1979

Oxygen and carbon dioxide effects on the photosynthetic and photorespiratory carbon pools in Glycine max

William D. Hitz
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Botany Commons, Plant Biology Commons, and the Plant Pathology Commons

Recommended Citation
https://lib.dr.iastate.edu/rtd/6607

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
INFORMATION TO USERS

This was produced from a copy of a document sent to us for microfilming. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help you understand markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure you of complete continuity.

2. When an image on the film is obliterated with a round black mark it is an indication that the film inspector noticed either blurred copy because of movement during exposure, or duplicate copy. Unless we meant to delete copyrighted materials that should not have been filmed, you will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed the photographer has followed a definite method in "sectioning" the material. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For any illustrations that cannot be reproduced satisfactorily by xerography, photographic prints can be purchased at additional cost and tipped into your xerographic copy. Requests can be made to our Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases we have filmed the best available copy.
HITZ, WILLIAM D.
OXYGEN AND CARBON-DIOXIDE EFFECTS ON THE
PHOTOSYNTHETIC AND PHOTORESPIRATORY CARBON
POOLS IN GLYCINE MAX.

IOWA STATE UNIVERSITY, PH.D., 1979
Oxygen and carbon dioxide effects on the photosynthetic and photorespiratory carbon pools in *Glycine max*

by

William D. Hitz

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Botany and Plant Pathology
Major: Botany (Physiology)

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1979
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>REVIEW OF LITERATURE</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>The Glycolate Pathway</td>
<td>3</td>
</tr>
<tr>
<td>The Formation of Glycolic Acid</td>
<td>5</td>
</tr>
<tr>
<td>The Photosynthetic Carbon Reduction and Photorespiratory Carbon Oxidation Cycles</td>
<td>6</td>
</tr>
<tr>
<td>Magnitude of Carbon Flow Through the PCO Cycle</td>
<td>8</td>
</tr>
<tr>
<td>Control and Partitioning of Carbon Between the PCR and PCO Cycles</td>
<td>10</td>
</tr>
<tr>
<td>Carbon Flow into the PCR and PCO Cycles</td>
<td>12</td>
</tr>
<tr>
<td>Depletion of PCR Cycle Intermediates in the Presence of O₂</td>
<td>13</td>
</tr>
</tbody>
</table>

| PART A. OXYGEN AND CARBON DIOXIDE EFFECTS ON THE POOL SIZE OF SOME PHOTOSYNTHETIC AND PHOTORESPIRATORY INTERMEDIATES IN GLYCINE MAX | 15 |

| INTRODUCTION | 16 |

<table>
<thead>
<tr>
<th>MATERIALS AND METHODS</th>
<th>19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant Materials</td>
<td>19</td>
</tr>
<tr>
<td>Gas Exchange</td>
<td>19</td>
</tr>
<tr>
<td>Preparation of Enzymes</td>
<td>26</td>
</tr>
<tr>
<td>Metabolite Extraction</td>
<td>30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RESULTS</th>
<th>36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf Chamber Evaluation</td>
<td>36</td>
</tr>
<tr>
<td>CO₂ Exchange Rates</td>
<td>39</td>
</tr>
<tr>
<td>The Steady-State Photosynthesis Level of RuBP</td>
<td>41</td>
</tr>
<tr>
<td>The Steady-State Photosynthesis Level of PGA</td>
<td>45</td>
</tr>
<tr>
<td>Steady-State Photosynthesis Level of Glycolic Acid</td>
<td>45</td>
</tr>
<tr>
<td>Steady-State Glycine and Serine Levels During Photosynthesis</td>
<td>47</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DISCUSSION</th>
<th>53</th>
</tr>
</thead>
</table>

| PART B. RATE OF CARBON DIOXIDE EVOLUTION AND POOL SIZES OF SOME PHOTOSYNTHETIC AND PHOTORESPIRATORY INTERMEDIATES DURING THE POST-ILLUMINATION PERIOD IN GLYCINE MAX | 67 |

| INTRODUCTION | 68 |

| MATERIALS AND METHODS | 70 |
REVIEW OF LITERATURE

Introduction

The related phenomena of photorespiration and oxygen inhibition of photosynthesis have been the subjects of many investigations in the relatively short period of time since their discovery. Earlier work in this area has been extensively reviewed and will be briefly considered here in an introductory manner only. For more detailed reviews of the broad areas of research concerning photorespiration, the following reviews may be consulted: Brown (1953), Goldsworthy (1970), Zelitch (1971), Gibbs (1971), Black (1973), Zelitch (1973), Chollet and Ogren (1975), and Kelly et al. (1976).

While the influence of light on the rate of respiration in photoautotrophic organisms had been a subject of study for many years, measurement of the quantum yield of photosynthesis in the later 1940's stimulated interest in the problem. Since most common methods for the measurement of photosynthetic rate measure only net exchange of either oxygen or carbon dioxide, the rate of both components of the net exchange must be known to assess the true rate of photosynthesis. Methods which eliminated the photosynthetic component by chemical inhibition or non-photosynthetic mutants indicated an unchanged respiration rate in the light. These methods suffered the criticism of being incapable of measuring effects due to the process of photosynthesis itself on respiration in the light.

This criticism was overcome by the isotopic separation of photosynthesis and respiration (Van Norman and Brown 1952, Brown 1953, Ozbun...
et al. 1964). These studies indicated equal rates of light and dark respiration, however, experimental conditions required $O_2$ partial pressures equivalent to 5% $O_2$ at the most, and $CO_2$ concentrations in the range of 0.1 to 1%.

Decker (1955) first interpreted a rapid rate of $CO_2$ evolution immediately following darkening of a photosynthesizing leaf as an indication of a possibly higher rate of respiration in the light. Decker (1958) further checked this possibility by extrapolating the rate of net $CO_2$ exchange vs $CO_2$ concentration to zero $CO_2$. The extrapolated rate was 3.5 times the rate of $CO_2$ evolution measured in the dark. The occurrence of the rapid $CO_2$ release upon darkening was shown to occur in several species (Decker 1958) and to be dependent upon previous irradiance in a manner similar to photosynthesis (Decker 1959).

Hock, Owens, and Kok (1963) subsequently refined $^{18}O_2$-$^{16}O_2$ measurements of photosynthesis and respiration to allow the use of air levels of $CO_2$ and from 0.2 to 0.4 atmospheres of $O_2$. Oxygen uptake in the light exceeded uptake in the dark under these conditions, and DCMU, at levels sufficient to inhibit photosynthesis, reduced $O_2$ uptake rates in the light to dark respiration levels. These results, along with those of Decker and the studies of $^{14}CO_2$ release of Goldsworthy (1966) and Tregunna et al. (1964), indicated a process separate from dark respiration which occurred in the light, utilized $O_2$, and released $CO_2$.

The increased rate of photorespiration at $CO_2$ concentrations below 0.1% $CO_2$ (Tregunna et al. 1961 and Forrester et al. 1966), along with the known similar conditions leading to rapid rates of glycolic acid synthesis (Wilson and Calvin 1955, Tolbert and Zill 1956, Bassham and
Kirk 1962, Zelitch 1958), led several workers (Goldsworthy 1966, Moss 1966, Coombs and Whittingham 1966) to speculate that the formation of glycolic acid and its subsequent metabolism were related to photorespiration.

The Glycolate Pathway

Glycolic acid was identified as an early product of photosynthesis by Benson and Calvin (1950). Shou et al. (1950) found that $^{14}$C-glycolic acid fed to Chlorella in the light gave labeled photosynthetic intermediates and products similar to $^{14}$CO$_2$ fixation. Rabson et al. (1962) found that glycine, serine, and glycerate were labeled when leaf segments were allowed to metabolize $^{14}$C-glycolate in the light. Further, labeled glyoxyxlate, glycine, and serine were shown to give labeled glucose when metabolized by wheat leaves (Wang and Waygood 1962) and in tobacco leaf discs (Jiminez et al. 1962). When C-2 labeled glycolate was fed, 2,3 labeled serine and 1,2,5,6 labeled hexose was formed; and, when C-1 labeled glycolate was fed, $^{14}$CO$_2$ was released and C-1 labeled serine was produced (Jiminez et al. 1962). The function of the pathway was investigated in photosynthesizing leaves by the use of $^{14}$CO$_2$ (Hess and Tolbert 1966). Glycolate and glycine were found to be uniformly labeled at short labeling times, and could have been formed in sequence. Serine was also uniformly labeled at C-1 and C-2, but C-3 remained of lower activity at short labeling time. This was taken as an indication that, while most serine might be formed in the glycolate pathway, a small portion was formed from a separate pool of 3 carbon compounds.
The glycine to serine conversion in the glycolate pathway was shown to be a major source of photorespiratory CO$_2$ by Kisaki and Tolbert (1970). Isonicotinyl hydrazide, which had earlier been shown to increase $^{14}$C in glycolate and glycine from $^{14}$CO$_2$ at the expense of serine (Coombs and Whittingham 1966) and to partially inhibit the conversion of glyoxylate to glycine (Pritchard et al. 1963), was shown to stop CO$_2$ evolution in illuminated tobacco leaf discs and to stop the labeling of serine.

