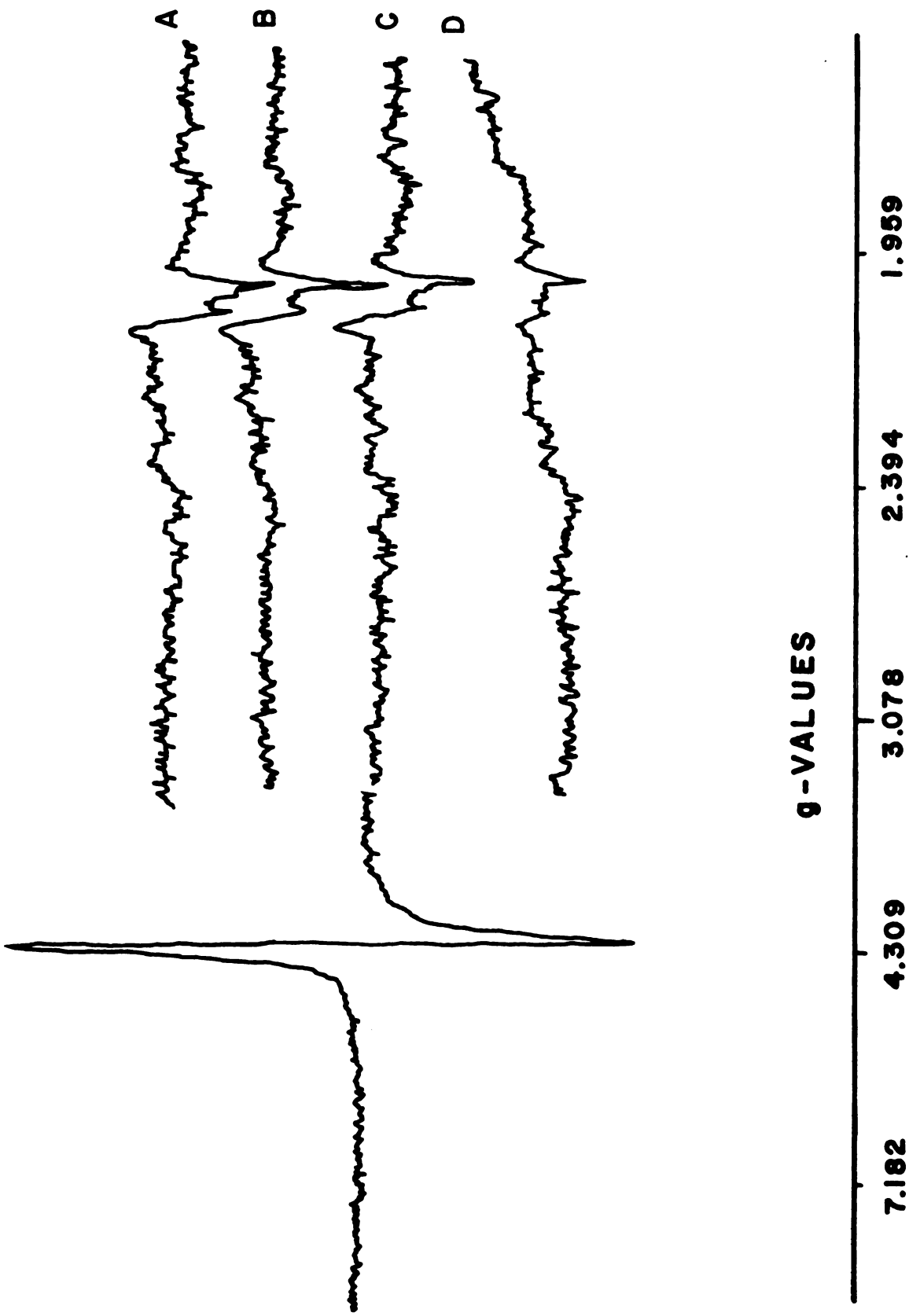

A
B
C
g-VALUES
2.072
2.033
1.996
1.959
1.924

totally inactivated the enzyme. c) An enzyme sample was treated with sufficient iodoacetic acid to totally inhibit the enzyme by modification of sulfhydryl groups at the active site. d) Since, hydroxylamine has been reported to inhibit this enzyme (165, see also Chapter VIII this thesis), it was mixed with the enzyme, incubated, and then $NaBH_4$ was added in an attempt to reduce any oxime which might have been formed during the incubation. The above portions of this experiment were carried out at Michigan State, the samples were frozen in EPR tubes in liquid nitrogen and sent to Orme-Johnson at the University of Wisconsin in a liquid nitrogen shipping dewar for the EPR spectroscopy. The EPR spectra of these samples are shown in Figure 16. The results were totally unexpected. The only treatment which altered the EPR radical signal (the $NH_2OH$, $NaBH_4$ treatment) was also the one which had no effect on the enzyme activity. Why the hydroxylamine produced this effect is unclear. It is possible that if this sample had been activated before the determination of the EPR spectrum the results could have been different.

Iron Content of the Enzyme

The discovery of a signal in the EPR spectrum corresponding to iron raised such questions as: was the iron simply a contaminant, could this iron be the required transition metal for the oxygenase, and was iron present in all sources of the enzyme. Experiments to answer these questions were carried out at Michigan State. The method used for quantitation of the iron was the analytical technique of Van de Bogart and Beinert (166). This method is basically a wet ash procedure with strong acids followed by reduction of any $Fe^{+++}$ to $Fe^{++}$, neutralization,

Figure 16. EPR spectra of ribulose-$P_2$ carboxylase after chemical modification. A) Treatment with iodoacetamide. The microwave frequency was 9.0475 GHz, the microwave power was 3 mW, all other conditions were as in Figure 14. B) Treatment with tetranitromethane. All conditions were as in A. C) Enzyme alone, no treatment. The microwave frequency was 9.0435, all other conditions were as in A. D) Treatment with hydroxylamine followed by treatment with sodium borohydride. All conditions were as in C.


A
B
C
D
g-VALUES
7.182
4.309
3.078
2.394
1.959

and extraction into isoamyl alcohol through the complexing of the iron with bathophenanthroline. The resulting pink color was read in the spectrophotomer at 535 nm against an isoamyl alcohol-bathophenanthroline blank. The standards were linear up to 25 nmol of iron, and 5 nmol gave an absorbance of approximately 0.100.

The stoichiometry of the iron proved to be 2 mol of iron/mol of enzyme, or one iron atom for every 4 active sites. This number was found in all samples examined and was confirmed independently in Dr. Orme-Johnson's lab where this particular technique is frequently used. This stoichiometry represents total iron, whereas the EPR data is only for $Fe^{+++}$ ($Fe^{++}$ is not paramagnetic). Usually $Fe^{+++}$ is readily reduced if it is free in solution, but up to 4 mM DTT had no effect on the EPR signal suggesting that this iron was bound to the protein, perhaps in a manner analogous to transferrin (167).

To evaluate whether the iron was present in the enzyme samples as a contamination, iron free water was obtained by passing glass distilled water over a Chelex 100 column and buffer through Dowex Chelating Resin. After the iron content of bicine was determined to be low this latter column was eliminated due to materials leaching out of the Dowex (see Chapter IV). Table 8 shows the amount of iron present in some of the reagents required for the ribulose-$P_2$ carboxylase assays. Iron free $MgCl_2$ was prepared by ion exchange chromatography of ultrapure $Mg(NO_3)_2$ on a Dowex-1-$Cl^-$ column prepared with ultrapure HCl. Buffers for assay were adjusted with ultrapure KOH. Dialysis of the enzyme at pH 8, against 10 changes of iron free buffer (1 to 100 volume ratio), resulted in no change in the iron content of the protein. The dialysis

Table 8

Iron Content of Reagents for Assay of Ribulose-$P_2$ Carboxylase/Oxygenase

| Reagent | Iron (nmol Fe/ μmol reagent) |
|---|---|
| Bicine | |
| Sigma | > 0.10 |
| Boehringer | > 0.10 |
| Ribulose-$P_2$ | > 0.10 |
| $MgCl_2$ | > 0.01 |
| $NaHCO_3$ | > 0.02 |

tubing had been prepared by boiling for 5-10 min in 5 mM EDTA. Similar results were obtained when the dialysis was conducted at pH 5.9 but this pH caused the protein to precipitate after several hours.

With many iron binding proteins and transferrin (167) the bound iron can be removed if it can first be reduced. Dr. Hall in Tolbert's lab had noticed that treatment of the enzyme with high concentrations of DTT produced a pink color which could be dialyzed away (135). Such a pink color has been attributed to iron-DTT complexes (Henry Lardy, personal communication). Because of this, an enzyme sample of 10 mg/ml was made 100 mM DTT for 4 hr and then dialyzed agasinst several changes of buffer containing 100 mM DTT. This procedure reduced the iron concentration from 2 mol/mol of enzyme to 0.7 mol/mol of enzyme. Assay of this enzyme in iron-free media revealed no change in either activity. In a related experiment, stirring the enzyme in 50 mM ascorbate over Chelex produced no change in the iron EPR signal.

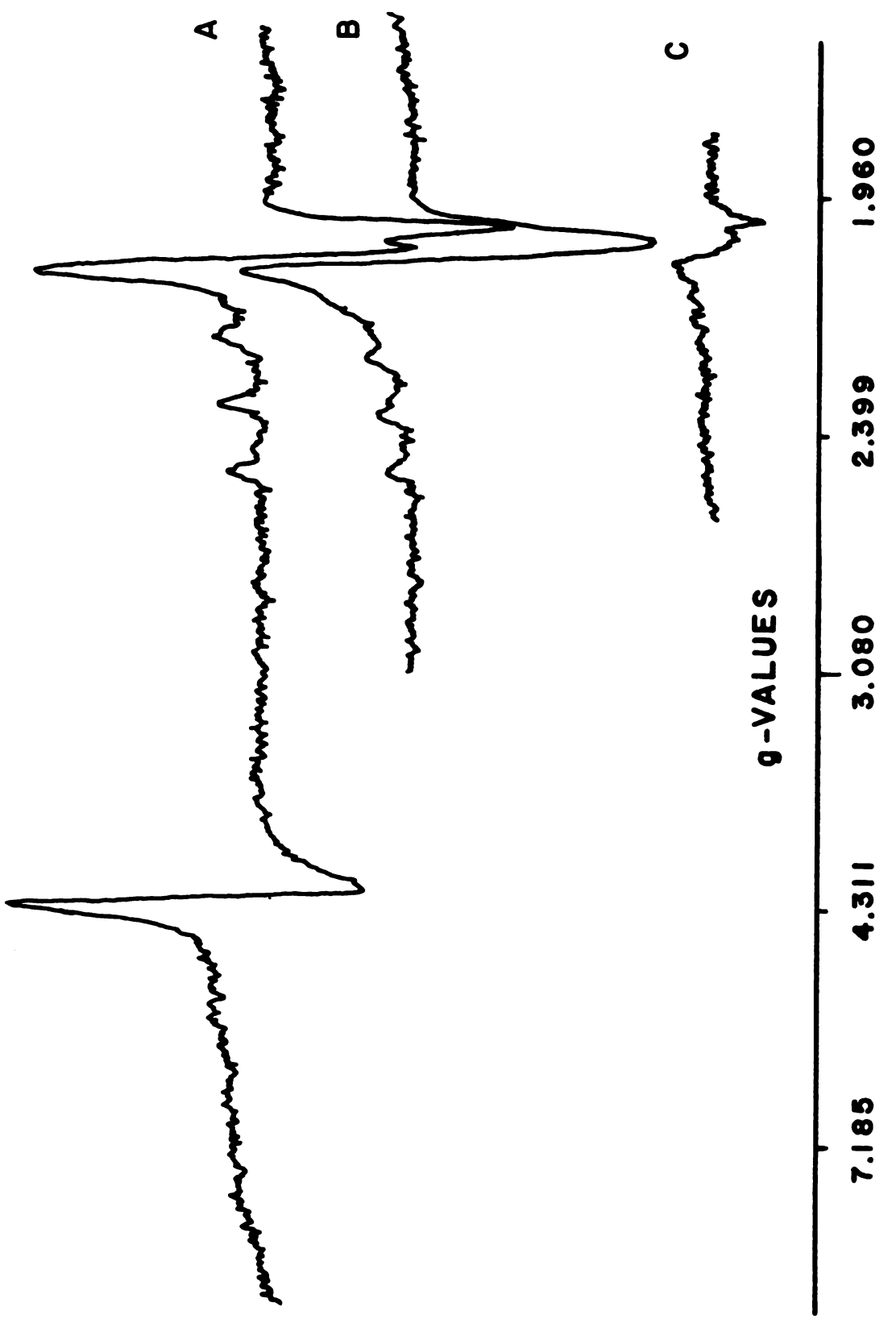
From these data, conclusions concerning the role of iron in the mechanism of this enzyme, if any, are impossible. Since removal of 60% of the iron had no effect on either of the two catalytic activities of the enzyme, and since the amount of iron present is quite small relative to the number of active sites, one is tempted to rule out any role for iron. On the other hand it is difficult to understand why different sources of enzyme all had the same amount of tightly bound iron if it doesn't have a role.

Copper Content of the Enzyme

The role of copper, if any, in ribulose-$P_2$ carboxylase has been extensively investigated and hotly debated. Lane's group reported that

copper was present, but removal of it had no effect on the carboxylase activity (174,168). Chollet et al (169) reported finding essentially no copper, iron, or flavin associated with crystalline tobacco enzyme. Lorimer et al (11) found similar results from spinach. On the other hand there is the report of Branden (44) which claims that the oxygenase is a copper protein and that it can be separated from the carboxylase by Sepharose 6B column chromatography (see Chapter VIII). During our first trip to Orme-Johnson's facility, we were able to use Michigan grown spinach as the source of the enzyme and saw no indication of copper in the EPR. Our second trip was made in the winter, and since the experiments were to require large amounts of enzyme, spinach was shipped in from California and Florida. The spinach from the two different sources were clearly different varieties, but their specific activities were quite similar, 1.8 μMol/min/mg for the enzyme from California spinach, and 1.6 μMol/min/mg for the enzyme from Florida spinach. The enzyme prepared from California spinach contained no copper signal in the EPR, but the enzyme prepared from Florida spinach did (Figure 17). No analytical determinations of copper were made and since only $Cu^{++}$ will show up on EPR, it is possible that the California enzyme contained $Cu^{+}$. The copper was easily reduced, addition of 2 mM DTT completely abolished the signal (Figure 17). Whether copper has a physiological role in the mechanism of this enzyme remains a moot question, but it is a potentially useful probe for the conformational state of the protein. The addition of $Mg^{++}$ and $CO_2$ for activation resulted in the splitting of the copper signal (Figure 17). This suggests that the copper is interacting with the protein in some region which changes with the activation process.

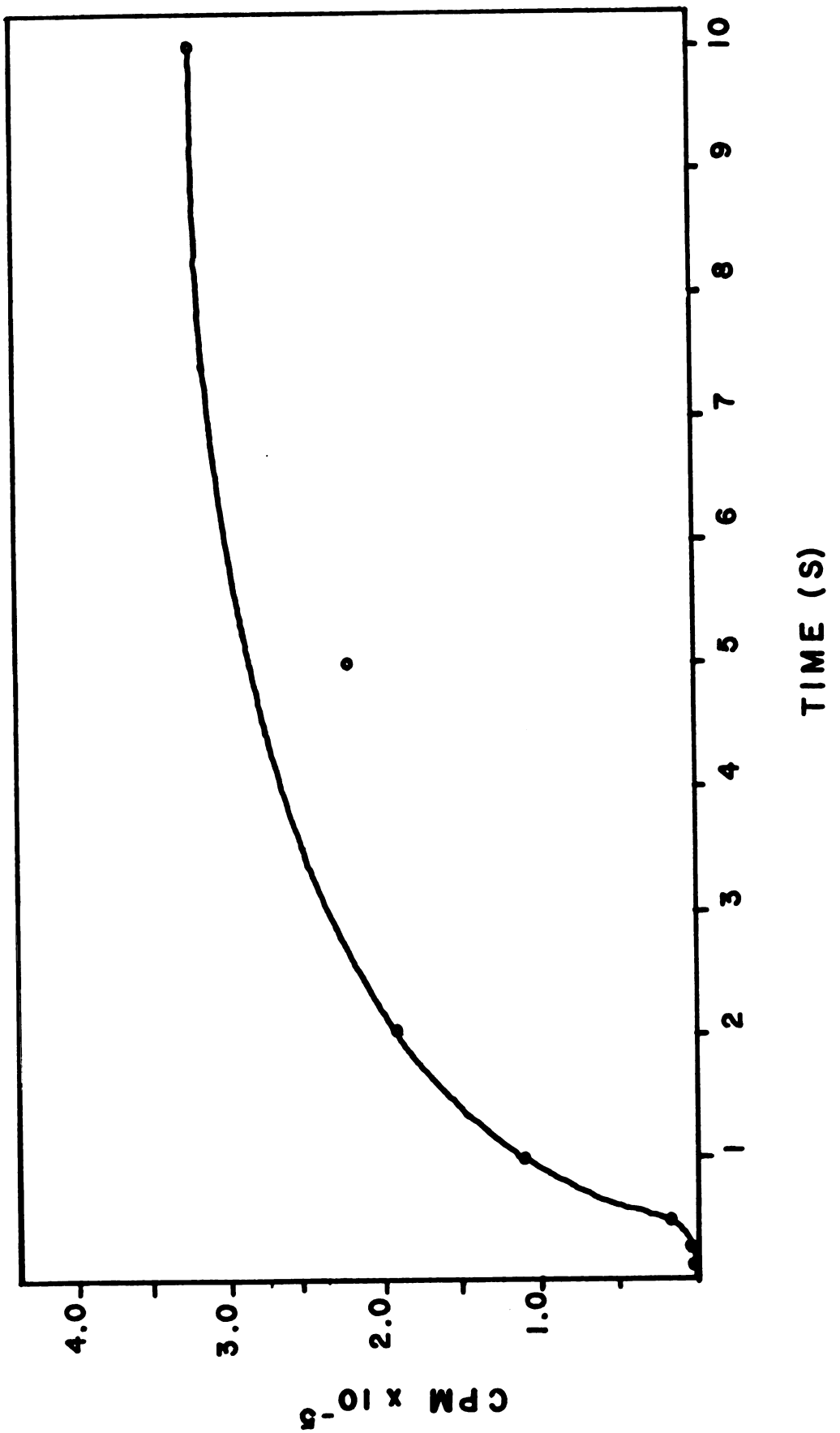
Figure 17. The EPR spectrum of ribulose-$P_2$ carboxylase purified from spinach obtained from Florida. The large signal and the associated hyperfines in the g=2 region are indicative of Cu(II). A) This enzyme sample was activated by $CO_2$ and $Mg^{++}$. $CO_2$ alone did not produce the split in the Cu signal, $Mg^{++}$ was required. The microwave power was 3 mW. The microwave frequency was 9.0494 GHz. All other conditions were the same as in Figure 14. B) Enzyme in absence of $Mg^{++}$. The microwave frequency was 9.0534 GHz. All other conditions were the same as in A. C) The same enzyme sample after incubation with 2 mM DTT for 20 min at room temperature. All conditions were the same as in B.


A
B
C
7.185
4.311
3.080
2.399
1.960
g-VALUES

## Rapid-Reaction Initial Kinetics

Access to the rapid-quench apparatus provided us with the opportunity of determining a rection progress curve for the carboxylase at very short times (Figure 18). The curve indicates a lag of 0.5 to 1 s in the reaction. While this seems negligible in terms of normal enzyme assay times, it raises some questions about the carboxylase mechanism. Unless these results can be attributed to some flaw in the experimental design (a repeat experiment gave the same results) they may indicate substrate activation (see Chapter V) or perhaps some form of positive cooperativity for the carboxylase reaction. These assays were performed at 10° with a very high enzyme concentration. As a result, the leveling off after a few seconds may be due to the lack of available $CO_2$ for the carboxylation reaction. This experiment should be extended with the addition of carbonic anhydrase to the reaction mix to speed up the $CO_2$-$HCO_3^-$ equilibrium. This topic will be pursued next year when I have regular access to the rapid-quench apparatus.

Figure 18. Reaction progress curve of ribulose-$P_2$ carboxylase for very short time assays. The reactions were killed by the injection of 2N HCl. The procedure is described in the text. The enzyme concentration was 20 mg/ml.


CPM x 10^-3
4.0
3.0
2.0
1.0
1
2
3
4
5
6
7
8
9
10
TIME (S)

# CHAPTER VII

# ACTIVE SITE MODIFICATION

## Introduction

Several laboratories have been successful in modifying amino acid residues in the active site region of ribulose-$P_2$ carboxylase. Cysteine (107), arginine (124,125), lysine (108,119), tyrosine (164) have all been shown to be in the active site region of ribulose-$P_2$ carboxylase. My efforts in the direction of active site modification were limited to trying to explain two other phenomena uncovered in the work of this thesis: a) what group on the protein is responsible for the tight binding of xylitol-$P_2$ and xylulose-$P_2$; and b) what group is responsible for the radical signal observable in the EPR?

## Materials

Tetranitromethane and dibromoacetophenone (p-bromophenacylbromide) were purchased from Sigma. $NaB^3H_4$ was obtained from New England Nuclear.

## Methods

The enzyme assays were performed in the usual manner. Assays with dibromoacetophenone were in 0.25 ml reaction volume and all other were 0.5 ml volume. The dibromoacetrophenone was dissolved in acetone before use. The reduction with $NaB^3H_4$ was carried out in a hood for the venting of the $^3H_2$ given off during the reaction.

Results and Discussion

One hypothesis offered for the strong inhibition by xylitol-$P_2$ and xylulose-$P_2$ is that the hydroxyl at C-3 reacts with an adjacent base in the active site to form a hydrogen bond. Histidine could be such a base, so modification of histidyl residues was attempted with dibromoacetophenone. The protocol was to incubate activated enzyme with dibromoacetophenone for varying periods of time, up to 6 hr, and then assay the carboxylase activity. The assumption was made that if such a modification occurred the enzyme would be catalytically inhibited as well as having a reduced affinity for the two inhibitors. Since the reagent is insoluble in water and had to be dissolved in acetone, acetone treated enzyme controls were also run. The dibromoacetophenone inhibitied the carboxylase reaction less than 10% and was indistinguishable from the acetone controls. The active site of phospholipase $A_2$ contains an essential histidine and while the pH optimum for catalysis is around 8, the pK for inactivation with dibromoacetophenone is 6.1 (170). I attempted modification of ribulose-$P_2$ carboxylase both at pH 7.0 and at pH 8.1, but there was no effect different from the acetone control. Either there are no reactive histidyl residues in the active site region, or this was not the reagent of choice. This later possibility is supported by the fact that C. Paech (personal communication) found a reaction between diethylpyrocarbonate and the carboxylase by following the reaction spectrophometrically, but he did not correlate this effect with enzyme activity.

Tetranitromethane is a powerful nitrosylating reagent which is rather specific for tyrosyl residues. Because of the radical signal in

the EPR spectrum of this enzyme, this compound was tested for its effect on enzyme activity. $C(NO_3)_4$ had no effect on the radical signal (Chapter VI), but it was a potent inhibitor of both activities. In one preliminary experiment, ribulose-$P_2$ was found to partially prevent the inactivation due to $C(NO_3)_4$. This was run at only one concentration of $C(NO_3)_4$. Before I had the opportunity to continue these experiments a paper appeared from Tabita's group (164) discussing the inhibition of the carboxylase activity in enzyme prepared from R. rubrum. They found $C(NO_3)_4$ to be a competitive inhibitor with respect to ribulose-$P_2$. Because of this and the negative results in the EPR experiments, futher work with tetranitromethane has been postponed.

After solid data was obained for a lysyl residue in the active site region, Dr. Paech and I made some abortive efforts to look for Schiff base formation between this residue and ribulose-$P_2$ and xylulose-$P_2$. The procedure was to incubate the enzyme in the presence of each of the keto diphosphates for 20 min and then add $NaB^3H_4$ in an effort to reduce the Schiff base if one was present. While there was incorporation of label into the protein, acid hydrolysis and chromatography of the residues revealed no incorporation of the sugar phosphates.

# CHAPTER VIII

## PROJECTS TO VERIFY THE WORK OF OTHERS

### Introduction

One of the basic requirements of science is that the experiments be reproducible and that the phenomena discovered must be features of the material under study rather than a feature of the way in which the experiment was performed. This has not been the case for many of the papers on ribulose-$P_2$ carboxylase. It is for this reason that experiments performed in one laboratory must be repeated in different laboratories and from different directions. In the few years that I have been associated with ribulose-$P_2$ carboxylase/oxygenase several papers have been published which have yet to be successfully confirmed in other laboratories. Some of these papers presented data of such potential significance that those of us in Tolbert's group felt it necessary to attempt to repeat the experiments. This chapter contains the results of five such projects.

### Ribulose-$P_2$ Carboxylase/Oxygenase, One Enzyme or Two

Since the discovery of the oxygenase activity of ribulose-$P_2$ carboxylase (8,9,10,11) many efforts have been made to determine whether both activities are really present in one protein. Many different laboratories have found the two activities to copurify through ammonium sulfate fractionation, Sepharose chromatography, ultracentrafugation, cocrystallization, ion exchange chromatography, electrophoresis,

isoelectric focusing, and hydroxyl apatite chromatography. In addition there is competitive inhibition between $CO_2$ and $O_2$. Numerous compounds have been tested, all of which (with the exception of $Mn^{++}$ (158)) inhibit both activities equally. Both activities have a break point in the Arrhenius plot at the same temperature (171). The compound 2-carboxyribitol-$P_2$, an analogue of the proposed reaction intermediate inhibits both activities equally. Because of this accumulation of data which suggested that both the carboxylation and oxygenation reactions occurred at the same active site, a report of the separation of these activities (44) was received with a great deal of interest. The decision was made to repeat this work, initial skepticism notwithstanding. Our results have been published (45) and are included in this thesis as Appendix 4.

We repeated Branden's (44) work as closely as possible, and talked to him to clear up specific points about the methods. We totally failed to reproduce his results. He was plagued with a high endogenous rate of oxygen consumpton and as a result, incubated the enzyme in the assay mix and initiated the reaction with ribulose-$P_2$. Our explanation for his data is that this failure to use activate enzyme caused the oxygenase activity to be undetectable in the column fractions which contained carboxylase. When we tried running the oxygenase assays in this manner our activity all but disappeared. Why he found a ribulose-$P_2$ dependent uptake of oxygen in a different region of the Sepharose profile remains unclear. This activity was associated with a large amount of copper (44) and iron (personal communication). We postulated that due to the instability of the substrate ribulose-$P_2$, a non-specific oxygen uptake

catalyzed by a $Cu^{++}$-protein complex would not be surprising. The role of copper in this enzyme, if any, remians to be determined (Chapter VI).

Oxygen Activation

Recently Wildner reported that oxygen was required in a non-catalytic capacity for both activities of this enzyme (172). His data were that enzyme prepared from spinach, in Tris-sulfate buffer, suffered a substantial loss of both activities after being gassed for several hours with Ar gas. Also, there was a substantial change in the intrinsic fluorescence of the protein. Both of these results were completely reversible following the reintroduction of $O_2$ to the system.

To repeat this work. R. Gee prepared the enzyme, gassed it with Ar, and determined the intrinsic fluoresence, N. Hall ran oxygenase assays, and I ran the carboxylase assays. In our hands, the sample treated with argon was exactly the same as the untreated control in all respects. We were unsuccessful in two attempts to confirm Wildner (172), but I can offer no explanation for the differences between his results and ours.

Differential Regulation

If one accepts the argument that photorespiration is purely a wasteful process and therefore should be eliminated, differential regulation of the carboxylase and oxygenase would be one way to achieve this goal. This section deals with claims from two different laboratories that oxygenase activity could be reduced by certain chemical treatments.

Wildner (87) reported that preincubation of the enzyme with

ribulose-$P_2$ and subsequent treatment with iodoacetamide resulted in complete retention of the carboxylase activity and a total loss of oxygenase activity. Omission of the ribulose-$P_2$ resulted in complete inactivation of both activities. I attempted to repeat this experiment on three occasions, and a sample of the results is shown in Table 9. Both activities were protected when the enzyme was preincubated with ribulose-$P_2$ before the IAA treatment, and complete inactivation of both activities occurred when this preincubation was not done. C. Paech also performed this experiment and obtained results identical to mine. N. Hall aided in the performance of the oxygenase assays.

Bhagwat and coworkers (165) have reported that 5 mM hydroxylamine completely inhibited the oxygenase without affecting the carboxylase activity. They did not use activated enzyme in their experiments. It is not clear from their paper whether the enzyme or the substrate was exposed to the $NH_2OH$ before the initiation of the reaction. In our effort to repeat this work, C. Paech and I used activated enzyme and studied the effect of $NH_2OH$ when it was preincubated with the enzyme, and when it was preincubated with the substrate (Table 10). Our data shows equivalent inhibition of both activities under each of these two conditions. The oxygenase may have been slightly more inhibitied than the carboxylase when the enzyme was preincubated with the hydroxylamine, but the carboxylase was certainly inhibited also. Since the appearance of this paper, there has been a second report of differential regulation with hydroxylamine (173). This later work was done with partially purified enzyme from Anabaena, and both activities were assayed in the same reaction vessel. While this seems like a good approach it means that it is impossible to optimize each of the activities. Treatment of

Table 9

Modification of Ribulose-$P_2$ Carboxylase/Oxygenase with Iodoacetamide

| | Carboxylase | Oxygenase |
|---|---|---|
| No Treatment | 100% | 100% |
| 5 mM iodoacetamide | 0% | 0% |
| 5 mM iodoacetamide after 20 min with 25 mM ribulose-$P_2$ | 85-95% | 60-75% |

Table 10

Treatment of Ribulose-$P_2$ Carboxylase/Oxygenase with Hydroxylamine

| | $NH_2OH$ Concentration mM | Carboxylase | Oxygenase |
|---|---|---|---|
| A | 0.00 | 100% | 100% |
| | 0.63 | 99% | ___ |
| | 1.25 | 97% | ___ |
| | 2.50 | 89% | ___ |
| | 5.00 | 85% | ___ |
| | 6.25 | 80% | 58% |
| | 10.00 | 69% | ___ |
| | 12.50 | 46% | 39% |
| | 25.00 | 11% | 23% |
| B | 0.00 | 100% | 100% |
| | 2.50 | 90% | 87% |
| | 5.00 | 84% | 84% |
| | 10.00 | ___ | 65% |
| | 25.00 | 41% | ___ |

In A, hydroxylamine was incubated with ribulose-$P_2$ for 6-10 min before initiation of the reaction by the addition of the enzyme. The reaction was run at the same concentration of hydroxylamine as the incubation.