Intracellular localization of the various steps in the glycolate pathway was determined by cell fraction studies and localization of enzymatic activities in various organelles. Kisaki and Tolbert (1969) showed that, while peroxisome containing fractions from green leaves of several species, contained glycolate oxidase, a glyoxylate aminotransferase, and were capable of converting glycolate to glycine, no CO$_2$ was evolved and serine was not produced. Chloroplast preparations were capable of oxidizing glyoxylate to CO$_2$ at low rates but could not metabolize glycolate.

Enzymes catalyzing a glycine decarboxylation reaction and subsequent serine formation were found in mitochondria from green leaves (Kisaki et al. 1971a). Kisaki et al. (1971b) characterized these enzymes as a glycine synthetase (E.C. 2.1.2.10) which is linked to serine hydroxymethyl transferase (E.C. 2.1.21). The linked enzymes catalyze the synthesis of 1 mole of serine from 2 moles of glycine, with the release of 1 mole of CO$_2$ and 1 mole of NH$_3$. The enzyme system is apparently similar to one found in bacteria and mammalian mitochondria. The decarboxylation system requires tetrahydrofolic acid and pyridoxal phosphate and is stimulated by NAD (Woo and Osmond 1976). The reaction required intact mitochondria
(Bird et al. 1972a, 1972b, Woo and Osmond 1976) and is apparently at least partially linked to ATP synthesis through the electron transport system (Bird et al. 1972a, Woo and Osmond 1976).

Woo and Osmond (1976) estimated the total glycine decarboxylation capacity in spinach leaves to be a minimum of 80 μmoles hr⁻¹ (mg Chl)⁻¹ at saturating glycine levels. This estimated capacity is more than sufficient to account for measured rates of photorespiration of 30 μmoles hr⁻¹ (mg Chl)⁻¹ (Ludwig and Canvin 1971).

The Formation of Glycolic Acid

While the existence of glycolic acid as an early product of photosynthesis has been known since the work of Benson and Calvin (1950), the mechanism of its synthesis has remained quite controversial.

Wilson and Calvin (1955) postulated that glycolate was formed by the loss of a 2 carbon fragment from thiamine pyrophosphate during the transketolase catalyzed transfer of carbons 1 and 2 of a hexose phosphate to a C-3 or C-5 sugar phosphate. The increased formation at low CO₂ concentration was assumed to be due to a slower rate of transfer of these 2 carbon groups due to slower flow through the cycle. Shain and Gibbs (1971) showed that broken chloroplasts in the light could synthesize glycolate in the presence of added transketolase and fructose-6-phosphate at a maximum rate of 10 μmoles hr⁻¹ (mg Chl)⁻¹.

An alternate pathway to glycolate production was suggested by Bassham and Kirk (1962), who noted that an oxidation of carbons 1 and 2 of RuBP to form phosphoglycolate followed by hydrolysis by phosphoglycolate phosphatase (Richardson and Tolbert 1961) could lead to glycolate.
The O₂ and CO₂ effects on the Warburg effect also led Tamiya and Huzisige (1949) to speculate an O₂ competition for CO₂ at the carboxylating enzyme. Ogren and Bowes (1971), in light of the O₂ and CO₂ concentration effects on glycolate production and the Warburg effect, postulated a competitive oxygenation of RuBP by RuBP carboxylase to produce phosphoglycolate. The proposed oxygenation was later shown to occur in purified preparations of RuBP carboxylase (Bowes et al. 1971, Bowes and Ogren 1972, Andrews et al. 1973).

Laing et al. (1974) calculated that, in air, the rate of phosphoglycolate formation by RuBP oxygenase was sufficient to account for a steady-state photorespiration rate of 30 μmoles hr⁻¹ (mg Chl)⁻¹ as measured by Ludwig and Canvin (1971).

The Photosynthetic Carbon Reduction and Photorespiratory Carbon Oxidation Cycles

Evidence that RuBP oxygenase may be the primary in vivo source of phosphoglycolate for further metabolism through the glycolate pathway comes principally from ¹⁸O labeling studies (Andrews et al. 1971, Berry et al. 1977). Andrews et al. (1971) demonstrated the incorporation of ¹⁸O from molecular oxygen into the carboxyl groups of glycine and serine by spinach leaves in the light. Such labeling should not take place if the oxygenation to form glycolate takes place by a peroxide catalyzed reaction (Tolbert and Ryan 1975). Purified RuBP oxygenase has been shown to incorporate ¹⁸O from ¹⁸O₂ into the carboxyl oxygen of phosphoglycolate (Lorimer et al. 1973). Berry et al. (1977) extended these studies to show that the order of appearance of ¹⁸O in intermediates of the glycolate pathway is glycolate first, glycine second, and
Early $^{14}$C labeling studies (Jiminez et al. 1962, Wang and Waygood 1962), as well as later studies by Waidyantha et al. 1975a, indicated that, under some conditions, sucrose was an end product of the glycolate pathway.

The discovery of hydroxypyruvate reductase in leaves (Stafford et al. 1954), its localization in peroxisomes (Tolbert et al. 1970), and the discovery of serine-glyoxylate amino transferase (Rehfeld and Tolbert 1972) allowed the possibility of glycerate synthesis in the peroxisome. The subsequent phosphorylation of this glycerate in the chloroplast allows regeneration of PGA and thus return of 75% of the carbon entering the glycolate pathway to the photosynthetic carbon reduction (PCR) cycle. Evidence for this return of carbon to the PCR cycle through PGA comes also from $^{18}$O labeling studies (Berry et al. 1977). PGA from spinach leaves at the CO$_2$ compensation point was found to be $^{18}$O labeled in the carboxyl group to a level 1/5 to 1/10 of that in glycolate. The ratio of RuBP carboxylation to oxygenation must be 1:2 at the CO$_2$ compensation point (Osmond and Bjorkman 1972); this oxygenation to carboxylation ratio gives 4 moles of PGA for each mole of phosphoglycolate produced by RuBP carboxylase/oxygenase (Andrews et al. 1973). Since each mole of glycolate entering the glycolate pathway may give 1/2 mole of PGA (Tolbert and Ryan 1975), one mole of $^{18}$O carboxyl labeled PGA from the glycolate pathway should be produced for each 4 moles of unlabeled PGA, giving a theoretical label in PGA of 1/5 that in glycolate.

This apparent cyclic interlocking of the PCR cycle and the glycolate pathway led Berry et al. (1977) to name the entire scheme the photosyn-
thetic carbon reduction and photorespiratory carbon oxidation cycles. The proposed pathways are shown in Figure 1 as adapted from Berry et al. (1977) and Tolbert and Ryan (1975).

Magnitude of Carbon Flow Through the PCO Cycle

The magnitude of carbon flow through glycolate during photosynthesis has been estimated at widely varying amounts. Zelitch (1971) estimated from studies on the inhibition of glycolate oxidase that a minimum of 50 μmoles of CO₂ hr⁻¹ (mg Chl)⁻¹ are released during photorespiration. Inhibition of glycolate oxidase in leaf cells of barley (Servaites et al. 1978) and in soybean leaf cells (Servaites and Ogren 1977) caused accumulation of about 50% of total ¹⁴CO₂ fixed at air levels of CO₂ and O₂ into glycolate.