In B, hydroxylamine was incubated with the enzyme for 20 min before initiation of the reaction with ribulose-$P_2$. The reactions were run at the same concentration of hydroxylamine as the incubation.

All assays were run as described in Chapter I.

the enzyme with $NH_2OH$ and $NaBH_4$ did produce a change in the radical signal present in the enzyme (Chapter VI), but this could not be correlated to any change in activity. Hopefully these differences in data will eventually be resolved.

Effect of Superoxide Dismutase

Since the discovery of the oxygenase reaction the possibility of the involvement of $O_2^-$ has been considered. Ryan and Tolbert (129) demonstrated that on polyacrylamide gels the enzyme acted as a superoxide dismutase. More recently, Wildner and Henkel (87) postulated that $O_2^-$ was the true substrate for the oxygenase reaction. Bhagwat and Sane (88) have recently offered data in support of this hypothesis. Their data suggested that the oxygenase was inhibited by the addition of superoxide dismutase or nitroblue tetrazolium to the assay mix. This is contrary to published work from Tolbert's lab (11). They purified their own SOD from bovine erythrocytes. No evidence was offered concerning the effect of SOD on the carboxylase reaction. Since $O_2^-$ is such a reactive species, its concentration in free solution is quite small. To account for observed rates of the ribulose-$P_2$ oxygenase reaction, the oxygenase would have to generate the $O_2^-$ from $O_2$, and for the oxygenase reaction to be inhibited by SOD this $O_2^-$ would have to be released from the enzyme before it was used.

C. Paech and I attempted to repeat this experiment using SOD from Sigma. The SOD was very active as checked by us in the xanthine oxidase-cytochrome C assay of Fridovitch (174). No inhibition of either ribulose-$P_2$ carboxylase or oxygenase activity was noted upon addition of 100 U of SOD to each assay mix. Using copper penicillimine, a small

molecular weight SOD Lorimer (personal communication) also found no effect on either activity. In a similar vein, we found no signal in the EPR indicative of $O_2^-$ (Chapter VI). Until the mechanism of the oxygenase is elucidated, the role of $O_2^-$, if any, will remain unclear.

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# APPENDICES

From: PHOTOSYNTHETIC CARBON ASSIMILATION
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# ACTIVE SITE OF RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE*

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The properties, distribution, biogenesis, function, and regulation of ribulose bisphosphate carboxylase/oxygenase, as described elsewhere in this Symposium, are mainly integrated around the mechanism of action of the carboxylase and oxygenase reactions. Recently, interest has been stimulated by the discovery of the oxygenase reaction (1, 2) and its role in the glycolate pathway of photorespiration (3), and by the desirability of differential regulation of the carboxylase and oxygenase activities, if possible, in favor of the former in order to increase photosynthetic productivity. A prerequisite for such regulation is a thorough knowledge of the relationships between structure and function in this protein.

RuBP carboxylase, in various molecular forms (4), is present in all photosynthetic organisms. To date, most of the work has been done either with the crystalline enzyme from tobacco leaves or with an enzyme from spinach whose ease of purification to homogeneity in large amounts has often been reported. Purified preparations can be obtained in one day by precipitation from the homogenate with polyethylene glycol followed by DEAE cellulose chromatography (N.P. Hall, unpublished). However, the complexity of this large protein and the inability to dissociate it into active subunits have been intimidating, and unfortunately little is known about RuBP carboxylase/oxygenase relative to other key enzymes.

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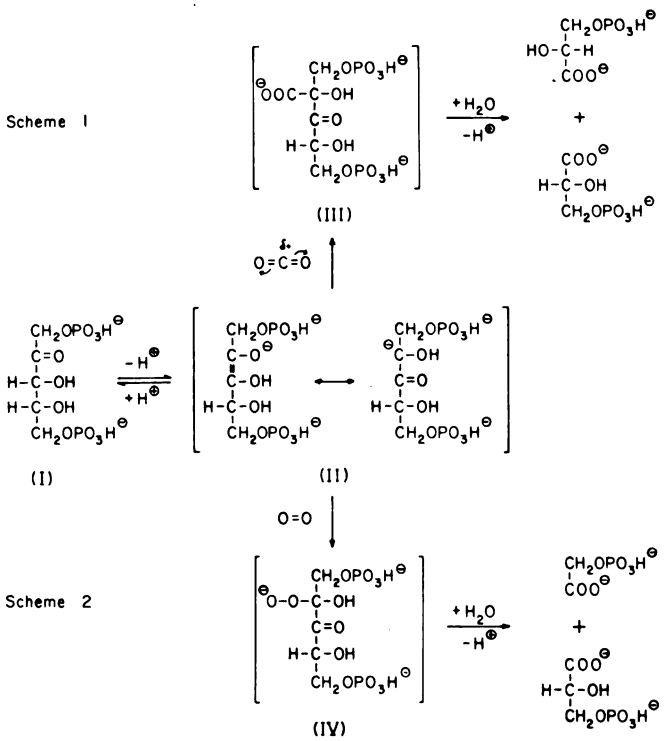
Abbreviations: RuBP, ribulose 1,5-bisphosphate; XuBP, xylulose 1,5-bisphosphate; xylitol BP, xylitol 1,5-bisphosphate; FBP, fructose 1,6-bisphosphate; CRBP, 2-carboxy-D-ribitol 1,5-bisphosphate; bromodihydroxybutanone BP, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate.

Scheme 1



Scheme 2



The chemical mechanism of the carboxylation reaction predicted by Calvin (5) (Scheme 1) even before discovery of the enzyme is soundly based on organic chemistry, but progress has been slow in showing how the functional groups at the active site enable the enzyme to catalyze this reaction. The initial step is a base-catalyzed enolization (II) at C3 of RuBP (I), which could be the rate-limiting step of the overall reaction (6). After addition of $CO_2$, which is the actual substrate for the carboxylase (7), to the intermediary carbanion, at C2 of RuBP (II), C-C bond cleavage of the intermediate β-keto acid (III) occurs between C2 and C3 (8). On the basis of the enzyme's sensitivity to sulfhydryl-modifying reagents, and in order to explain unsuccessful attempts to isolate the hypothetical β-keto acid (III), a catalytic mechanism involving hemimercaptal formation of a sulfhydryl group with RuBP at carbon 2 was proposed (9). Later, support for the existence of the β-keto intermediate (III) was sought by attempts at its synthesis (10, 11). The finding that the stable transition-state analog of (III), 2-carboxy-D-ribitol 1,5-bisphosphate (CRBP) inhibits by competition with RuBP (12, 13) has been considered proof for intermediate (III) in the reaction.

Calvin's original hypothesis for the mechanism of the carboxylase reaction was also used to explain the $^{18}O$-labeling pattern in

phosphoglycolate produced by the oxygenase reaction (2) (Scheme 2). Although this concept is appealing, the enzyme does not contain any of the cofactors normally associated with oxygenase essential for activating the $O_2$ (2, 13), such as metal ions or flavins. $O_2$ is normally in the triplet state, which is 22 kcal/mol lower in energy than singlet $O_2$. A direct reaction of triplet $O_2$ with a singlet molecule (II) is a spin-forbidden process. Therefore, the enzymic mechanism for the oxygenation of RuBP must circumvent the mechanism described by Scheme 2, or use some other one, and it remains unknown.

Pon et al. (14) showed in 1963 that preincubation with $CO_2$ and $Mg^{2+}$ was essential to the enzyme assay, and this requirement has been further ascribed to a homotropic effect of $CO_2$ (15, 16). Pon et al. also noted that the reaction had to be initiated with RuBP in order to get a maximal rate. But it was not clearly established until 1976 that $CO_2$ and $Mg^{2+}$ activation is a necessity for obtaining a realistic $K_m(CO_2)$ of 10 to 20 μM and a $K_m(O_2)$ of about 200 μM, and that without $CO_2$ and $Mg^{2+}$ pretreatment the enzyme is active (17, 18).

The activity of RuBP carboxylase *in vivo* is thought to be controlled also by several other mechanisms such as shifts in stromal pH and $Mg^{2+}$ concentration due to the $H^+$ gradient created by electron transport in the light. In addition, some of the intermediates of the reductive and oxidative pentose phosphate pathways are effectors for isolated RuBP carboxylase/oxygenase. Among these are 6-phosphogluconate (see Bassham et al., this volume), fructose 1,6-bisphosphate (FBP), ribulose 5-phosphate, ribose 5-phosphate, and NADPH. The physiological function of these effectors is uncertain, and their mechanism and site of action on the carboxylase are unknown.

A working model for the active site (Figure 1) of RuBP carboxylase/oxygenase must take into account a binding site for RuBP and the mechanism of the base-catalyzed enolization, a $CO_2$ substrate site near carbon 2 of RuBP, and the possiblity of an $O_2$ site, as well as amino acid residues essential for catalysis. The $CO_2$ activator site must bind both $CO_2$ and $Mg^{2+}$ in a manner to activate or affect the catalytic site. In addition, there is a need for effector sites.

## ESSENTIAL LYSYL RESIDUES AND SULFHYDRYL GROUPS

Pyridoxal 5'-phosphate has been used as a probe to demonstrate that ε-amino groups of lysyl residues are important for the mechanism of RuBP carboxylase/oxygenase from spinach (19-21) and *Rhodospirillum rubrum* (22-24). Pyridoxal phosphate has some advantage over other affinity labels employed so far (25-29) (see Hartman et al., this volume) in that it not only reacts reversibly with RuBP carboxylase forming a Schiff base, but also can be used as an irreversible inhibitor through subsequent $NaBH_4$ reduction. In addition to the characteristic spectral properties of both the Schiff base and its reduced form, a radioactive label can be easily introduced

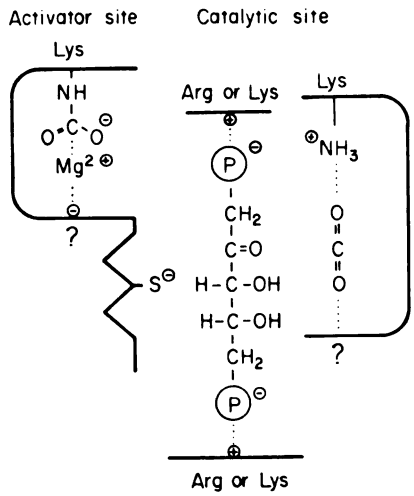



Figure 1. Working model for active site of RuBP carboxylase/oxygenase.

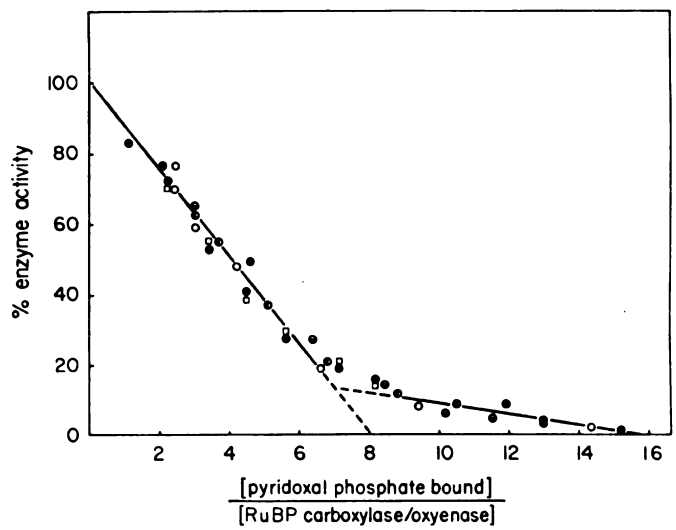



Figure 2. Activity of RuBP carboxylase/oxygenase with increasing amounts of bound pyridoxal phosphate after $NaBH_4$ reduction. Open circles represent carboxylase activity and squares, oxygenase activity. The enzyme was incubated with increasing concentrations of pyridoxal phosphate and reduced with $NaBH_4$, and the amount of bound pyridoxal phosphate was determined spectrophotometrically (21).

by $NaB^3H_4$ reduction, but the stoichiometry is subject to an isotope effect (C. Paech, unpublished). Pyridoxal phosphate appears to have an exclusive specificity for primary amino groups, whereas the affinity labels 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate and N-bromoacetylethanolamine phosphate exhibit greatest activity with sulfhydryl groups (26, 28).

RuBP carboxylase/oxygenase from spinach, which has an $A_8B_8$ structure, was completely inactivated when 16 lysyl residues were blocked by pyridoxal phosphate (21). The stoichiometry of pyridoxal phosphate incorporation was determined spectrophotometrically with an extinction coefficient of 4800 $M^{-1}$ $cm^{-1}$ for the reduced Schiff base, as derived for this system (21). This is different from the coefficient used for this purpose as found in the literature, but it is in the same range as that derived for aspartate transcarbamylase (30). This value for the reduced Schiff base should not be confused with the value 5800 $M^{-1}$ $cm^{-1}$ derived for the Schiff base between RuBP carboxylase and pyridoxal phosphate. Loss of enzyme activity with increasing amounts of irreversibly bound pyridoxal phosphate was linear but biphasic (Figure 2). The two linear portions extrapolated to 8 and 16 mols of bound pyridoxal phosphate per mol of enzyme. Complete inactivation was not reached until all 16 mols of pyridoxal phosphate were bound. The carboxylase and the oxygenase activities declined in parallel.

SDS gel electrophoresis of enzyme-pyridoxal phosphate complexes reduced with $NaB^3H_4$ revealed no tritium incorporation into the small subunit under conditions producing complete inactivation. Introduction of covalently bound pyridoxal phosphate resulted in a net charge change on the subunits, so that specificity of pyridoxal phosphate incorporation could also be monitored directly by gel electrophoresis in 8 M urea. Even under conditions such that 16 pyridoxal phosphate molecules were bound, only the mobility of the large subunit was changed. For both activities, modification of the enzyme decreased $V_{max}$ values but did not change $K_m$ values, which indicates only a decrease in the amount of catalytically active enzyme and not a change in the catalytic properties of the enzyme.

Bicarbonate and $Mg^{2+}$ competitively reduced the pyridoxal phosphate inhibition of the spinach carboxylase (Figure 2 of ref. 20). This is in contrast to the enhancement of inhibition reported for the enzyme from R. rubrum (23). In the absence of $Mg^{2+}$, RuBP, alone or with bicarbonate, provided full protection against pyridoxal phosphate inactivation. Although the number of pyridoxal phosphate molecules incorporated was reduced by 16, a small amount of nonspecific binding of pyridoxal phosphate still occurred (21). RuBP was at first reported to protect about half the reactive lysyl residues against pyridoxal phosphate (20). This was an error caused by the experiments being done in the presence of $Mg^{2+}$ and incubated for 20 min to establish the Schiff base equilibrium. Under these conditions the RuBP was slowly lost through catalytic reaction with $O_2$ and $CO_2$, which had not been excluded from the incubation mixture, and therefore the protective effect was lost.