Direct measurements of photorespiration at air levels of CO₂ and O₂ indicate a value near 30 μmoles hr⁻¹ (mg Chl)⁻¹ or about 20% of the rate of true photosynthesis (Canvin et al. 1975). If all or most of this CO₂ arises from the glycine to serine conversion in the PCO cycle, carbon flux through the cycle must be at least 80% of the rate of true CO₂ fixation since 1 carbon in 4 entering the PCO cycle is lost as CO₂.

This magnitude of flow has been further verified in measurements of flux rates from glycine to serine during photosynthesis (Mahon et al. 1974, Waidyantha et al. 1975b, Kumarasinghe et al. 1977). The flux rate, depending upon O₂ and CO₂ conditions, was from 80% to 100% of total carbon fixed.
Figure 1. The Photosynthetic Carbon Reduction (PCR) and Photorespiratory Carbon Oxidation (PCO) cycles, adapted from Tolbert and Ryan (1975) and Berry et al. (1977)
Control and Partitioning of Carbon Between the PCR and PCO Cycles

With the discovery of the oxygenase capability of RuBP carboxylase/oxygenase came the suggestion that the relative rates of carboxylation and oxygenation and, therefore, photosynthesis, and photorespiration could be controlled by the kinetic properties of the enzyme (Laing et al. 1974). In this and other studies (Lorimer et al. 1973) it was shown that CO₂ acted as a competitive inhibitor of RuBP oxygenase and O₂ acted as a competitive inhibitor of RuBP carboxylase.

Servaites et al. (1978) and Servaites and Ogren (1977) found that ¹⁴CO₂ incorporation into glycolate in leaf cells treated with an inhibitor of glycolate oxidase was controlled by the solution concentration ratio of O₂/CO₂ over a wide range of ratios. The incorporation remained nearly constant, however, from O₂/CO₂ about 40 to 100. At air levels of O₂ and CO₂, the ratio is approximately 32.

Comparison of the temperature responses of leaf photosynthesis and RuBP carboxylase to oxygen inhibition are similar (Laing et al. 1974). This similarity has been considered further evidence that the in vivo velocities of the carboxylation and oxygenation reactions are controlled by the ratio of O₂ to CO₂.

Ku and Edwards (1977) determined the Michaelis-Menton binding constant for CO₂ and inhibitor constant for O₂ in intact leaves. The values for both constants were slightly below measured values for the activated, purified carboxylase. Using these kinetic constants, and assuming that RuBP carboxylase/oxygenase controls the rates of photosynthesis and photorespiration, gives prediction of true photosynthesis vs CO₂ equivalent to shifting the apparent photosynthesis vs CO₂ from
the compensation point to the origin. In such a model, photorespiration is determined as the difference between true and apparent photosynthesis. The model predicts increasing photorespiration at decreased CO₂ concentration.

The calculated oxygenase to carboxylase ratio at air levels of O₂ and CO₂ is about 0.25 for both the high (Laing et al. 1974) and low Km (Bahr and Jensen 1974) forms of RuBP carboxylase/oxygenase. This ratio at air levels of O₂ and CO₂ predicts a ratio of about 4 at 50 μl l⁻¹ of CO₂ and 21% O₂, just above the CO₂ compensation point.

While predictions of decreased flow through the PCO cycle at high concentration of CO₂ and 21% O₂ apparently hold true at CO₂ concentrations above about 900 μl l⁻¹, from 400 μl l⁻¹ CO₂ to 0, they do not. Ludwig and Canvin (1971) and D'Aoust and Canvin (1973) measured rates of true and apparent photosynthesis by a differential ¹⁴CO₂⁻¹²CO₂ uptake technique. Photorespiration rates calculated from the difference between true and apparent photosynthesis measured in this manner show no CO₂ dependence from 50 to 350 μl l⁻¹ CO₂. Initial rates of ¹⁴CO₂ efflux during ¹²CO₂ flushing also showed nearly equal rates of photorespiration at 280 and 620 μl l⁻¹ CO₂, though the rate of efflux was decreased by 50% at 1330 μl l⁻¹ CO₂ (Parnik et al. 1972). These observations are verified by the fact that extrapolation of the apparent photosynthesis rate vs CO₂ to 0 CO₂ predicts the initial rate of CO₂ release by illuminated leaves into CO₂ free air (Forrester et al. 1966).

Few attempts at directly measuring changes in the flow of carbon within the PCO cycle from 500 μl l⁻¹ CO₂ to 0 have been made. Snyder and Tolbert (1974) showed that in sugar beet and tobacco leaves the
per cent of $^{14}\text{CO}_2$ fixed into glycine and serine remained unchanged at 50 and 300 $\mu\text{l} \text{l}^{-1} \text{CO}_2$ in 21% $\text{O}_2$. On the other hand, Lee and Whittingham (1974) found that in tomato leaves $^{14}\text{C}$ in glycine after 3 minutes of fixation more than doubled while decreasing $\text{CO}_2$ from 300 to 100 $\mu\text{l} \text{l}^{-1}$. In this study, serine was labeled only to a small extent and label increased little at decreased $\text{CO}_2$. Osmond and Björkman (1972) also concluded from studies of label incorporation into glycolate, glycine, and serine that carbon flow through the glycolate pathway was influenced little by changing $\text{CO}_2$ concentration below 400 $\mu\text{l} \text{l}^{-1}$.

Mahon et al. (1974) estimated carbon flux from glycine to serine from changes in $^{14}\text{C}$ specific activity. Flux changed very little between 115 $\mu\text{l} \text{l}^{-1} \text{CO}_2$ and 400 $\mu\text{l} \text{l}^{-1} \text{CO}_2$. Carbon flux was decreased slightly at 915 $\mu\text{l} \text{l}^{-1} \text{CO}_2$.

Carbon Flow into the PCR and PCO Cycles

At external $\text{CO}_2$ concentrations below the $\text{CO}_2$ compensation point, $\text{CO}_2$ evolution through the photorespiratory cycle continues (Goldsworthy 1966, Moss 1966). In this situation, carbon must enter the combined PCR-PCO cycles from stored products. Evidence exists in the literature which confirms this observation.

Isolated chloroplasts illuminated in the presence of oxygen and limiting $\text{CO}_2$ are capable of converting label from exogenously fed PGA to glycolate (Kirk and Heber 1976, Robinson et al. 1977). Bundle sheath cells from maize were shown to form glycolate from ribose-5-phosphate in $\text{CO}_2$ free air (Chollet 1974) and Chlorella illuminated in $\text{CO}_2$ free $\text{O}_2$, converted labeled exogenous glucose to $\text{CO}_2$ and glycolate.
That this influx of stored carbon occurs in leaves of higher plants is shown from two sources. Lewanty and Maleszewski (1976) demonstrated that leaves of *Phaseolus* maintained in the light and CO\(_2\) free O\(_2\) after a period of \(^{14}\)CO\(_2\) fixation, lost label from both sucrose and starch as \(^{14}\)CO\(_2\). Ludwig and Canvin (1971) and D'Aoust and Canvin (1972) noted that the specific radioactivity of evolved CO\(_2\) in the light and during the postillumination CO\(_2\) burst was dependent upon the conditions of CO\(_2\) fixation. Specific radioactivity in the evolved CO\(_2\) decreased as CO\(_2\) concentration in the atmosphere during fixation decreased. The observation was explained as either direct dilution of the evolved CO\(_2\) from a carbon source remote from the photosynthetic pool, or dilution of the precursors of the photorespiratory CO\(_2\) from such a pool.

**Depletion of PCR Cycle Intermediates in the Presence of O\(_2\)**

Depletion of PCR cycle intermediates due to the export of glycolate from the chloroplast during photosynthesis in the presence of O\(_2\) has been suggested as a casual factor of the Warburg effect in isolated chloroplasts (Ellyard and Gibbs 1969). Inhibition of photosynthesis in isolated leaf cells treated with inhibitors of enzymes in the PCO cycle has also been attributed to such carbon depletion (Servaites and Ogren 1977, Servaites et al. 1978). Kirk and Heber (1976) and Robinson et al. (1977) have both suggested that, at low CO\(_2\) concentrations, the level of intermediates in the PCR cycle, in particular RuBP, may limit both the rate of CO\(_2\) fixation and of glycolate synthesis.
From the measured specific activity of CO$_2$ evolved in the light (Ludwig and Canvin 1971) and from the specific activity of various PCR and PCO cycle intermediates at differing CO$_2$ concentrations (Mahon et al. 1974), it appears that, at CO$_2$ concentrations below 250 to 300 µl l$^{-1}$, carbon from stored sources continually enters these interlocking cycles. Direct evidence as to whether or not this carbon influx is of sufficient maximum rate to maintain the level of PCR cycle intermediates high enough to provide maximal rates of glycolate synthesis at low CO$_2$ is not available. Indirect evidence is apparently contradictive. The maximum rate of CO$_2$ evolution into CO$_2$ free air in the light is transient, lasting only 2 to 5 minutes (D'Aoust and Canvin 1972). On the other hand, the lower, steady-state rate of CO$_2$ efflux into CO$_2$ free atmospheres may be increased by increasing O$_2$ concentration (Forrester et al. 1966).