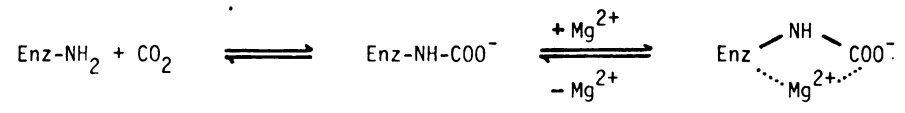
On the basis of these data, the simplest explanation is that the 16 essential amino groups are located in the active-site region. A more complicated explanation would be that RuBP exerts its protective effect both at the catalytic site directly and at an allosteric site through a proposed RuBP-induced conformational change of the enzyme.

To gain further information about the function of the two lysyl groups, the reactivation pattern of the pyridoxal phosphate-inhibited enzyme during dissociation of the Schiff base complex was studied (21). From low initial inhibition, reactivation proceeded in a first-order reaction, independent of whether enzyme activity was measured directly during reactivation or after samples taken at different times had been fixed by $NaBH_4$ reduction. From high levels of pyridoxal phosphate inhibition, the reactivation followed first-order kinetics with the same rate constant only in the experiment that included $NaBH_4$ reduction. Direct assay showed a clear delay in the reactivation process, as if a rate-limiting step were imposed on the overall process. This suggested that the lysyl groups reacting only at high pyridoxal phosphate concentrations are at the activator sites, and that the delay in regaining activity is due to slow activation of the enzyme with $CO_2$ and $Mg^{2+}$ after liberation of the pyridoxal phosphate. This has to take place before any particular subunit is catalytically active. The 8 amino groups reacting at low pyridoxal phosphate concentration appear to be at the catalytic sites. This is consistent with the reactivation kinetics and with the unaltered $K_m$ values of the pyridoxal phosphate-modified enzyme. The structural similarity between RuBP and pyridoxal phosphate, i.e., that the carbonyl group and one phosphate are 3 carbons apart, may account for a higher affinity for pyridoxal phosphate at the substrate site.

Differential labeling of the enzyme first with p-chloromercuribenzoic acid and then with pyridoxal phosphate indicated that one group of 8 of the lysyl residues was protected from the pyridoxal phosphate. Removal of the mercuribenzoate group by dithiothreitol left the enzyme inactive with only 8 pyridoxal phosphate molecules bound to it. Although both p-chloromercuribenzoic acid and iodoacetamide inactivate the enzyme, and the binding of each can be prevented by RuBP, only the large p-chloromercuribenzoate group blocks pyridoxal phosphate binding. Since modification of the sulfhydryl group(s) prevents binding of RuBP, a sulfhydryl group(s) must be very close to the binding site for RuBP. Therefore, the 8 lysyl groups excluded from reaction with pyridoxal phosphate in the presence of p-chloromercuribenzoic acid are thought to be located at the catalytic site, and the other group of 8 that are still available for Schiff base formation are possibly located in the vicinity of the activator site.

The function of the $\epsilon$-amino group of lysine at the catalytic site is most likely noncovalent binding of $CO_2$, since pyridoxal phosphate appears as a competitive inhibitor for $CO_2$ (20). However, lysyl groups, there, may also be involved in proton transfer and

binding of phosphate groups. An ε-amino group of lysine at the activator sites could provide the requirements for allosteric binding of $CO_2$ and $Mg^{2+}$ that would be exothermic, reversible, and pH dependent according to the model in Scheme 3, which is based on the model for $CO_2$ activation (17):




Supporting the model in Figure 1 for two types of primary amino groups is the establishment of two different lysyl residues, also, with the use of bromodihydroxybutanone bisphosphate (29). Since this affinity label is one carbon atom shorter than RuBP, it may have greater mobility at the active site and therefore react with either lysyl group; but, once bound to one of the two, the first molecule may preclude binding of another to the second amino group because of a high charge density through the phosphate groups. This does not explain the low stoichiometry of 4 to 5 moles of reagent per mole of enzyme for inhibition, when the stoichiometry of inhibition with CRBP clearly indicates the requirement of 8 equivalents per mole of enzyme (11). The determination of bound bromodihydroxybutanone BP is based either on $^{32}P$ measurement of the incorporated affinity label or on $^{3}H$ measurements after $NaB^{3}H_4$ reduction of the enzyme-inhibitor complex. Both methods are subject to errors due to the known lability of the affinity label (loss of one or two phosphate groups) (28) and to possible isotopic effects during reduction with $NaB^{3}H_4$.

The $A_2$ enzyme from R. rubrum binds two pyridoxal phosphate molecules per molecule or one per subunit, but is inactivated after blocking of the first catalytic site (24). This agrees with data obtained with bromodihydroxybutanone BP for the same enzyme (25), but is in contrast to the finding that two pyridoxal phosphate molecules must be bound to each subunit of the spinach enzyme. One suggested reason for this difference is that RuBP carboxylase from R. rubrum may behave like a half-site enzyme. Although the amino acid compositions of the large subunits of the carboxylase from all its various sources are apparently similar, there should be some differences between the $A_2$ and the $A_8B_8$ enzyme, expressed in catalytic and regulatory properties.

## ARGININE AT BINDING SITE FOR RuBP

Arginine residues in enzymes serve a general function as binding sites for negatively charged groups such as phosphate or carboxylate ions (31). Similarly, RuBP carboxylase/oxygenase is

inactivated by reagents specific for arginyl residues, i.e., 2,3-butanedione and phenylglyoxal. McFadden's group (32), using 2,3-butanedione with borate to bind a third of the total arginines, noted enzyme inhibition that was partially protected against by the product 3-phosphoglycerate, as if the modified arginines were at the active site. Phenylglyoxal, which does not require borate, appears to be a better reagent for arginyl residues in this enzyme. Inactivation follows saturation kinetics, and ~ 25 to 30 arginyl residues are blocked, as judged by amino acid analysis. RuBP has given about 70% protection against phenylglyoxal inhibition (C. Paech and M. Spellman, unpublished), although Hartman's group (33) reports finding no such protection.

These preliminary results indicate that one or more arginyl residues at the active site probably function in binding the phosphate of RuBP. Further work is necessary to decide whether both phosphate groups of RuBP are bound by arginine or whether some other cationic group such as a lysyl $\epsilon$-amino group may also be involved.

## STUDIES OF THE RuBP BINDING SITE WITH SUBSTRATE ANALOGS

RuBP carboxylase/oxygenase is specific for RuBP as a substrate for carboxylation and oxygenation. A very low dissociation constant for the enzyme-RuBP complex has been estimated: 0.5 $\mu$M (34) or $\leq$1 $\mu$M (12). In fact, the RuBP-enzyme complex can be separated from excess RuBP by gel filtration, and the bound RuBP subsequently detected by the carboxylase reaction. Whereas most sugar mono- and bisphosphates are poor inhibitors, xylitol BP (S. D. McCurry, unpublished, but see ref. 35) and xylulose bisphosphate (XuBP) (36) are powerful, competitive inhibitors with respect to RuBP. The maximum inhibitory effect of either one is manifested after ~20 min, and the binding constant for xylitol BP is <1 $\mu$M (McCurry, unpublished). Like CRBP, $^3$H-xylitol BP could not be dialyzed away from the enzyme. XuBP and xylitol BP differ from RuBP stereochemically at C3, and their severe inhibitory effect may reside in hydrogen-bonding between that hydroxyl group and the base at the catalytic site that participates in enediol formation for RuBP. The energy required to break this hydrogen bond could contribute substantially to stability of the enzyme-inhibitor complex, as evidenced by ribitol BP being a poor inhibitor (F. J. Ryan, unpublished).

XuBP has not been reported to occur in nature, but it could be readily formed *in situ*, and it is prepared by aldolase *in vitro* from dihydroxyacetone phosphate and glycolaldehyde phosphate. Apparently the formation of free glycolaldehyde phosphate would be lethal, and this could explain why all $C_2$ transformations in carbohydrate metabolism involve a glycolaldehyde-thiamin pyrophosphate complex with transketolase rather than free glycolaldehyde phosphate. Conceivably XuBP could be formed by epimerization from RuBP in the active site of the carboxylase from the enediol intermediate if it were allowed to revert, but the absence of such reversion is evidenced

by the lack of $^3H$ exchange in the absence of $CO_2$ with RuBP labeled with $^3H$ at C3 (6). The question of whether or not such an epimerization would be permitted by the enzyme remains moot.

The most effective inhibitor known for carboxylase/oxygenase is the transition-state analog CRBP (11, 12), a competitive, time-dependent, irreversible inhibitor. Each enzyme molecule has 8 binding sites for this compound, and a divalent cation is required for binding. Synthesis of CRBP from cyanide and RuBP produces two epimers, CRBP and 2-carboxy-D-arabinitol 1,5-bisphosphate, and the proportion of inhibition due to each is unknown. These two compounds have been synthesized and purified in our laboratory (J. Pierce, unpublished), and their effects on RuBP carboxylase/oxygenase are under investigation. In addition, the compounds 2-carboxy-D-xylitol 1,5-bisphosphate and 2-carboxy-D-lyxitol 1,5-bisphosphate have been synthesized and purified. Determination of the relative effects of these compounds on the enzyme, coupled with knowledge of the stereochemistry of known inhibitors of the enzyme, may allow estimation of the geometry of the active site.

It has been suggested that carboxylase inhibition by RuBP is an allosteric effect (37) or is due to an interaction with lysine at the catalytic site (19). Attempts to detect a Schiff base between either RuBP or XuBP at a lysine by reduction with $NaB^3H_4$ have not been successful (Paech and McCurry, unpublished). Summarized below is new evidence that the RuBP inhibition is caused by other inhibitory compounds, leaving unanswered the question whether RuBP itself has any regulatory control of enzyme activity.

## RuBP DEGRADATION PRODUCTS AS INHIBITORS

Inhibition of the initial rate of carboxylase activity by high concentrations of RuBP has been known for many years (14). Pre-incubation of the enzyme with RuBP in the absence of $CO_2$ results in inhibition. Therefore the enzyme assay is initiated with RuBP after first activating with $CO_2$ and $Mg^{2+}$. Although RuBP inhibition has been ascribed to an allosteric effect (37) or to an interaction at the active site (19), it does not occur in assays with whole chloroplasts (38). We have obtained data indicating that the "substrate inhibition" phenomenon produced with solutions of RuBP is due to the presence of inhibitors formed from the RuBP by epimerization at C3 or by β-elimination of the C1 phosphate (39).

The idea that an inhibitory compound was present in RuBP arose from the observation that different batches of RuBP had different initial reaction rates although all contained nearly the same amount of RuBP as judged by enzyme, phosphate, and carbohydrate analyses. The initial catalysis rate, when a reaction is started with RuBP, is almost unaffected by the suspected impurities; but, with time or in the absence of RuBP after the substrate is used up (Figure 3), time-dependent inhibition of these impurities becomes apparent. Addition of a second aliquot of substrate to the same enzyme results

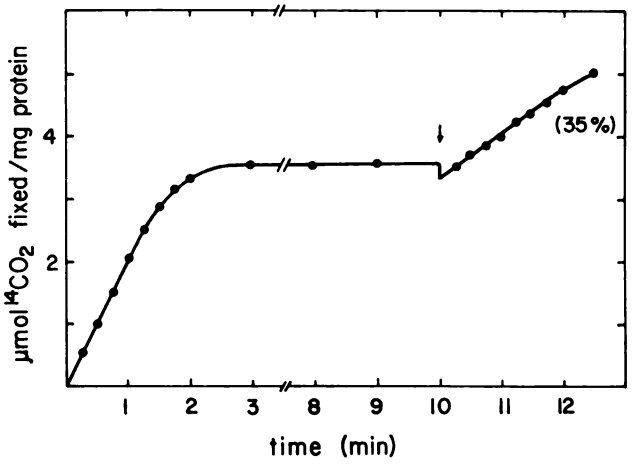
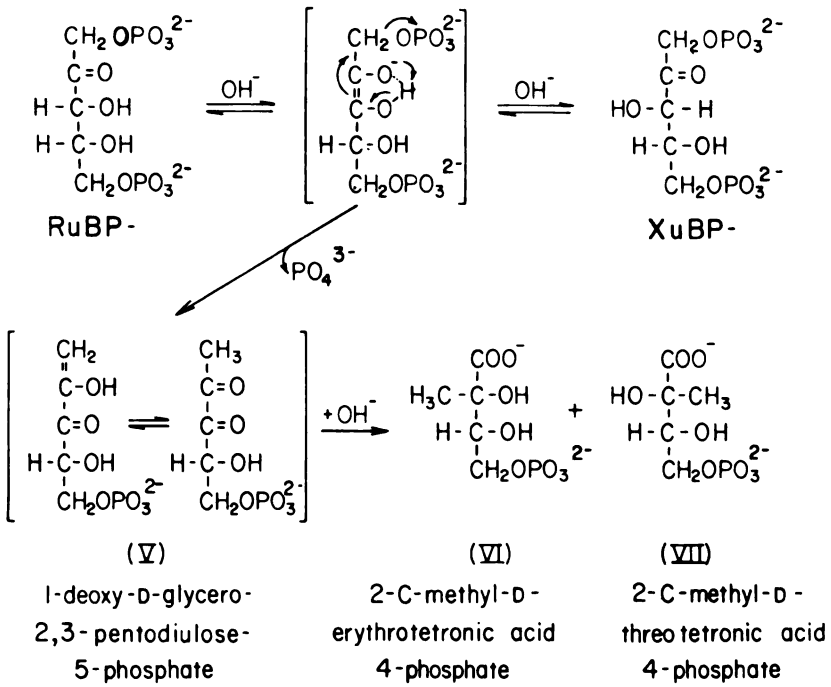



Figure 3. Time course of RuBP carboxylase reaction with a limiting amount of RuBP. After the reaction had gone to completion, a second aliquot of substrate was added, and the change in the initial rate was recorded as percent inhibition (39).

in a much slower, or inhibited, rate of carboxylation. Product inhibition was ruled out (39). In RuBP solutions, the inhibitors build up with increasing storage time, particularly at pH above 8 or elevated temperatures.

Incubation of RuBP solutions at pH 11 and 30° results in total loss of phosphate at carbon 1. Under the condition of the carboxylase assay, RuBP decomposes at the rate of 1.25% per hr. Mild base treatment causes substantial inhibition without substantial loss of substrate. Fresh solutions of enzymatically synthesized RuBP have essentially no inhibitory components, but RuBP from commercial sources is inhibitory because of degradation products formed during the isolation steps.

Two inhibitory products have been found in RuBP solutions. One is XuBP, which arises from nonenzymic epimerization, as shown in reaction Scheme 4:




Proof of its presence and structure has been obtained by thin-layer and gas-liquid chromatography after reduction and phosphatase treatments. XuBP preparations used as substrate with isolated enzyme cause some initial $CO_2$ incorporation into 3-phosphoglycerate (39), but this catalysis is due to the presence of a little RuBP arising from epimerization rather than to XuBP acting as a substrate.

The other inhibitory compound arising from RuBP appears to be a diketo degradation product formed from one or the other or both of the bisphosphates by β-elimination. Its structure has not been unequivocally established, but the UV absorption spectra, NMR spectra, and phosphate elimination support the idea that it is 1-deoxy-D-glycero-2,3-pentodiulose 5-phosphate. Additional support comes from treatment of RuBP solutions with o-phenylenediamine resulting in partial removal of the inhibitory effect: compound V is relatively unstable and probably undergoes rearrangement to a stable end product having properties consistent with those of a branched-chain compound (Scheme 4), clearly recognizable by NMR spectroscopy after extensive base treatment of RuBP. Since the diketo compound, which appears to be the inhibitory component, is rather unstable, it accumulates to only a small extent and does not persist in the reaction mixture.

The physiological implication of these data is that RuBP is an unstable substrate with toxic degradation products, and therefore

free RuBP must not occur in chloroplasts. Under normal conditions in chloroplasts the estimated concentration of RuBP is 0.2 to 0.4 mM but that of RuBP binding sites in the carboxylase is 8 times as high: 3 mM (38, 40). Therefore, because of the very low dissociation constant (0.5 μM) for the enzyme-RuBP complex (11), it is unlikely that this labile substrate ever exists free in solution in the chloroplast. In chloroplasts, only 40 to 60% of the carboxylase is estimated to be active at any one time (41). Our hypothesis is that one function for the large amounts of the carboxylase protein in chloroplasts is storage of RuBP to prevent its breakdown to inhibitory products.

## CARBOXYLASE/OXYGENASE RATIO AND $O_2$ SITE

Since RuBP carboxylase activity initiates the photosynthetic carbon cycle and RuBP oxygenase activity diverts carbon flow into phosphoglycolate formation and photorespiration, the regulation and control of these two overlapping and competing carbon pathways appears to be a major consideration for net photosynthesis. Comparison with other systems suggests that regulation may depend on a combination of substrate availability and alteration in enzyme activity for reactions that initiate metabolic pathways or are at branch points. Indeed, compelling evidence indicates that RuBP carboxylase and oxygenase activities are competitively dependent on the $CO_2$ and $O_2$ substrate concentrations (42), but some evidence suggests that the amount of carbon flow through the glycolate pathway in the leaf is in part independent of the $CO_2/O_2$ ratio.

A change in enzyme activity at branch points is the general way of dealing with alterations in limiting amounts of protein, through feedback regulants or effectors. The carboxylase/oxygenase, however, is present in large excess. Effectors for this enzyme have been reported, but they alter the carboxylase and oxygenase activities similarly (43). All the inhibitors or substrate analogs so far examined also affect the carboxylase and oxygenase activities similarly. So far there is no confirmed evidence for any alteration in the carboxylase/oxygenase ratio by any mechanism other than $CO_2$ and $O_2$ competition. We cannot confirm the claim that RuBP carboxylase and RuBP oxygenase are two different proteins (44) (manuscript in preparation). There have been reports of variations in the activity ratio of preparations from different stages of leaf development, from leaves versus fruit, from different plants or mutants, after treatment with different inhibitors or effectors, and at different degrees of purification (literature not reviewed). However, many of these reports cannot be confirmed; until such reports are confirmed by several groups, a change in the carboxylase/oxygenase ratio must be considered unproven. One of the difficulties is that the oxygenase assay is relatively insensitive compared with the carboxylase assay. For instance, the enzyme must be $CO_2$ activated, but $CO_2$ is an inhibitor of the oxygenase. The $CO_2$ and

$Mg^{2+}$-activated enzyme, upon dilution into a $CO_2$-free oxygenase assay medium, loses activity with a half-life of 1 min (N. P. Hall, unpublished).

The nature of the RuBP oxygenase reaction, the possible existence of an $O_2$ site or an undiscovered cofactor for the oxygenase, and whether the reaction can be regulated are critical unanswered questions. It is very unlikely that the lysyl $CO_2$-binding sites could bind or activate $O_2$. Despite intensive search, no cofactor has been found that is required for the oxygenase reaction. An early report that $Cu^{2+}$ is present in the enzyme but has no effect on the carboxylase activity (45) has twice been reexamined without finding $Cu^{2+}$ or any effect of $Cu^{2+}$ on oxygenase activity (2, 13). The speculation that inherent amino acid functions of the enzyme, such as sulfhydryl groups, could serve as activator and/or binding site for the oxygen (46) was based on a claim that differential regulation of carboxylase and oxygenase could be achieved by modifying sulfhydryl groups with glycidate or iodoacetamide; but this could not be confirmed by several laboratories including ours. The basis for $CO_2$ and $O_2$ being competitive inhibitors of each other may be competition for a reaction with the other substrate, RuBP, rather than for a reactive-site component. In fact the most important property of this enzyme may be that it catalyzes the carboxylation reaction in the face of an unavoidable oxygenase activity.

The unknown nature of the oxygenase is of great interest mechanistically as well as physiologically. RuBP oxidation is an enzymic property of the RuBP carboxylase/oxygenase protein. Without the activated enzyme RuBP oxidation is relatively slow. Not only does RuBP rearrange and decompose to inhibitory products as described above, but it may also be attacked by strong oxidants equivalent to those found in the chloroplasts (F. J. Ryan, unpublished; J. Pierce, unpublished; N. P. Hall, unpublished). Just as we are proposing that RuBP is stabilized against rearrangement in the chloroplast by being stored at the RuBP binding site on the enzyme, it may also need protection against oxidation in the chloroplasts. The enzyme-RuBP storage complex is inactive as either a carboxylase or an oxygenase, but $CO_2$ and $Mg^{2+}$ activate both activities (18). This activation by $CO_2$ or by effectors, which is necessary for catalysis, is to be avoided at other times in the presence of limiting amounts of $CO_2$ because of the oxygenase reaction. Upon activation, the $O_2$ attack may be an essential part of the overall photosynthetic process, or it may be an unavoidable reaction of an intermediary enolate form of the RuBP in the active site of the enzyme. But exactly how the oxygenase reaction occurs is not known.

## SUMMARY

The catalytic region of each large subunit of RuBP carboxylase/oxygenase from spinach must contain two anionic binding sites for RuBP, of which one or both appear to be arginine. This site may

serve for storage of labile RuBP and as a catalytic site upon activation. The $CO_2$ substrate site includes a lysyl residue which might take part in orienting the $CO_2$ adjacent to carbon 2 of the RuBP. The lack of evidence for an $O_2$ binding site suggests that the oxygenase activity may proceed by direct oxidation of the activated RuBP-enzyme complex. The $CO_2/Mg^{2+}$ activator site is also in the large subunit and involves another lysyl residue for the formation of a carbonate-$Mg^{2+}$ complex. The conformational change induced during the formation of this complex is conceptually an orientation of the base opposite C3 of RuBP for catalyzing the rate-limiting enolization step.

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## DISCUSSION

McFADDEN: There are about 80 to 100 sulfhydryl groups per molecule of the higher plant enzyme. How extensively modified by mercurial reagent (PCMB) was your RuBP carboxylase when tested for RuBP binding? If extensive modification had occurred, it would be important to probe directly for RuBP binding (i.e., by gel filtration or equilibrium dialysis). Was this done?

PAECH: The enzyme was incubated with a 50-fold molar excess of p-chloromercuribenzoic acid for 20 min. This resulted in modification of ~30 SH groups, according to T. Sugiyama et al. (Arch. Biochem. Biophys. 125, 98-106), and complete loss of enzyme activity. RuBP binding was determined directly by gel filtration (see ref. 21).

MIZIORKO: You mentioned that a very tight complex is formed with both XuBP and xylitol BP. Is this type of complex stable to treatment by passage over a Sephadex column?

McCURRY: Yes, very stable.

MIZIORKO: Is there a divalent ion requirement for formation of the complex?

McCURRY: No.

MIZIORKO: Is a covalent adduct formed in either or both cases?

McCURRY: We cannot envision one. We tried to reduce the complex, as it had been suggested that RuBP might form a Schiff base in the active site with carbonyl, and we thought of applying the same hypothesis to XuBP. We could not show any reduction, but that is no guarantee it will not occur.

MIZIORKO: Do you have to age the incubation mixture in order to get a tight complex that is stable to Sephadex chromatography or can you just do an initial mixing?

McCURRY: Do you mean running over the column within 1 or 2 min?

MIZIORKO: Yes. You mentioned that it was a slow process.

McCURRY: Yes, ~90% inhibition occurs within 2 min but complete inhibition takes 20 min.

MIZIORKO: According to a model which requires two $CO_2$ sites per subunit of enzyme, modification of either the substrate $CO_2$ site or the activator $CO_2$ site should result in loss of catalytic activity. The modification data clearly show two types of site, with substantial enzyme activity remaining in the region of pyridoxal phosphate titration where virtually complete modification of one type of site is expected. Is the residual activity consistent with the two-site model?

TOLBERT: According to our proposal, binding of the first group of pyridoxal phosphate molecules is facilitated because pyridoxal phosphate is structurally similar to RuBP. At higher pyridoxal phosphate concentrations, significant amounts also begin to form Schiff base at the second site. Because of the increasing competition between the two groups of pyridoxal phosphate binding sites, which becomes significant after 6 pyridoxal phosphate molecules

are bound to the enzyme, the reaction with the first set of amino groups would not go to completion when 8 pyridoxal phosphate molecules were bound, nor would full inactivation occur until saturation of all 16 amino groups with pyridoxal phosphate.

HARTMAN: The lack of protection by bicarbonate against modification by pyridoxal phosphate appears inconsistent with your hypothesis that the site labeled is involved in binding of $CO_2$.

PAECH: The data from kinetic experiments in Figure 2 of ref. 20 clearly demonstrate protection by bicarbonate against pyridoxal phosphate inhibition. In order to measure spectrophotometrically Schiff base formation between the enzyme and pyridoxal phosphate, the enzyme was equilibrated at 1 mg/ml with 0.5 mM pyridoxal phosphate and 0 to 300 mM bicarbonate. Although dissociation constants are not available for the enzyme-$CO_2$ complex and the enzyme-pyridoxal phosphate complex, it is clear from the kinetic experiments in ref. 20 that the two competing ligands were present in an unfavorable ratio, i.e., too little bicarbonate. Therefore, this second research approach indicated incorrectly a lack of protection by $CO_2$.

WILDNER: What is the chemical evidence that the activation of RuBP carboxylase is based on carbamate formation at the lysine of the activation site?

TOLBERT: Carbamate formation with a basic amino group, in this case ε-amino group of lysine, has been the mechanism generally cited for $CO_2$ modification of enzyme activity. As pointed out in the text, carbamate formation is exothermic and pH sensitive, as required for RuBP carboxylase activation. Further chemical and physical proof for this carbamate formation will be forthcoming.

CHOLLET: Are the products of RuBP degradation irreversible inhibitors? If you assay the enzyme after passage over a Sephadex G25 column, or after dilution, is it still inhibited?

TOLBERT: The xylitol BP reaction is essentially irreversible.

CHOLLET: In doing chemical modification studies, we, and perhaps the Oak Ridge people and Bob Divita, have used substrate protection, as you have. If we use control enzymes with no modifier, plus or minus 5 mM RuBP, preincubated for 1 to 1½ hr, then dilute out or pass over a Sephadex G25 column, the two enzymes (plus or minus RuBP) have identical activity. Since the time is sufficient for degradation products to form, either they are readily reversible or we do not see them; the data of Fred Hartman and Bob Divita indicate that they do not see them either.

PIERCE: It has not been shown whether or not the diketo compounds are reversible. Enzyme incubated with what we have proposed to be inhibitors arising from RuBP have not been passed over columns.

HARTMAN: I am not aware of any evidence suggesting that ionized sulfhydryl is the essential base obstructing the C3 proton.

TOLBERT: We do not know what base is there. The only thing we do know is the sulfhydryl protection. It has been proposed that there are two sulfhydryl groups.

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# INHIBITION OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE BY RIBULOSE-1,5-BISPHOSPHATE EPIMERIZATION AND DEGRADATION PRODUCTS

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Summary: Xylulose-1,5-bisphosphate in preparations of ribulose-1,5-bisphosphate (ribulose-$P_2$) arises from non-enzymic epimerization and inhibits the enzyme. Another inhibitor, a diketo degradation product from ribulose-$P_2$, is also present. Both compounds simulate the substrate inhibition of ribulose-$P_2$ carboxylase/oxygenase previously reported for ribulose-$P_2$. Freshly prepared ribulose-$P_2$ had little inhibitory activity. The instability of ribulose-$P_2$ may be one reason for a high level of ribulose-$P_2$ carboxylase in chloroplasts where the molarity of active sites exceeds that of ribulose-$P_2$. Because the $K_D$ of the enzyme/substrate complex is $\leq 1$ μM, all ribulose-$P_2$ generated *in situ* may be stored as this complex to prevent decomposition.

The hysteretic substrate inhibition of ribulose-$P_2$ carboxylase/oxygenase (EC 4.1.1.39) by ribulose-$P_2$ (1-4) has been attributed to an allosteric binding site (3) or competitive binding for ribulose-$P_2$ and $CO_2$ (5), either of which could produce a conformational change (6,7). However purified carboxylase cannot be fully reactivated upon addition of $Mg^{2+}$ and bicarbonate once it has been preincubated with ribulose-$P_2$ (3). Partial inactivation does not occur for enzyme activity in whole chloroplasts where ribulose-$P_2$ may accumulate in the absence of $CO_2$ to a 16 fold molar excess over the carboxylase binding sites, but the carboxylase activity is fully restored within 10 min after addition of bicarbonate (8). Much of the substrate inhibitory effect and the difference between the isolated enzyme and the chloroplasts can be explained by 2 impurities which we find in ribulose-$P_2$ preparations used for *in vitro* assays. One is xylulose-$P_2$, an inhibitor of the enzyme (9), and the other apparently is a diketo compound, most likely 1-deoxy-D-*glycero*-2,3-pentodiulose-5-phosphate. Other α-dicarbonyl compounds are also inhibitors of this enzyme (10,11). The carboxylase inhibition by newly prepared ribulose-$P_2$ was small, as if there had been insufficient time for inhibitor formation.

Abbreviations: ribulose-$P_2$,ribulose-1,5-bisphosphate; deoxypentodiulose-5-P, 1-deoxy-D-*glycero*-2,3-pentodiulose-5-phosphate; xylulose-$P_2$, xylulose-1,5-bisphosphate.

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## MATERIALS AND METHODS

Ribulose-$P_2$ carboxylase/oxygenase from spinach leaves was prepared as described elsewhere (12). Ribulose-$P_2$ was prepared enzymatically from ribose-5-P (13). Xylulose-$P_2$ was synthesized with rabbit muscle aldolase from glycolaldehyde-P and dihydroxyacetone-P (14). Proof of structure and purification have been described (9). (1-$^{13}$C) xylulose-$P_2$ was synthesized from (1-$^{13}$C) fructose-1,6-$P_2$, which was a gift from A. S. Serianni of Mich. State Univ., and glycolaldehyde-P by aldolase. $NaH^{14}CO_3$ and $NaB^3H_4$ were from Amersham-Searle; other chemicals were from Sigma Chemical Co. and were used without further purification. Ribulose-$P_2$ carboxylase activity was determined using a radiometric assay (15). The enzyme was incubated at 2mg/ml for at least 1 h in the assay buffer composed of 0.1 M N,N-bis(2-hydroxyethyl)glycine (Sigma, "Bicine") at pH 8.0 and 30°, 10 mM $MgCl_2$, 0.2 mM $Na_2$-EDTA, and then made to 10 mM $NaHCO_3$ and kept for 30 min at 30°. Aliquots from this stock solution were equilibrated in the assay buffer at 20 mM $NaH^{14}CO_3$ (0.16 Ci/mol) and a final protein concentration of 80 µg/ml (0.14µM). The reaction was initiated by addition of ribulose-$P_2$ to a final concentration of 0.5 mM and was run for 20 to 60 sec, stopped by acid and the fixed $^{14}$C counted as described elsewhere (5). Under these conditions the specific activity of the enzyme preparations was 1.0 to 1.4 µmol $CO_2$ fixed per min per mg protein. Protein determinations were performed according to a modified Lowry procedure (16). Carbon-13 n.m.r. spectra were obtained with a Bruker WP-60, 15.08 MHz Fourier-transform spectrometer equipped with quadrature detection. The spectrometer was locked to the resonance of $D_2O$ in a capillary. Chemical shifts are given relative to external tetramethylsilane (0 ppm) and are accurate to within 0.1 ppm.