While measurements of the level of PCR cycle intermediates during photosynthesis have been made in intact chloroplasts (Ellyard and Gibbs 1969) and RuBP has been measured in leaf discs (Laber et al. 1974), no complete study of the effects of O$_2$ and CO$_2$ concentration of the levels of PCR and PCO cycle intermediates has been made for intact leaves.
PART A. OXYGEN AND CARBON DIOXIDE EFFECTS ON THE POOL SIZE OF SOME PHOTOSYNTHETIC AND PHOTORESPIRATORY INTERMEDIATES IN GLYCINE MAX
INTRODUCTION

Since the discovery of the oxygenase activity of RuBP carboxylase/oxygenase (Ogren and Bowes 1971), attempts have been made to model the response of net photosynthesis to CO$_2$ concentration on the basis of the kinetic constants of the enzyme (Laing et al. 1974, Ku and Edwards 1977). These models predict the rate of net photosynthesis and the total oxygen inhibition of photosynthesis but they also predict increasing photorespiration with decreasing CO$_2$ concentration below air levels of CO$_2$.

That photorespiration remains constant below about 400 µl l$^{-1}$ CO$_2$ in air has been shown by measurement of photorespiration during photosynthesis (Ludwig and Canvin 1971, D'Aoust and Canvin 1973) and is indicated by the extent of labeling in glycolate pathway intermediates at various CO$_2$ concentrations (Snyder and Tolbert 1974, Osmond and Björkman 1972) and carbon flux through the glycolate pathway (Mahon et al. 1974).

It has been suggested (Kirk and Heber 1976, Robinson et al. 1977) that a relatively constant glycolate synthesis rate, and therefore photorespiration, may be due to rate limiting levels of RuBP at low CO$_2$ concentrations. This low, rate limiting, RuBP level in the chloroplast could be due to increased rates of glycolate synthesis and export relative to CO$_2$ fixation at low CO$_2$ in the presence of O$_2$. Photosynthesizing chloroplasts have been shown to deplete intermediates of the photosynthetic carbon reduction (PCR) or Calvin cycle at low CO$_2$ (Kirk and Heber 1976, Robinson et al. 1977). In these studies it was also shown, however, that, under conditions of low CO$_2$ in O$_2$, chloroplasts are capable
of incorporating carbon from external sources into PCR cycle intermediates and further exporting glycolate.

Intact leaves apparently import carbon into the combined PCR-photorespiratory carbon oxidation (PCO) cycles (Figure 1) during photosynthesis in $O_2$ at low $CO_2$. The specific radioactivity of evolved $CO_2$ (Ludwig and Canvin 1971) and of some PCR and PCO cycle intermediates (Mahon et al. 1974) remains less than that of the fed $^{14}CO_2$ in the presence of $O_2$ at low $CO_2$. If all photorespiratory $CO_2$ arises from the glycolate pathway, this result is most simply explained by a flux of stored carbohydrate into the PCR-PCO cycles during photorespiratory $CO_2$ loss (Ludwig and Canvin 1971). Lewanty and Maleszewski (1976) demonstrated the loss of $^{14}C$ from sucrose and starch as $^{14}CO_2$ during illumination of Phaseolus leaves in $CO_2$ free $O_2$.

The rate of stored carbohydrate influx into the PCR-PCO cycles can, under some circumstances, limit photorespiratory $CO_2$ loss. The initial rate of $CO_2$ evolution into $CO_2$ free air is transient, lasting some two to five minutes (D'Aoust and Canvin 1972). Inhibition of the PCO cycle in the presence of oxygen greatly increases total carbon loss from the PCR cycle, and inhibits photosynthesis, apparently due to depletion of intermediates (Servaites and Ogren 1977, Servaites et al. 1978).

In an attempt to determine whether or not the loss of photorespiratory $CO_2$ at $CO_2$ concentrations near or below the $CO_2$ compensation point is sufficient to reduce the level of PCR and PCO cycle intermediates, we have simultaneously measured the steady-state photosynthesis pool size of some PCR and PCO cycle intermediates and net $CO_2$ exchange rate in soybean leaves. Pool size measurements were made at three oxygen
concentrations and three to five CO$_2$ concentrations. Each intermediate was assayed directly to avoid problems of labeling to known specific activity. While carbon flux cannot be directly measured by this method, concentration of metabolites at enzyme-single substrate reactions should give an indication of relative flux at differing O$_2$-CO$_2$ concentrations. Knowledge of absolute pool sizes of these intermediates and of changes in these pool sizes at differing CO$_2$ and O$_2$ concentrations should aid in determining controlling factors in the rate of photorespiration at and below air levels of CO$_2$. 
MATERIALS AND METHODS

Plant Materials

Soybeans (Glycine max L. Merr.) c.v. Amsoy 71, were grown in 6 inch pots with nonsterile soil. Plants were started 4 per pot and thinned to the 3 most vigorous seedlings after emergence. Pots were watered from below until after emergence. After expansion of the first trifoliate, pots were watered every second day with 200 ml of double strength, modified Hoagland's solution (Johnson et al. 1957). Micronutrients were added according to Evans (Evans et al. 1972). Iron was supplied as the chelate of ethylenediamine-Di (o-hydroxyphenylacetic acid) (Sigma Chemical Co., St. Louis), at the rate of 2 μmole Fe per liter of nutrient solution.

Growth chamber conditions were 27° days, 21° nights at 70-85% relative humidity. Daylength was 16 hr and irradiance was 60 nEinsteins cm⁻² sec⁻¹ (400-700 nm) from fluorescent and incandescent lights.

The center leaf of the fourth trifoliate, numbering from the primary leaves upward, was used during the fourth week after planting.

Gas Exchange

Carbon dioxide exchange rates were measured with an open gas exchange system, using a Beckman model IR-215A infrared gas analyzer (Beckman Instruments Inc., Fullerton, Calif.).

The leaf cuvette was designed to enclose the whole center leaf of the trifoliate after removal of the two outside leaves. Drawings of the cuvette design are shown in Figure 2. The petiole was passed through a gap in the tygon O-ring and sealed with silicone stop-cock
Figure 2. Side and lower half surface views of the leaf gas exchange chamber. Scale 1:1
grease before the two halves of the chamber were clamped together.

Resistance to water vapor exchange in the chamber was measured by the method of Parkinson (1968). A psychrometer consisting of wet bulb and dry bulb thermometers mounted through a rubber stopper and placed in a 75 ml flask was placed in the outlet air line from the chamber. Temperature of the psychrometer was controlled by placing the flask in the same temperature control bath which regulated the temperature of both the leaf chamber jacket and the inlet air stream. The psychrometer was calibrated at different gas flow rates by passing air saturated with water at known temperatures through the empty leaf chamber and psychrometer.

Resistance to laminar flow in the cuvette was determined by measuring the water vapor pressure of the outlet gas when a filter paper leaf trace 47.4 cm$^2$ in area and saturated with water was in the chamber and the inlet air was passed through a magnesium perchlorate drying column.

Inlet gas to the system was mixed from bottled nitrogen, oxygen, and a mixture of approximately 0.12% CO$_2$ in N$_2$. The CO$_2$/N$_2$ mixture was made by releasing 0.088 moles (2.14 l at 25$^\circ$) of CO$_2$ into an evacuated gas cylinder (1.55 ft$^3$, 28.4 l). The CO$_2$ was released by slowly adding 50% sulfuric acid to 0.088 moles of NaHCO$_3$ in solution, and flushing the CO$_2$ released into the evacuated cylinder with an N$_2$ stream bubbled through the acidified solution. The cylinder was then pressurized to 900 pounds per in$^2$ (61.2 atm, 1737 l at 25$^\circ$) by direct connection to a fully pressurized N$_2$ cylinder.