## RESULTS AND DISSCUSSION

*Evidence for Inhibitors in Ribulose-$P_2$ Preparations-* Early reports (1,2) of ribulose-$P_2$ inhibition of the carboxylase indicated that saturating or higher concentrations had to be used. In our experience the extent of inhibition was not reproducible. Different batches of ribulose-$P_2$ solutions at apparently identical concentrations, as judged by phosphate (17), carbohydrate (18), and enzymatic analyses with an excess of ribulose-$P_2$ carboxylase and $NaH^{14}CO_3$ of a known specific activity gave different initial rates of carboxylation (data not shown). These differences became insignificant when ribulose-$P_2$ concentrations below 5 times the $K_m$ were used in the assay. At these low concentrations, short reaction times of 10-40 sec are required, and the inhibition, which is time-dependent, is not significant. Nevertheless the inhibitory effect from low concentrations of ribulose-$P_2$ can be demonstrated (Fig. 1). After complete utilization of a known amount of substrate, severe inhibition occurred during a second reaction period initiated by adding ribulose-$P_2$ to the same concentration as in the first stage. Several sources of inhibition could be ruled out. Product inhibition under these conditions was negligible; 1 mM 3-P-glycerate had no effect and 0.5 mM P-gycolate caused a 4% inhibition of the inital rate. Also mono- and divalent cations (e.g.

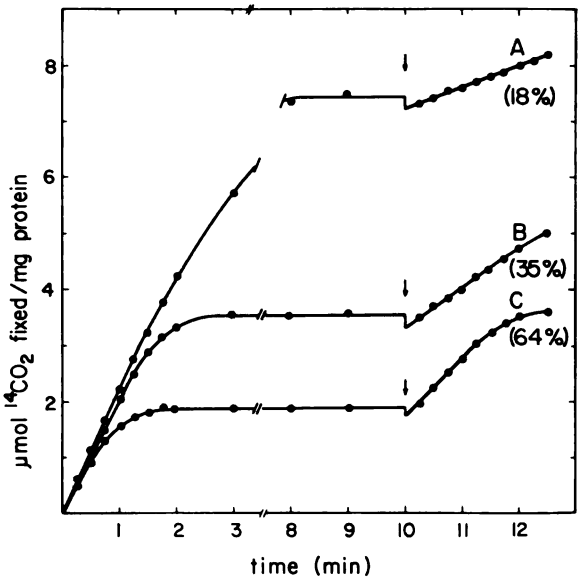



Figure 1. Progress curve of ribulose-$P_2$ carboxylase reactions with (A) 0.5, (B) 0.25, (C) 0.125 mM ribulose-$P_2$. 0.3 ml of a ribulose-P solution was added to 7.2 ml of the assay buffer containing the enzyme at 80 µg/ml and $NaH^{14}CO_3$ at 20 mM final concentration. Ten aliquots of 0.25 ml were withdrawn at indicated times and added to 0.2 ml 2 N HCl. The arrows indicate initiation of the second progress curve by addition of a similar amount of ribulose-$P_2$ (0.2 ml into 4.8 ml) as in the first reaction. A corresponding volume was removed prior to addition of ribulose-$P_2$ in the second run. Thus, the zero point is 4% lower and a maximum of 96% of the first initial rate in the second progress curve would represent no inhibition. The values in parentheses represent the observed percent inhibition of the first initial velocity.

$Ba^{2+}$, $Ca^{2+}$, $Sr^{2+}$) as well as $NaVO_3$ (19) were not inhibitory up to 10 mM.

*Stability of Ribulose-$P_2$* - Treatment of ribulose-$P_2$ solutions at 30° and pH 11 increased the inhibition to a maximum of about 70%, presumably from reaching an inhibitor/substrate equilibrium. Prolonged base treatment or more elevated temperature caused more rapid substrate loss and appearance of orthophosphate, but no further inhibition (Fig. 2). Loss of ribulose-$P_2$ during alkaline treatment correlated with the formation of inorganic phosphate (20) (Fig. 3). When ribulose-$P_2$ was no longer detectable enzymatically, approximately 50% of its total phosphate had been liberated. No ribulose-$P_2$ was regenerated by incubating samples, taken during the time course of alkaline treatment, with a mixture of phosphoriboisomerase, phosphoribulokinase, and ATP; therefore neither ribulose-5-P nor ribose-5-P were formed during the alkaline treatment.

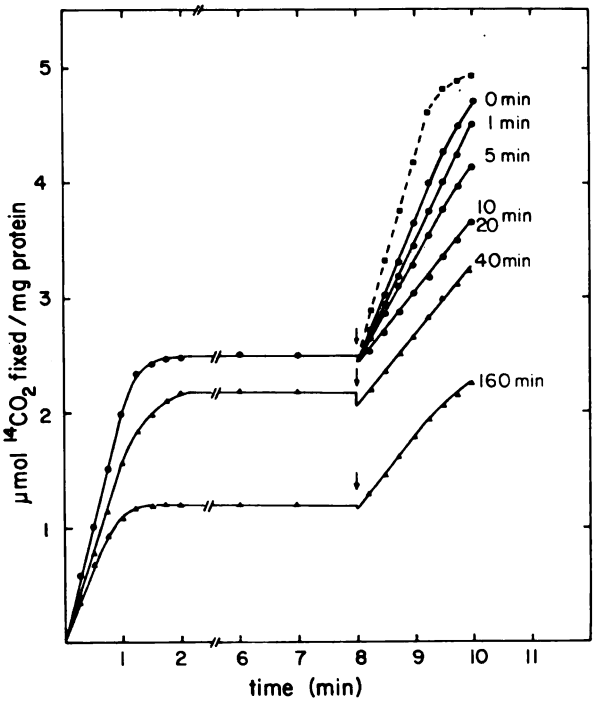


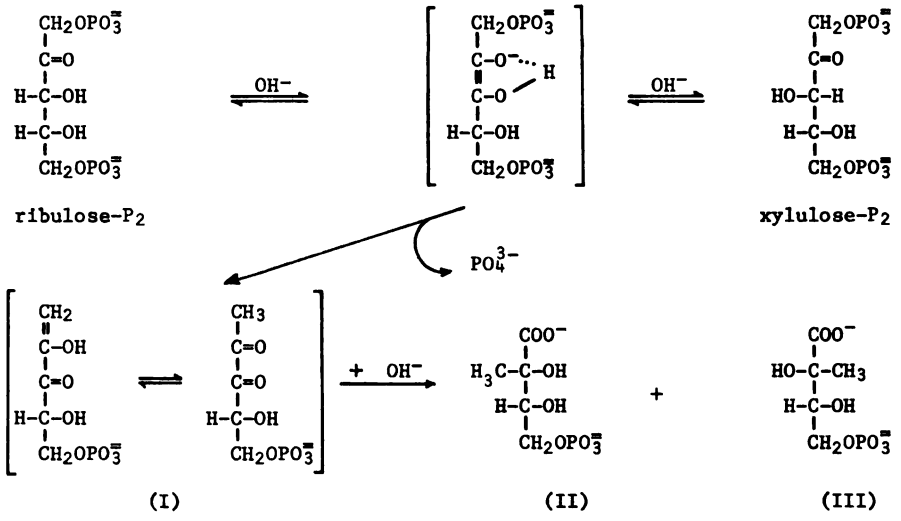

Figure 2. Time course of the ribulose-$P_2$ carboxylase reaction with 0.2 mM ribulose-$P_2$. Ribulose-$P_2$, 5 mM, was incubated at pH 11 and 30° for 1, 5, 10, and 20 min as indicated. The pH was then adjusted to 8.0 and an aliquot was used to run two consecutive progress curves (●——●) as described in the legend to Fig. 1. Another sample was incubated for 40 and 160 min at pH 11 and 40° and used for the same experiment (▲——▲). When ribulose-$P_2$ was generated from ribose-5-P enzymatically, and used directly the initial rate in the second progress curve reached 88% of the initial rate, which because of the dilution was over 90% of the maximum expected (■---■).

Even at pH 8.3 and 30° (the enzyme assay conditions) loss of ribulose-$P_2$ amounted to 1.25% per hour (data not shown).

Ribulose-$P_2$ solutions essentially free of inhibitor were obtained in a coupled assay in which ribulose-$P_2$ was generated from ribose-5-P or ribulose-5-P with phosphoriboisomerase, phosphoribulokinase, ATP and used immediately (■----■ in Fig. 2). In this case there was little effect from inhibitor accumulation during the second cycle of the repeat assay.

*Mechanism of Degradation and Identification of Inhibitors* - Ribulose-$P_2$ is usually prepared enzymatically from ribose-5-P and stored as the barium salt which is unstable even at 4° (13). The usual way to prepare the potassium or sodium salt is by mixing the barium salt with Dowex 50/$H^+$, filtering, and neutralizing with 6 N KOH. During neutralization, epimerization at C-3 induced by hydroxyl ions would form xylulose-$P_2$, which is a potent inhibitor

of ribulose-$P_2$ carboxylase (9). Alkaline treatment may also lead to phosphate elimination at $C_1$ from the enediol intermediate formed from either ribulose-$P_2$ or xylulose-$P_2$, to yield deoxypentodiulose-5-P. This unstable compound (I) should undergo a rearrangement to form (II) and (III).




(I) 1-deoxy-D-*glycero*-2,3-pentodiulose-5-phosphate
(II) 2-C-methyl-D-*erythro*-tetronic acid 4-phosphate
(III) 2-C-methyl-D-*threo*-tetronic acid 4-phosphate

Evidence for a phosphate elimination mechanism was the appearance of ortho phosphate (Fig. 3) and a time course of degradation of (1-$^{13}$C) xylulose-$P_2$ at pH 11 and 40° (Fig. 4). The starting compound was 91% $^{13}$C enriched at C-1. As the reaction proceeded, the area under the doublet, centered about 69.4 ppm, decreased as methyl resonances at 24.0 and 23.4 ppm increased, reaching their maximum values when no further xylulose-$P_2$ remained. The methyl resonances are assumed to arise from the two major degradation products of the proposed diketo compound via the described mechanism. Similar mechanisms for non-phosphorylated sugars have been proposed (21).

To identify the inhibitors, samples of ribulose-$P_2$ were reduced with $NaBH_4$, pH 7, and treated with acid phosphatase. The products were converted to the pentaacetyl pentitols and subjected to gas liquid chromatography (22). The major peaks were identified as ribitol and arabinitol, present in approximately the same quantities. These peaks were followed by a small xylitol peak. After treatment with $NaB^3H_4$ and phosphate ester cleavage, the tritiated reduction products were separated by thin layer chromatography on

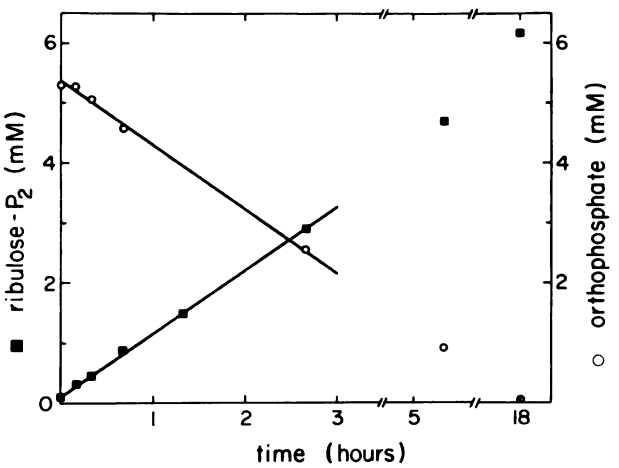



Figure 3. Loss of ribulose-$P_2$ at pH 11 and 40°, and formation of inorganic phosphate as a function of time. The ribulose-$P_2$ content was measured enzymatically with a pure preparation of ribulose-P carboxylase and $NaH^{14}CO_3$ of a known specific activity.

borate-impregnated silica gel plates. The two major labeled spots corresponded to arabinitol and ribitol, and a minor spot corresponded to xylitol. These compounds were recrystallized with the corresponding unlabeled alcohols to constant specific activity. The presence of approximately 1% xylulose-$P_2$ in preparations of ribulose-$P_2$ could account for the inhibitory phenomenon of ribulose-$P_2$ on carboxylase activity. That xylulose-$P_2$ solutions may also epimerize to ribulose-$P_2$ was demonstrated by assaying them with the carboxylase. 3-P-Glycerate was formed, but the reaction reached completion with most of the xylulose-$P_2$ remaining. Thus it is unlikely that xylulose-$P_2$ was acting as a substrate for ribulose-$P_2$ carboxylase.