A schematic of the gas exchange system is shown in Figure 3. Each inlet gas was passed through a separate needle valve for flow regulation.
Figure 3. The gas inlet system and CO₂ exchange rate monitoring system
The combined gas streams were mixed and humidified by bubbling through a water-containing flask at 25°, followed by a smaller flask at 23°. The gas stream, saturated with water at 23°, was rewarmed to 25° by passing through a 60 cm coil of copper tubing immersed in the 25° bath.

Water vapor was removed from the leaf cuvette outlet gas stream by passage through a cold tube immersed in an ice bath followed by a magnesium perchlorate column (1.5 cm x 24 cm). Flow rate was measured with a ball type gas flow meter (Matheson Gas Products, Joliet, Ill.) directly ahead of the infrared gas analyzer.

Volume of the system with the cold trap in the outlet line was 197 ml. Without the cold trap and connecting tubing, the volume was 160 ml. The leaf chamber volume was 49 ml and the volume of the sample cell of the infrared analyzer was 97.5 ml.

Leaf cuvette temperature, the first humidifier bath, and the gas inlet temperatures were controlled with a Hakke Temperature Controller (Hakke Ltd.). Water from the temperature controller was circulated through the water jacket of the leaf cuvette.

Oxygen content of the inlet gas mixture was determined with an oxygen electrode (Yellow Springs Instruments, Yellow Springs, Ohio) immersed in the 25° humidifier flask. The electrode was calibrated before each experimental run by adjusting the scale to read 100% saturation in O₂ while passing O₂ from the O₂ cylinder through the system. Zero percent saturation was checked after up scale calibration by passing N₂ through the system.

The infrared gas analyzer was calibrated against CO₂ in N₂ standards supplied by Air Products Co. (Tamaqua, Penn.) and by Matheson Gas
Products (Joliet, Ill.). Carbon dioxide analysis supplied with the standards was checked using an arbitrarily chosen standard and the standard curve supplied with the instrument. Some deviation from the manufacturer's analysis was indicated and the standards were adjusted to be consistent with the arbitrary standard.

The gas analyzer was used in the differential mode by filling the reference cell with the downscale gas, closing the inlet and outlet valves, then setting zero on the scale of the chart recorder connected to the infrared analyzer while the downscale gas was passed through the exchange system and the sample cell of the analyzer at the operating flow rate. The scale was set to 100 as an upscale gas was flowed through the system in a like manner. Standards were chosen to give scales of 1 to 2 \( \mu l \text{l}^{-1} \) CO\(_2\) per scale division. Measurements were made by setting the inlet gases to the desired flow rate and \( \text{N}_2/\text{O}_2/\text{CO}_2 \) concentration with the leaf chamber bypassed, then measuring the concentration change, in \( \mu l \text{CO}_2 \) per l of gas, which occurred with the leaf in place and multiplying by the measured flow rate. Net \( \text{CO}_2 \) exchange rate was expressed as nmoles of \( \text{CO}_2 \) exchanged per second per mg chlorophyll calculated from the following formula:

\[
\text{NCO}_2\text{ER} = [\text{CO}_2] \mu l \text{l}^{-1} \times \text{Flow Rate l sec}^{-1} \times \frac{273^0}{298^0} \times 1000 \text{nMoles (22.4 \mu l)}^{-1}
\]

Irradiance in the cuvette was provided by two 150 watt flood lamps placed above two 7 cm water filters. Irradiance was measured by removing the lower half of the cuvette and placing the sensing head of a photon flux density meter (Lambda Instruments, Lincoln, Neb.) below the upper half of the chamber. Irradiance could be decreased by placing
translucent filters in sequence above the leaf chamber.

Temperature of the lower leaf surface was estimated with a micro iron-constantan thermocouple pressed against the lower surface.

Preparation of Enzymes

Ribulose-1,5-bisphosphate carboxylase (E.C. 4.1.1.39) was partially purified by a modification of the method of Racker (1962). Spinach was purchased locally, the stems removed and the leaves deribbed and washed. The washed leaves (165 g) were homogenized for 3 min at high speed in a Waring blender with 400 ml of cold 0.01 M potassium phosphate buffer, pH 7.4. The homogenate was strained through 8 layers of cheesecloth and the extract was readjusted to pH 7.4 by the addition of dilute ammonium hydroxide. Solid ammonium sulfate was added to 18% saturation at 0° (10.6 g/100 ml) and the solution was stirred in an ice bath for 20 min then centrifuged at 13,000 x g for 20 min.

The supernatant was decanted and solid ammonium sulfate was added to give 37% saturation at 0° (17.5 g/100 ml). The solution was stirred for 20 min in an ice bath, and a large amount of green precipitate was removed by centrifugation for 20 min at 13,000 x g. The supernatant was discarded and the pellet dissolved in 40 ml of 0.1 M potassium phosphate buffer pH 7.4 which was 5 mM in EDTA, 2 mM in 2-mercaptoethanol, and 6 mM in MgCl₂.

The solution was rapidly brought to 60° by immersion in an 80° water bath with constant stirring. The temperature was maintained at 60° for 3 min by transferring the solution to a 60° water bath. A large amount of green precipitate was removed by centrifugation for 20 min at
27,000 x g and discarded.

The supernatant from the heat treated solution was brought to 45% saturation in ammonium sulfate at 0° by slowly adding saturated ammonium sulfate solution at 0°. The solution was stirred for 10 min, then centrifuged for 20 min at 27,000 x g.

The supernatant was discarded and the pellet dissolved in 7 ml of the 0.1 M phosphate buffer. The dark yellow protein solution was applied to a Sephadex G-25 column (1.5 x 45 cm) previously equilibrated with distilled water and eluted with distilled water. The protein containing fraction emerged very shortly after the void volume (32 ml) and ahead of a dark yellow band. Eight ml of a 300 mM Tris-HCl buffer pH 7.8 was added to the protein fraction from the column (30 ml) giving a solution 8 mM in Tris-HCl at pH 7.8.

The ribulose-1,5-bisphosphate carboxylase preparation was stable when stored desiccated at -20° for 12 months. Specific activity was 0.01 μmole RuBP converted to PGA min⁻¹ (mg protein)⁻¹ at 5 mM NaHCO₃, 10 μM RuBP, 10 mM MgCl₂, and pH 7.3. The assay was run by the coupled enzyme method similar to that described by Racker (1962). The preparation was shown to be free of phosphoenolpyruvate carboxylase contamination by the absence of acid stable ¹⁴C after incubation of the enzyme solution with 20 mM phosphoenolpyruvate and 5 mM NaH¹⁴CO₃ at pH 8.2.

Glycolic acid oxidase (glycolate: oxygen oxidoreductase E.C. 1.1.3.1) was isolated by the method of Kerr and Groves (1975), except that the purification was not carried through the gel filtration step. All steps were performed as near zero degrees as possible. Protein was determined as the absorbance at 280 nm.
Leaves and some stem material (200 g) from 3-week-old pea plants \textit{(Pisum sativum)} were ground with a Waring blender in 800 ml of cold
0.1 M potassium phosphate buffer 0.1 mM in EDTA and 5 mM in 2-mercapto-
ethanol at pH 7.6. Sixty g of insoluble polyvinyl pyrrolidone was
included with the grinding media.

The homogenate was strained through 6 layers of cheesecloth and
centrifuged at 10,000 x g for 10 min. The pellet was discarded and the
supernatant brought to 25% saturation \textit{(at 0°)} in ammonium sulfate by
the addition of solid ammonium sulfate \textit{(13.4 g/100 ml)}. The solution
was stirred for 20 min, then centrifuged at 10,000 x g for 10 min.
The pellet was again discarded and the supernatant brought to 45%
saturation \textit{(at 0°)} in ammonium sulfate by the addition of solid ammonium
sulfate \textit{(11.5 g/100 ml)}. The solution was again centrifuged at 10,000
x g for 10 min, the supernatant discarded, and the precipitate redis-
solved in 25 ml of 20 mM Tris-HCl, pH 8.3 and 5 mM in 2-mercaptoethanol.
Two ml of 2% protamine sulfate was added, the solution was stirred for
30 min, then centrifuged at 29,000 x g for 30 min.

The clarified protein solution was desalted by passage through a
Sephadex G-25 column \textit{(1.5 x 45 cm)} equilibrated with 5 mM Tris-HCl at
pH 8.3. The protein band emerged immediately after the void volume
\textit{(32 ml)}. The first 35 ml after the void volume was collected and loaded
onto a DEAE-cellulose column \textit{(2.8 x 18 cm, ~Cl form)}, which was equili-
brated also with 5 mM Tris-HCl pH 8.3. The column was eluted with the
same buffer at 30 ml/hr.

Glycolic acid oxidase appeared in the column wash of the DEAE
column. Most contaminating protein remained bound to the DEAE column.
at this pH. Glycolic acid oxidase was shown by Kerr and Groves (1975) to have a pI of not less than 9.6 and so is substantially nonionized at pH 8.3. The glycolic acid oxidase containing fractions were pooled, brought to 50% saturation in ammonium sulfate, and centrifuged at 27,000 x g for 20 min. The precipitate was resuspended in 5 ml of 50 mM HEPES-K buffer (pH 8.3), which was 20 µM in reduced flavin-mononucleotide and 50% saturated with ammonium sulfate. The precipitate was stored at 4°.

The preparation was free of RuBP carboxylose activity and the specific activity was 21 µmoles glycolate oxidized min⁻¹ (mg protein)⁻¹ when assayed as described.

Glycolic acid oxidase was assayed by a method similar to that described by Servaites et al. (1978). To a 25 ml conical centrifuge tube was added 0.2 ml HEPES-K buffer (50 mM pH 8.3), 0.1 ml glycolate (10 mM in buffer), and 200 units catalase preparation in 0.1 ml buffer. The reaction was started by the addition of 0.05 ml of the column fraction to be assayed, and the reaction mixture incubated for either 5 or 10 min at 30°. The reaction was stopped by the addition of 0.2 ml of 25 mM phenylhydrazine-HCl in 0.5 N HCl. The reaction tubes were then cooled on ice, and 0.5 ml of concentrated HCl was added followed by 0.3 ml of 50 mM potassium ferricyanide. The tubes were then vortex mixed, centrifuged, and allowed to stand at room temperature for 20 min before A₅₅₀ was determined.
Metabolite Extraction

The center leaf of the fourth trifoliate of plants 4 weeks old was used. The two outside leaves of the trifoliate were removed and the petiole placed through a slot in the gas seal of the upper half of the cuvette. The area around the petiole was sealed with silicone stopcock grease and the lower half clamped in place. The leaf was allowed to photosynthesize under the desired atmosphere until a steady rate of net CO₂ exchange was attained.

Leaves were killed 5 min after attaining a maximum rate of net CO₂ exchange in a given O₂-CO₂ atmosphere by first cutting the petiole, then removing the clamps holding the lower half of the cuvette, and opening the chamber very slightly. The detached leaf was then held with a forceps, the lower half of the cuvette was allowed to drop away, and the leaf was very rapidly plunged into liquified freon-12 which was held at just above its freezing point (-156°) by partial immersion in liquid N₂.

Frozen leaves were transferred to individual envelopes and held at -20° until they were freeze-dried. The dried leaves were ground to a fine powder in a mortar and pestle with a small amount of ground glass. Approximately 2% of the ground mixture was weighed and set aside for chlorophyll determination. The remaining ground material was weighed and transferred to a 15 ml nalgene centrifuge tube.

Samples for analysis of RuBP and PGA were extracted with 4 ml of 1 N formic acid. The mixture was sonicated for 30 sec at 0°, then centrifuged at 27,000 x g for 20 min prior to ion exchange fractionation.
Three ml of the extract was added to a Dowex-1 Cl⁻ column (0.7 x 3 cm). The column was washed with 8 ml of water, then 5 ml of 0.02 N HCl. Both RuBP and PGA were eluted from the column with 5 ml of 0.5 N HCl. The 0.5 N HCl fraction was immediately frozen and lyophilized. The lyophilized fractions were redissolved in 1 ml of 100 mM Tris-HCl, pH 7.8, which was also 10 mM in MgCl₂.

Samples for the analysis of glycolic acid, glycine, and serine were extracted with 4 ml of 0.5 N HClO₄, sonicated and centrifuged as were the RuBP-PGA samples. The residue from extraction was reextracted with a second 4 ml portion of 0.5 N HClO₄. The combined extracts were neutralized with 1 N KHCO₃ at 0°. The precipitated KClO₄ was removed by centrifugation, and the supernatant passed through a Dowex-50 H⁺ column (0.7 x 3 cm). The column was washed with 10 ml of water. The amino acids were removed from the column with 7 ml of 2 N NH₄OH. The column wash fraction which contained glycolate was made basic with NH₄OH, then both the wash fraction and the 2 N NH₄OH fraction were frozen and lyophilized.

RuBP was analyzed by the ¹⁴CO₂ method of Ellyard and Gibbs (1969). RuBP carboxylase was activated prior to the assay as follows: 8 mg of the enzyme preparation (7.2 mg protein) was dissolved in 0.9 ml of pH 8.2 Tris-HCl buffer (25 mM, 10mM in MgCl₂, 5 mM in dithiothreitol) which had been flushed with N₂. The solution was made 10 mM in NaHCO₃ by the addition of 0.1 ml of 0.1 M NaH⁻¹⁴CO₃ 0.25 μCi (μmole)⁻¹. The enzyme-NaHCO₃ solution was allowed to stand in a sealed tube 15 min before use. To 0.1 ml of either the redissolved acid fraction from leaf extracts or RuBP standards in scintillation vials was added 0.1 ml
of the activated enzyme solution. The vials were flushed briefly with N₂, sealed, and held at 30° for two hr. The reaction was stopped by the addition of 0.3 ml of glacial acetic acid, and the solution was dried under a stream of dry air. The residue was dissolved in 0.5 ml of 0.1 M NaH¹²CO₃; 0.3 ml of glacial acetic acid was again added, and the contents again evaporated to dryness.

Acid stable ¹⁴C was determined by liquid scintillation spectroscopy after dissolving the residue remaining in the vial in 0.1 ml of water, adding 2 ml of ethylene glycol monomethyl ether and 8 ml of scintillation fluid (100 ml Beckman Bio-Solv, 0.5 g 2-(4'-Biphenyl)-6-phenylbenzoxazole and 8 g 2-(4'- tert butylphenyl)-5-(4'-b phenyl)-1,3,4 oxadiazole in 1 liter toluene).

A standard curve was run with each set of analysis. RuBP was purchased (Sigma Chemical Co.) as the dibarium salt. The salt was dissolved in a small volume of 0.05 N HCl and washed through a Dowex-50 H⁺ column (0.7 x 2 cm) to remove the barium. The column effluent was neutralized with Tris base. RuBP content of the resulting solution was determined by the coupled enzyme method of Racker (1962). Approximately 0.01 µmole of RuBP was added to 0.85 ml of the complete PGA assay media described below. The reaction was started by the addition of 0.1 ml of the activated RuBP carboxylase preparation which was 10 mM in MgCl₂ and 50 mM in NaHCO₃. The reaction was allowed to run to completion as determined by the stabilization of the absorbance at 340 nm. Total RuBP added was calculated as follows:

\[ \text{RuBP (nmoles)} = \frac{A_{340}}{6.27 \times 10^{-3} \text{ nmoles}^{-1} \text{ ml}^{-1}} \times 0.95 \text{ ml} \times \frac{1}{2} \left( \frac{\text{moles RuBP}}{\text{moles NADH}} \right) . \]
PGA was analyzed from an aliquot of the same column fraction. Decrease in the absorbance of NADH at 340 nm was measured as PGA was reduced to 3-phosphoglyceraldehyde by glyceraldehyde 3-phosphate dehydrogenase. The method used was that described by Lowry and Passonneau (1972). To 0.7 ml of imidazole buffer (50 mM, pH 7.1) which was also 2 mM in MgCl₂, 2 mM in 2-mercaptoethanol, and 20 mM in NaCl was added 0.1 ml of the redissolved acid fraction from leaf extracts, 0.1 ml of NADH (1 mg/ml) and 0.1 ml of ATP (20 mM). The reaction was initiated by the addition of 0.017 units each of 3-phosphoglycerate phosphokinase and glyceraldehyde 3-phosphate dehydrogenase (purchased from Sigma Chemical Co. as the mixed enzymes) in 0.01 ml of the imidazole buffer. Total reaction volume was 0.91 ml. Total PGA in the aliquot was calculated as follows:

\[
PGA \text{ (nmoles)} = \frac{A_{340}}{6.27 \times 10^{-3} \text{ nmoles}^{-1} \text{ ml}^{-1}} \times 0.91 \text{ ml}
\]