The β-elimination product from ribulose-$P_2$ has not been characterized unequivocally but is proposed to be 1-deoxy-D- *glycero*- 2,3-pentodiulose-5-phosphate for the following reasons: (a) β-elimination is kinetically favored over hydrolysis under alkaline conditions. The deoxypentodiulose-5-P would not accumulate and its further degradation to the end product shown in the scheme would explain why the inhibitory effect from alkaline treatment did not further increase. (b) Incubation of ribulose-$P_2$ preparations with o-penylenediamine to complex the dicarbonyl compound reduced inhibition of the enzyme by the ribulose-$P_2$ preparation. (c) Dicarbonyl derivatives of carbohydrates are sensitive to oxygen under alkaline conditions and undergo rapid degradation and rearrangement (23). (d) Deoxypentodiulose-5-P belongs to a class of reagents specific for arginyl residues in enzymes (24), and it has been shown that

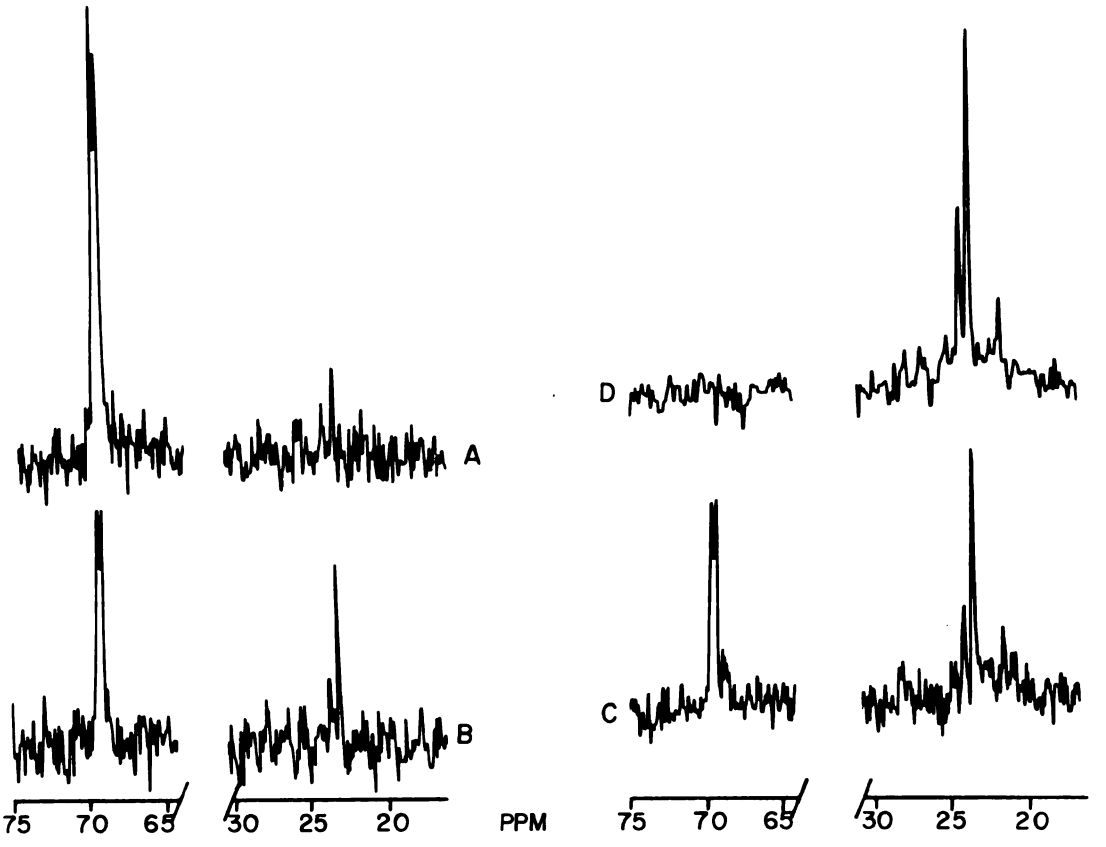



Figure 4. Time course of degradation of (1-$^{13}$C) xylulose-$P_2$ at pH 11 and 40°. (A) 4-16 min, (B) 47-58 min, (C) 125-160 min, (D) 245-295 min. The doublet centered about 69.4 ppm is due to coupling of the $^{31}$P nucleus with the $^{13}$C nucleus at C-1 of (1-$^{13}$C) xylulose-$P_2$ ($J_{C-P}$ = 4.4 Hz).

ribulose-$P_2$ carboxylase is inhibited by such compounds, e.g. phenylglyoxal and 2,3-butanedione (10,11). (e) Dicarbonyl compounds have characteristic absorption spectra which are pH dependent and include peaks in the region 400-460 nm (25). Such spectral properties have been observed with ribulose-$P_2$ solutions (data not shown).

*Physiological Implications* - The rapid formation of inhibitors in solutions of ribulose-$P_2$ is important with respect to kinetic studies and the carboxylase assay. Recognition of these inhibitors may also be the key to some yet unsolved questions concerning the enzyme *in vivo*. The average concentration of ribulose-$P_2$ in chloroplasts is less than the total concentration of binding sites of ribulose-$P_2$ carboxylase (26). Therefore, ribulose-$P_2$ probably never occurs in free solution in the chloroplasts (R.G. Jensen, personal

communication). Conditions for chemical epimerization or β-elimination of ribulose-$P_2$ could occur in the chloroplasts, for example during the day when temperatures may reach 40° and the pH over 8. The presence of a large excess of protein (ribulose-$P_2$ carboxylase) would account for the binding of almost all the ribulose-$P_2$ as soon as it is formed by phosphoribulokinase, as the dissociation constant for the enzyme/ribulose-$P_2$ complex is $\leq 1$ μM (5,27).

If xylulose-$P_2$ and deoxypentodiulose-5-P were formed *in situ* there would be sufficient ribulose-$P_2$ carboxylase to bind these inhibitors without any loss in rate of the whole photosynthetic carbon cycle. Bahr and Jensen (28) have estimated that in the chloroplast only 40 to 60% of the carboxylase is functioning at any given time; the rest may be a storage reserve or trap for ribulose-$P_2$ or these inhibitory products, respectively. The deoxypentodiulose-5-P seems too unstable to accumulate in chloroplasts, so that the removal of xylulose-$P_2$ would be of more serious concern. A phosphatase catalyzing the conversion of xylulose-$P_2$ to xylulose-5-P has not been reported, but such an enzyme would effectively recycle the inhibitor through the photosynthetic carbon cylce.

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# Communication

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# Inhibition of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase by Xylulose 1,5-Bisphosphate*

(Received for publication, July 25, 1977, and in revised form, October 18, 1977)

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## SUMMARY

**Xylulose 1,5-bisphosphate is a potent inhibitor of ribulose-1,5-bisphosphate carboxylase/oxygenase. The enzyme was inhibited 50% at a xylulose 1,5-bisphosphate concentration of 0.56 μM and an enzyme concentration of 0.14 μM. When both ribulose 1,5-bisphosphate and xylulose 1,5-bisphosphate were added simultaneously to the enzyme, this inhibition appeared to be competitive. A preincubation of 20 to 30 min was needed for maximum inhibition. The inhibitory effect of this compound is probably exerted through its binding at the ribulose 1,5-bisphosphate active site, since both the carboxylase and oxygenase activities were inhibited similarly.**

Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) catalyzes the addition of $CO_2$ to ribulose-$P_2$[1] to give 2 molecules of 3-phosphoglycerate, as well as the addition of $O_2$ to ribulose-$P_2$ to give 1 molecule of 3-phosphoglycerate and 1 of phosphoglycolate. These two reactions compete to divide carbon flow between photosynthesis and photorespiration, and therefore become a logical point for control. Studies on the mechanism of action of ribulose-$P_2$ carboxylase/oxygenase contribute to the determination of whether differential regulation of the two activities is possible. Xylulose-$P_2$ was tested because of its similarity to ribulose-$P_2$, for the two differ only in the stereochemistry at carbon 3. Both the carboxylase and oxygenase functions are known to be inhibited by ribulose-$P_2$ (1, 2) and xylitol-$P_2$ (3). Likewise, we have now found that xylulose-$P_2$ is a competitive inhibitor with respect to ribulose-$P_2$, and thus both the carboxylase and oxygenase activities were inhibited.

## MATERIALS AND METHODS

Ribulose-$P_2$ carboxylase/oxygenase was prepared from spinach plants grown in the greenhouse on a 10-h day as described previously (3) and stored at 4° in 50% saturated $(NH_4)_2SO_4$. The purified and desalted enzyme was diluted to a 2 mg/ml stock solution for assay.

The enzymatic preparation of ribulose-$P_2$ has been described previously (4). Xylulose-$P_2$ was prepared by an aldolase-catalyzed condensation of glycolaldehyde phosphate (Calbiochem) and dihydroxyacetone phosphate (Calbiochem) (5). The proof of structure of xylulose-$P_2$ was established by a $NaBH_4$ reduction of the aldolase condensation product to form a mixture of epimeric pentitol 1,5-bisphosphates, presumably xylitol-$P_2$ and arabitol-$P_2$. After acid phosphatase (Sigma) treatment, the free pentitols were chromatographed against known standards on silica gel plates impregnated with 0.1 M boric acid. The spots were visualized by alkaline permanganate (6) and coincided with xylitol ($R_f$ 0.08) and arabitol ($R_f$ 0.15) standards (gifts from R. Barker). The concentration of xylulose-$P_2$ was determined by total phosphate analysis (7).

Carboxylase assays were run in stoppered scintillation vials at 30° with 0.25 ml final volume composed of 100 mM *N,N*-bis(2-hydroxyethyl)glycine (Sigma, Bicine) at pH 8.0, 10 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (252 cpm/nmol), 0.25 mM EDTA, 1 mM dithiothreitol, 20 μg of enzyme (0.14 μM), and 0.5 mM ribulose-$P_2$ unless otherwise indicated.

The enzyme was activated in a stock solution composed of 2 mg of protein/ml of 50 mM *N,N*-bis(2-hydroxyethyl)glycine at pH 8.0, 0.40 mM EDTA, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 10 mM $NaHCO_3$ for at least 10 min prior to its use. The reaction was initiated by ribulose-$P_2$ and after 20 s to 1 min was stopped by the addition of 0.2 ml of 2 N HCl. Activity was expressed as total counts per min or nanomoles of $^{14}CO_2$ fixed per min into acid-stable reaction product (carbon 1 of 3-phosphoglycerate).

The oxygenase assays were run in a 0.5-ml volume in the reaction chamber of a Rank oxygen electrode at 30° with a continuous recording of $O_2$ uptake. The assay solution was 100 mM *N,N*-bis(2-hydroxyethyl)glycine at pH 8.3, 20 mM $MgCl_2$, 0.5 mM ribulose-$P_2$, 0.25 mM EDTA, 1 mM $NaHCO_3$, 1 mM dithiothreitol, enzyme, and atmospheric oxygen. The enzyme was preincubated as above with 10 mM $NaHCO_3$ for at least 10 min, and then assayed at a concentration of 400 μg/ml by initiation with the ribulose-$P_2$.

## RESULTS AND DISCUSSION

A Dixon plot for xylulose-$P_2$ inhibition of ribulose-$P_2$ carboxylase, when the enzyme was preincubated with the inhibitor for 20 min, is presented in Fig. 1. When the molar ratio of inhibitor to enzyme was equivalent to 4 inhibitor molecules per 8 active sites, the carboxylase was about 50% inhibited. The Dixon plot is biphasic, in that it consists of two different slopes. This implies two types of inhibition. For given amounts of xylulose-$P_2$, the inhibition of the carboxylase reached a maximum after 20 to 30 min of preincubation (Fig. 2*A*). We do not know if this hysteretic response to the inhibitor is an opposite effect from the hysteretic $CO_2$ activation. A plot of log residual activity *versus* time (Fig. 2*B*) was linear and indicated that the inactivation was an apparent first order process. This line did not pass through the point for 100% activity at zero time, as if there were a very fast initial inhibition phase or an initially inadequate mixing of the inhibitor and the enzyme.

When xylulose-$P_2$ and ribulose-$P_2$ were added simultaneously, the inhibition was apparently competitive with respect to ribulose-$P_2$ as shown by double reciprocal plots (Fig. 3). When the data are replotted as the slope *versus* [xylulose-$P_2$] (Fig. 3, *inset*), a straight line is obtained, indicative of pure competitive inhibition. Similarly, a plot of reaction velocity *versus* [xylulose-$P_2$] at fixed [ribulose-$P_2$] also indicates pure competitive inhibition (figure not shown). Experiments run after preincubation of enzyme for 20 min with similar concentrations of xylulose-$P_2$, before addition of ribulose-$P_2$, produced 90% inhibition. Since this long preincubation was required for equilibrium of enzyme and xylulose-$P_2$, one cannot expect

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[1] The abbreviations used are: ribulose-$P_2$, ribulose 1,5-bisphosphate; xylulose-$P_2$, xylulose 1,5-biphosphate; xylitol-$P_2$, xylitol 1,5-biphosphate; arabitol-$P_2$, arabitol 1,5-bisphosphate.

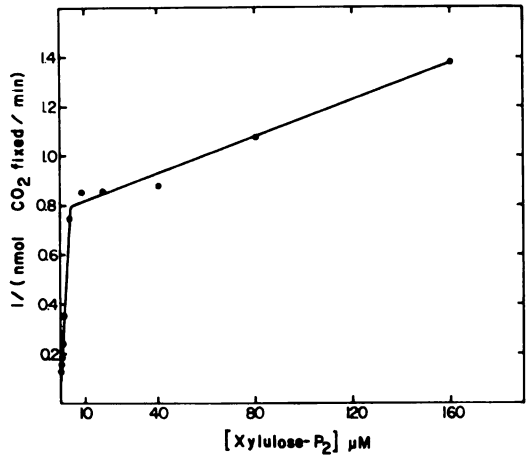



FIG. 1. Xylulose-$P_2$ inhibition of ribulose-$P_2$ carboxylase after a 20-min preincubation with xylulose-$P_2$.

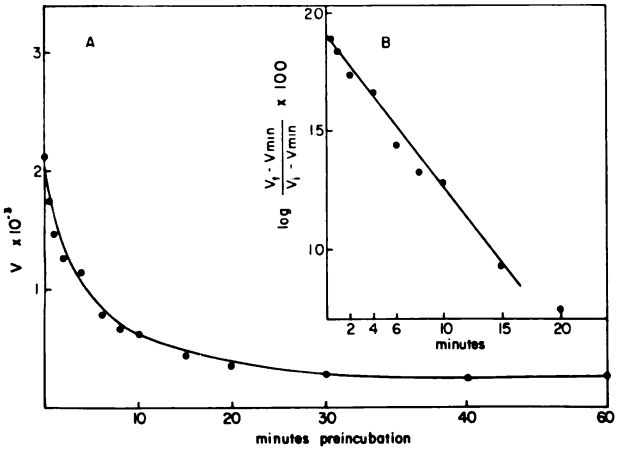



FIG. 2. *A*, time course of inhibition. Enzyme was preincubated in 8 μM xylulose-$P_2$ for indicated times and then assayed for 1 min. *V* is measured as counts per min of $^{14}C$ fixed per assay. *B*, first order plot of the inhibition, log $((V_i - V_{min})/(V_i - V_{min})) \times 100$ *versus* time of preincubation. $V_i$ = velocity at any time; $V_{min}$ = the minimum velocity at this xylulose-$P_2$ concentration; $V_i$ = the initial velocity.

ribulose-$P_2$ and xylulose-$P_2$ to come to equilibrium with the enzyme during a 1-min assay. Because of the hysteretic response of the carboxylase to the inhibitor, it is expected that the kinetics would appear noncompetitive after a 20-min preincubation, and this has been observed with xylitol-$P_2$ (data not shown and Fig. 3 of Ref. 3). Since $CO_2$ is needed for activation of the enzyme, it is not possible to preincubate the enzyme with ribulose-$P_2$ and xylulose-$P_2$ and then use $CO_2$ to initiate the reaction.

Xylulose-$P_2$ inhibited the ribulose-$P_2$ oxygenase activity with approximately the same stoichiometry as it did the carboxylase. The inhibition was also dependent upon the duration of the preincubation. Inhibition of both the carboxylase and oxygenase activity is to be expected if xylulose-$P_2$ and ribulose-$P_2$ are competing at the same site on the protein.

Xylulose-$P_2$ has been considered as a substrate for ribulose-$P_2$ carboxylase (9), but being inactive, it was apparently not further investigated. Its structure makes it almost indistinguishable from ribulose-$P_2$ by the paper chromatographic techniques used originally to identify the components of the

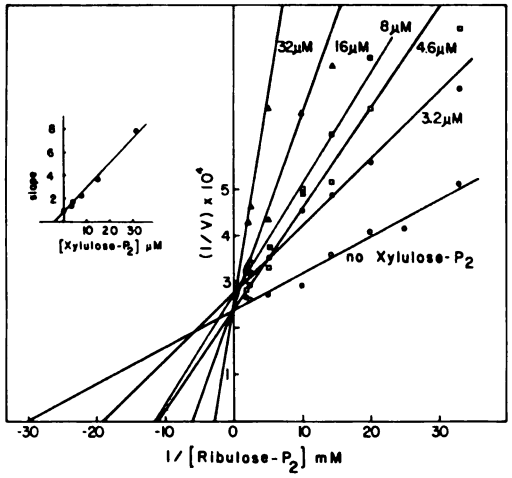
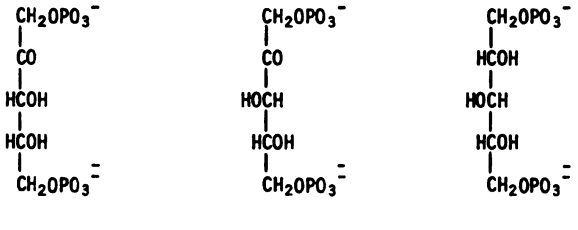



FIG. 3. Double reciprocal plot of activity *versus* ribulose-$P_2$ concentration when xylulose-$P_2$ and ribulose-$P_2$ were added simultaneously. The lines were calculated by a computer program (8). *V* is measured as counts per min of $^{14}C$ fixed per assay.

photosynthetic carbon cycle. While xylulose-$P_2$ can be easily synthesized by aldolase, we know of no reports concerning the natural occurrence of this compound. This apparent absence of xylulose-$P_2$ is consistent with the lack of confirmed reports of the occurrence of glycolaldehyde phosphate in nature.