Glycine and serine were analyzed in the lyophylized 2 N NH₄OH fraction from the Dowex 50-columns. The residue was redissolved in 2 ml of 2 N HCl and held at 100° for 2 hr to hydrolyze amides. The hydrolyzed samples were reduced to dryness under a stream of dry air, redissolved in 1 ml of water, and again taken to dryness. The residue was dissolved in 3 ml of water and taken just to neutrality with dilute NH₄OH. The neutralized samples were passed through a Dowex-1 Cl⁻ column (0.7 x 2 cm) to decolorize and remove acidic amino acids (includes departate, glutamate, glutamine, and aspartate originally in leaf). The columns were washed with 10 ml of water, and the combined through-puys were frozen and lyophylized. The residue was redissolved in 0.1 ml of
water and an aliquot (0.025 ml) was spotted on a thin layer cellulose plate (0.5 mm cellulose) along with $1 \times 10^{-9}$ mole each of $^{14}$C labeled glycine and serine (5 mCi/mmol). The amino acids were separated using the solvent systems of Haworth and Heathcote (1969). The plates were developed twice in the second solvent system to give complete separation of glycine and serine.

Autoradiograms of the developed plates were made by 3 days exposure to X-ray film (Kodak type XL). The film was developed and the outline of the radioactive spots transferred to the TLC plates. The outlined spots were removed by covering the outlined area with stripping mixture as described by Redgwell et al. (1974). To each of the removed spots in acid washed tubes was added 0.5 ml of borate buffer (Connell et al. 1955). The buffer solution was evaporated to dryness under vacuum over NaOH and H$_2$SO$_4$ to remove traces of ammonia. To the dried residue was added 0.5 ml of water and 2 ml of ninhydrin-hydrinantin reagent (Moore and Stein 1954). Color was developed in a boiling water bath for 15 min. The tubes were then cooled and diluted with 10 ml of 50% (v/v) ethanol. Glycine and serine standards were developed in the same manner. The developed tubes were mixed by inversion and clarified by centrifugation. Absorbance at 570 nm was determined.

Glycolic acid was determined in the acid fraction from 0.5 N HClO$_4$ extracted leaf residue. The neutralized lyophilized residue from the throughputs of the Dowex-50 columns was redissolved in 1 ml of 50 mM HEPES-K buffer, pH 8.3. Glycolic acid was determined by a method similar to that described by Laing (1974). To an aliquot of the glycolic acid containing fraction (0.1 ml) in a conical centrifuge tube was added
1 unit of glycolate oxidase and 20 units of catalase (Sigma Chemical Co., St. Louis) in 0.2 ml of HEPES-K buffer (pH 8.3, 50 mM). The reaction mixture was incubated for 30 min at 30°, 0.2 ml of phenylhydrazine-HCl (25 mM in 0.5 N HCl) was added, and the mixture incubated for 10 min. The tubes were cooled on ice and 0.5 ml of concentrated HCl was added, followed by 0.3 ml of 50 mM potassium ferricyanide. The tubes were vortex mixed then centrifuged for 10 min and allowed to stand at room temperature for an additional 20 min. The A_{550} of the glyoxylate-biphenyl formazan formed was determined. Blanks were prepared in the above manner with the enzyme solution added after the concentrated HCl.

Chlorophyll in the dried and ground leaf residue was determined by the method of Arnon (1949) after extraction with 5 ml of 80% (v/v) acetone and centrifugation to precipitate residue.
RESULTS

Leaf Chamber Evaluation

Resistance to laminar flow in the leaf cuvette was determined by the method of Parkinson (1968) using the following formula:

\[ r = \frac{mB H_2O \text{ at surface} - mB H_2O \text{ inlet} \cdot 2 \cdot \text{surface area (cm}^2)}{mB H_2O \text{ outlet} - mB H_2O \text{ inlet} \cdot \text{flow rate (cm}^3 \text{ sec}^{-1})} \]

Resistance to laminar flow was flow rate dependent and to some extent dependent upon the area of the evaporative surface. At flow rates below 1 1 min\(^{-1}\), resistance was unacceptably high. At 1 1 min\(^{-1}\), the measured resistance was 0.68 sec cm\(^{-1}\) with the 47 cm\(^2\) trace and dropped to 0.4 sec cm\(^{-1}\) at 2 1 min\(^{-1}\) flow rate. The resistance at 1 1 min\(^{-1}\) flow rate and a filter paper trace of 23 cm\(^2\) was 0.23 sec cm\(^{-1}\). A maximum resistance to laminar flow of 0.5 sec cm\(^{-1}\) has been suggested by Canvin and Fock (1972) as a requirement for leaf chamber design.

To further check chamber efficiency, net CO\(_2\) exchange rates in air and at saturating irradiance were measured at different flow rates. The results, shown in Figure 4, indicate that flow rates of 1 1 and above give little further increase in net carbon exchange rate. To maintain a smaller CO\(_2\) differential between the cuvette inlet and outlet, and to minimize resistance to laminar flow in the chamber, all subsequent studies were carried out using a flow rate of 2 1 min\(^{-1}\).

The effect of measured irradiance on net CO\(_2\) exchange rate was checked also. A plot of exchange rate vs irradiance is shown in Figure 5.
Figure 4. Net CO$_2$ exchange rate vs outlet gas flow rate from the leaf chamber
Figure 5. Net CO$_2$ exchange rate vs irradiance
Lower leaf surface temperature was measured also at each irradiance. Below 60 nEinsteins sec\(^{-1}\) cm\(^{-2}\), the lower leaf surface temperature remained very near the inlet air and chamber jacket temperatures of 25°. At higher irradiance, the leaf temperature increased and values of 29-30° were measured at 120 nEinsteins sec\(^{-1}\) cm\(^{-2}\). All subsequent rates were measured at this irradiance since the leaf temperature was felt to be acceptable and the irradiance was well-above the region of the curve giving linear rate increase with increased irradiance. Similar irradiance has been shown to approach light saturation in soybean (Dornhoff and Shibles 1970).

**CO\(_2\) Exchange Rates**

Net CO\(_2\) exchange rates in 20% O\(_2\) and 60% O\(_2\) increased linearly with increasing CO\(_2\) concentration within the range of 50 to 450 μl l\(^{-1}\) (Figure 6). A linear increase has been reported by previous investigators (e.g., Forrester et al. 1966, Ludwig and Canvin 1971). In 1% O\(_2\), net CO\(_2\) exchange rate vs CO\(_2\) concentration was curvilinear. In 1% O\(_2\), the slope of the net exchange rate vs CO\(_2\) between 50 μl l\(^{-1}\) and 130 μl l\(^{-1}\) was 0.25 nmoles CO\(_2\) sec\(^{-1}\) (mg Chl\(^{-1}\)) (μl CO\(_2\) l\(^{-1}\))\(^{-1}\) while from 280 to 410 μl l\(^{-1}\) the slope was 0.128 nmoles CO\(_2\) sec\(^{-1}\) (mg Chl\(^{-1}\)) (μl CO\(_2\) l\(^{-1}\))\(^{-1}\). In 20% O\(_2\), the slopes over corresponding CO\(_2\) concentrations were 0.125 and 0.124 nmoles CO\(_2\) sec\(^{-1}\) (mg Chl\(^{-1}\)) (μl CO\(_2\) l\(^{-1}\))\(^{-1}\), respectively.

The average rates of net CO\(_2\) exchange at 300 μl CO\(_2\) l\(^{-1}\) were 35.5 nmoles CO\(_2\) sec\(^{-1}\) (mg Chl\(^{-1}\)) in 1% O\(_2\), 25.0 nmoles CO\(_2\) sec\(^{-1}\) (mg Chl\(^{-1}\)) in 20% O\(_2\), and 6.5 nmoles CO\(_2\) sec\(^{-1}\) (mg Chl\(^{-1}\)) in 60% O\(_2\).
Figure 6. Net CO$_2$ exchange rate vs CO$_2$ concentration at 1%, 20%, and 60% O$_2$. 