Xylulose-$P_2$ and xylitol-$P_2$ are potent inhibitors of ribulose-$P_2$ carboxylase/oxygenase, because of their structural similarities to the substrate.




Xylulose-$P_2$ has a potential for three types of interactions at the ribulose-$P_2$ active site. (*a*) The phosphate groups can be bound in a manner similar to the binding of ribulose-$P_2$. (*b*) The hydroxyl at carbon 3 may interact with a base in the active site which is essential for enediol formation of ribulose-$P_2$ during catalysis. This binding is probably the unique characteristic of both xylulose-$P_2$ and xylitol-$P_2$ inhibition. (*c*) The carbonyl group at carbon 2 of xylulose-$P_2$ may interact with or form a Schiff base with an essential ε amino group of a lysyl residue which is involved in $CO_2$ binding (10). This possibility is supported by the fact that xylulose-$P_2$ partially blocks the reaction of pyridoxal 5'-phosphate with this lysyl residue (11).

*Acknowledgments*—W. A. Wood advised us on the synthesis and chromatographic identification of xylulose-$P_2$. We thank Christian Paech and Frederick J. Ryan for discussions of the experiments.

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# RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE FROM PARSLEY

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## SUMMARY

Ribulose-1,5-bisphosphate carboxylase/oxygenase from parsley leaves was purified by Sepharose 6B gel filtration at pH 8.3 as a single, colorless peak containing both activities. Approximately 0.2 g atom copper per mole enzyme was detected by atomic absorption spectroscopy, but this copper was not detectable by EPR spectrometry.

## INTRODUCTION

Since the discovery of the oxygenase activity of ribulose-$P_2$ carboxylase/oxygenase (E.C. 4.1.1.39), much evidence has been accumulated which indicates that both the carboxylase and oxygenase activities are due to one enzyme. Therefore, the recent report (1) that the two activities could be separated by gel filtration on Sepharose 6B at pH 8.3, and that the oxygenase fraction was blue and contained copper required further investigation. Small and varying amounts of copper have been reported to be asociated with the $A_8B_8$ enzyme from spinach leaves: 1 g atom/mole (2), 0.11 to 0.14 g atom/mole (3), 0.2 g atom/mole (4), and in this report 0.18 g atom in the enzyme from parsley. An EPR spectrum of Cu(II) bound to the spinach enzyme has been published (2). This paper presents unsuccessful attempts to separate the oxygenase from the carboxylase activities by the procedure of Brändén (1). The specific activity of ribulose-$P_2$ carboxylase/oxygenase prepared from parsley was similar to that obtained from spinach or tobacco leaves.

## MATERIALS AND METHODS

Ribulose-$P_2$ carboxylase/oxygenase was prepared from the leaves of parsley (from local grocery store) according to Brändén (1). One hundred g of

Abbreviations: Ribulose-$P_2$ for ribulose-1,5-bisphosphate.

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tissue was ground in a Waring blender at high speed for 60 s with 200 ml of 50 mM Tris-HCl at pH 8.3 and 4°. This buffer was prepared in 2 ways for different experiments; in one the pH was adjusted at 4°, the other at room temperature (22-24°) according to Brändén (personal communication). The homogenate was filtered through several layers of cheese cloth and centrifuged at 11,000xg for 30 min. The supernatant was filtered through Miracloth and solid $(NH_4)_2SO_4$ was added slowly, with constant stirring, to 30% saturation (16.4 g/100 ml). The suspension was stirred for 15 min, and stood for 45 min at 4° before centrifuging at 11,000xg for 40 min. This precipitate was discarded, and the supernatant was made 50% saturated with solid $(NH_4)_2SO_4$ (11.8 g/100 ml) to precipitate the enzyme. The stirring, standing, and centrifugation were repeated. The 30-50% $(NH_4)_2SO_4$ precipitate was resuspended in 25 ml or less of buffer. In one case the buffer was 50 mM Tris, pH 8.3 (adjusted at 4°), and in the second case the buffer was 5 mM Tris, adjusted at room temperature to pH 8.3. Usually the resuspended and redissolved precipitate was clarified by centrifugation at 25,000xg for 15 min. The supernatant was filtered through a Sephadex G-25 (medium), 6 x 25 cm column, which had been previously equilibrated with the resuspension buffer. The peak fractions, as determined by absorbance at 280 nm, were pooled and concentrated to 5-7 ml with a PM 30 membrane in an Amicon ultrafiltration chamber, pressurized with argon at 60 psi. The concentrate was chromatographed on a Sepharose 6B column (2.6 x 90 cm) equilibrated with the same resuspension buffer. All steps were carried out at 4°. The entire procedure was repeated with 2 mM β-mercaptoethanol in all buffers. The spinach enzyme was prepared by our usual procedure (5).

Ribulose-$P_2$ was prepared enzymatically (6). Sephadex G-25 and Sepharose 6B were from Pharmacia Fine Chemicals, $NaH^{14}CO_3$ from Amersham and other reagents from Sigma.

The enzyme was activated by adjusting preparations to 20 mM $MgCl_2$ and 10 mM $NaHCO_3$ and incubating them at 30° for at least 10 min prior to assaying. The carboxylase assays were run at 30° for 1 min and initiated by the addition of 10 µl aliquots of activated enzyme to 240 µl of 100 mM N,N-bis-(2-hydroxyethyl)glycine (Sigma, Bicine) at pH 8.2, 20 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$, 1 mM dithiothreitol, and 0.5 mM ribulose-$P_2$. The $^{14}C$ acid stable product was measured by scintillation counting. The oxygenase assay was based on the ribulose-$P_2$ dependent oxygen uptake in a Rank Brothers oxygen electrode. The reactions were normally initiated by the addition of 20 µl of activated enzyme to 480 µl of 100 mM N,N-bis(2-hydroxyethyl)glycine at pH 8.2, 20 mM $MgCl_2$, 1 mM dithiothreitol, and 0.5 mM ribulose-$P_2$. The reactions were monitored for 50-60s at 30°. Assays were also run according to Brändén (1) by initiating with ribulose-$P_2$, after a 5 to 10 min incubation of the enzyme in the assay buffer in the oxygen electrode chamber.

A Varian Model 175 Spectrophotometer was used for the atomic absorption spectroscopy using copper sulfate as a standard. EPR spectra were recorded at 77K with a Varian E-4 spectrometer operating at 9.22 GHz; microwave power, 20 mwatts; modulation frequency, 100 KHz; modulation amplitude, 5 gauss; field set, 2900 gauss; field sweep, 2000 gauss; time constant, 0.1 sec; instrumental gain, 2 x $10^3$.

## RESULTS AND DISCUSSION

Ribulose-$P_2$ carboxylase and ribulose-$P_2$ oxygenase activity from parsley leaves chromatographed as a single peak on Sepharose 6B at 4° in 50 mM Tris

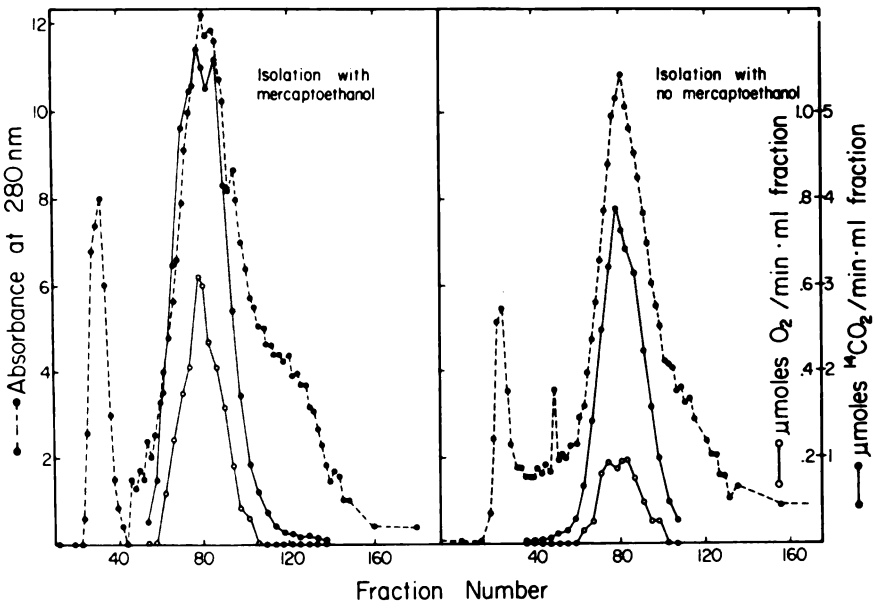


Figure 1. Separation of ribulose-$P_2$ carboxylase/oxygenase from parsley leaves on a sepharose 6B column. The buffer was 50 mM Tris adjusted to pH 8.3 at 4°.

buffer at pH 8.3 (Fig. 1). Similar results were obtained with or without 2 mM β-mercaptoethanol. These procedures and results are similar to those previously observed for enzyme for spinach or tobacco leaves (5), as well as parsley (Brändén, personal communication). To separate the two activities Brändén used 5 mM Tris, adjusted to pH 8.3 at room temperature, for the Sepharose 6B filtration step. This modification of the procedure with or without β-mercaptoethanol, did not result in the separation of the carboxylase/oxygenase activities in our experiments (Fig. 2). When the clarification step preceding the G-25 column was omitted (Methods), to bring the present procedure in line with that in reference 1, a shoulder absorbing at 280 nm, presumably nucleic acids, preceded the enzyme activity off the Sepharose 6B column (Fig. 2). This omission had no influence on the subsequent distribution of the carboxylase and oxygenase activities.

Brändén's oxygenase assays were plagued by a substantial endogenous rate of oxygen consumption which was not dependent on the presence of

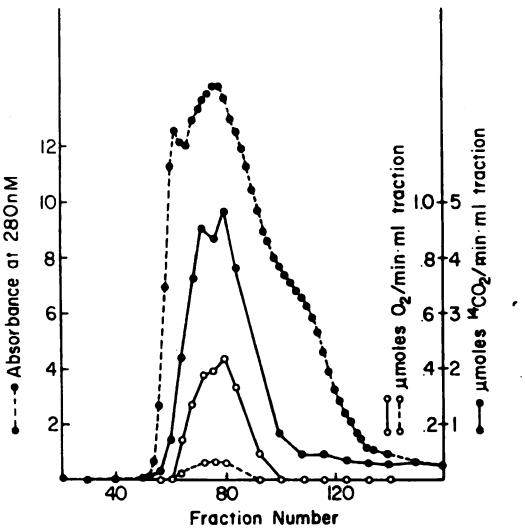


Figure 2. Separation of ribulose-$P_2$ carboxylase/oxygenase from parsley leaves on a sepharose 6B column. The buffer was 5 mM Tris adjusted to pH 8.3 at 22°. The oxygenase assays were initiated by the addition of activated enzyme to the assay mix containing ribulose-$P_2$ (o—o) or alternatively by the addition of ribulose-$P_2$ to oxygenase preincubated in the assay mix for 10 min (o- -o).

ribulose-$P_2$. This forced him to use a 5-10 min preincubation of the enzyme in the assay buffer before initiation of the reaction with ribulose-$P_2$. With partially purified enzyme preparations from spinach, tobacco, and now parsley, we did not observe an endogenous rate of $O_2$ uptake. Brändén (personal communication) has attributed his endogenous rate to oxidation of the dithiothreitol in the activating and assay buffers. Copper has been reported to accelerate the oxidation of dithiothreitol (7), but addition of small amounts of Cu(II) to our system did not produce a significantly faster endogenous rate, so we are at loss to explain his endogenous rate in the assay.

In our experiments ribulose-$P_2$ oxygenase assays were initiated by addition of an aliquot of the concentrated, activated enzyme into an assay buffer without $NaHCO_3$. An initially linear, substrate and enzyme dependent rate of $O_2$ uptake was measured over the first 30 s. Without substrate or before adding enzyme the endogenous rate was zero. If a

purified enzyme from spinach leaves was first activated by preincubation with 10 mM $NaHCO_3$ and $MgCl_2$, and then assayed for oxygenase activity at various times after its addition to the assay buffer, rapid inactivation occurred with a half time of approximately 1 min (data not shown). This rapid decline in activity has been analyzed by Laing and Christeller (8) and Lorimer et al. (9) and attributed to a dissociation of the active enzyme-$CO_2$-$Mg^{2+}$ complex in the final assay mixture which contained only 0.4 mM $NaHCO_3$ from carryover in the aliquot of activated enzyme in 10 mM $NaHCO_3$. Performance of the oxygenase assay in the manner described by Brändén (1) resulted in much lower activites due to dissociation of the activated complex. Thus 10 min after dilution of the activated enzyme from parsley into the assay buffer, the oxygenase activity was severely reduced (o--o in Fig. 2). Nevertheless, this small amount of remaining ribulose-$P_2$ oxygenase coincided with the ribulose-$P_2$ carboxylase peak from the Sepharose 6B column.

Additional observations by Brändén (1) were a blue color in the fractions with oxygenase activity and a strong EPR signal characteristic of "blue" oxidases. Our atomic absorption spectroscopy revealed trace amounts of copper, 0.18 g atom/mole enzyme, in the peak fractions (assuming a molecular weight of 550,000) and a similar amount of copper per mg protein in the shoulder fractions. No EPR signal characteristic of copper was found in any of the Sepharose 6B column fractions with carboxylase/oxygenase activity that contained approximately 10 μM copper by atomic absorption, even though the limits of detection in the EPR experiments were estimated to be between 1-2 μM for Cu(II). Addition of 1 mM ferricyanide to convert Cu(I) to Cu(II) was without effect on the EPR spectrum. Thus it seems that any Cu(I) present in the protein samples was inaccessible to ferricyanide or that the copper was EPR inactive. Cu(II) (10 μM) was added to fractions from Fig. 2, but it had no effect on the rate or on the location of the oxygenase activity.

In conclusion, ribulose-$P_2$ carboxylase/oxygenase from parsley leaves seems to be a single protein similar to that from spinach and tobacco leaves. Brändén's (1) reported separation of these two activities has not been confirmed. We also emphasize that in the ribulose-$P_2$ oxygenase assay, the rate must be determined quickly before the enzyme is inactivated in the absence of $CO_2$. The difference in the copper content of the preparations may be in the source of parsley. In view of the instability of ribulose-$P_2$ (10), a nonspecific copper-protein complex might also oxidize this substrate. Until the mechanism of action and the cofactor requirement for ribulose-$P_2$ oxygenase are known, a continuing concern about copper in the enzyme can be expected.

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