NET CO$_2$ EXCHANGE RATE (mg Chl$^{-1}$) vs CO$_2$ CONCENTRATION (µl l$^{-1}$)

- 1% O$_2$
- 20% O$_2$
- 60% O$_2$
The CO₂ compensation point increased with increasing O₂ in the atmosphere, from approximately 2 µl 1⁻¹ in 1% O₂ to 60 µl 1⁻¹ in 20% O₂ and to 170 µl 1⁻¹ in 60% O₂.

The Steady-State Photosynthesis Level of RuBP

Several extraction methods were examined before the method described above was adopted. While extraction of either chloroplast suspensions or frozen tissue with HClO₄ has been described (Ellyard and Gibbs 1969, Laber et al. 1974) for the extraction of RuBP, several difficulties arose when adapting the method to analysis of whole leaves. First, it was necessary to quantitate the chlorophyll as a basis of expression for both the metabolite level and the CO₂ exchange rate; and, second, the use of HClO₄ caused problems in later ion exchange purification steps. Extraction of frozen leaf tissue with various ethanol-water combinations up to 50% water yielded no RuBP. A variation of organic solvent extraction used with some success was removal of pigments by acetone extraction prior to aqueous acid extraction of the RuBP.

Extraction of frozen leaves also caused problems of maintaining the tissue frozen until the solvent used for extraction completely inactivated the enzymes present. Lyophilization before extraction, as suggested by Lowry and Passonneau (1972), allowed extraction of a separate aliquot for chlorophyll determination and insured precipitation of protein as the extracting solvent was added.

RuBP was assayed after partial purification by ion exchange chromatography to allow use of NaH¹⁴CO₃ of lower specific activity than would be required in assays of crude extracts. Sugar diphosphates bind
to anion exchange resins in the presence of moderate concentrations of weak acids (Atkins and Canvin 1971), while neutral, basic, and weak acid components pass through. The RuBP containing fraction was removed with a volatile strong acid which could be removed during the subsequent lyophilization. Little loss of either RuBP or PGA should occur during elution or further handling at low temperature due to the acid stability of both phospho-ester bonds of RuBP and the phospho-ester bond of PGA (Horecker et al. 1956). This partial purification removed extracted compounds and salts which might interfere with the carboxylase reaction at high concentration, removed pigments which caused variable quenching during $^{14}C$ counting by liquid scintillation methods and allowed control of RuBP concentration in the assay.

The RuBP assay conditions were adjusted so that RuBP standards (0 to 25 nmole RuBP per assay) were completely converted to PGA within 30 min. Aliquots of leaf extracts contained less than 20 nmole RuBP per assay and cpm of $^{14}C$ per nmole of RuBP converted, checked closely with the specific activity of the added NaH$^{14}CO_3$. Recovery of known quantities of RuBP added to extracts of leaves which were frozen after 10 min in the dark gave recoveries of 72-76% through the extraction and analysis procedure.

The measured RuBP level at 20% O$_2$ and 300 $\mu$1 1$^{-1}$ CO$_2$ was about 15 nmole (mg Chl)$^{-1}$. RuBP levels near 7 nmole (mg Chl)$^{-1}$ have been reported in photosynthesizing chloroplasts (Kirk and Heber 1976, Ellyard and Gibbs 1969) and 20 nmole (mg Chl)$^{-1}$ was reported in spinach leaf discs in air (Laber et al. 1974).
Each data point represents an individual leaf and plant. Some variation is likely due to biological variation. In addition, the turnover rate of the RuBP pool, as calculated from the net $CO_2$ exchange rate, is approximately two times per second at higher $CO_2$ concentrations. The ATP pool size is likely even smaller as Cockburn (1974) measured 3 to 4 nmoles ATP (mg Chl)$^{-1}$ in photosynthesizing chloroplasts. This turnover rate and limited capacity for RuBP production after darkening allows substantial error toward lower values if leaves are not frozen very rapidly. Liquified freon was used when making leaf kills to minimize freezing time. It is still possible that variation in kill time may account for some of the lower RuBP levels measured.

The influence of atmospheric $CO_2$ concentration on RuBP level during steady-state photosynthesis at the three $O_2$ concentrations tested is shown in Figure 7. The average RuBP level was 15 nmoles (mg Chl)$^{-1}$, and the regression line of RuBP level vs $CO_2$ concentration at 20% $O_2$ shows no measurable interaction over the range of $CO_2$ concentrations tested.

While transient increases in RuBP level have been reported upon decreasing $CO_2$ concentration in air (Benson and Calvin 1950, Bassham and Kirk 1962), both reports indicate RuBP levels at low $CO_2$ equal to or slightly less than at high $CO_2$ in longer term experiments.

Levels of RuBP at 60% $O_2$ were somewhat lower than those obtained at 20% $O_2$ (9 nmoles (mg Chl)$^{-1}$ at 300 $\mu l$ $l^{-1}$ $CO_2$) and showed a tendency toward decreasing at low $CO_2$ concentrations. It should be noted that, due to the increased $CO_2$ compensation point in 60% $O_2$, leaves photosynthesizing in atmospheres containing less than 150 $\mu l$ $l^{-1}$ $CO_2$ were undergoing net carbon loss.
Figure 7. The steady-state photosynthesis level of RuBP vs CO₂ concentration at 1%, 20%, and 60% O₂.
RuBP level in leaves photosynthesizing in atmospheres containing 1% O₂ and 300 to 500 μl l⁻¹ CO₂ was nearly equal to the level in leaves at 20% O₂. The steady-state RuBP level in leaves from atmospheres containing 1% O₂ and less than 300 μl l⁻¹ CO₂ increased to 60 n mole (mg Chl)⁻¹ as CO₂ concentration was decreased to 50 μl l⁻¹.

The Steady-State Photosynthesis Level of PGA

PGA elutes in the acid II fraction of Atkins and Canvin (1971) along with RuBP and was present in sufficient quantity to allow its assay by spectrophotometric means.

At 300 μl l⁻¹ CO₂, PGA level was 125-140 n moles (mg Chl)⁻¹ and was effected only slightly by O₂ concentration as shown in Figure 8. In the presence of either 20% or 60% O₂, the steady-state level of PGA during photosynthesis decreased as the CO₂ concentration was lowered to the compensation point and below in the case of 60% O₂. The PGA level in 1% O₂ also decreased slightly in going from 300 μl l⁻¹ CO₂ to 50 μl l⁻¹ CO₂, although to a much lesser extent than in the presence of higher O₂ concentrations.

Steady-State Photosynthesis Level of Glycolic Acid

Glycolic acid was measured in the acid extract of frozen and dried leaves after removal of basic components and some interfering pigments by passage through a cation exchange column. It was necessary to maintain all solutions at neutrality or higher pH during concentration, even by freeze-drying, to prevent loss of glycolic acid by evaporation. The assay as described had a lower limit of approximately 5 n moles glycolate per assay, and the response remained linear to at least
Figure 8. The steady-state photosynthesis level of PGA vs CO₂ concentration at 1%, 20%, and 60% O₂.
50 nmoles per assay. Blank readings were low and were apparently more influenced by the presence of pigments remaining in the extract than to the presence of reactive carbonyls other than the glyoxylate formed by the oxidation of glycolate.

The measured glycolate levels in nmoles (mg Chl)\(^{-1}\) as a function of \(\mathrm{CO}_2\) concentration at the 3 \(\mathrm{O}_2\) levels tested are shown in Figure 9. At \(\mathrm{CO}_2\) concentrations above 300 \(\mu\)l \(1^{-1}\), increasing atmospheric \(\mathrm{O}_2\) concentration increased the steady-state glycolic acid level slightly. Even at 1\% \(\mathrm{O}_2\), a significant glycolic acid pool remained, as the glycolic acid pool size after 10 min of darkness in air was 18 to 20 n mole (mg Chl)\(^{-1}\), or less than half that measured in the light at 1\% \(\mathrm{O}_2\). The effect of decreasing \(\mathrm{CO}_2\) concentration in the presence of \(\mathrm{O}_2\) was similar to the effect observed on PGA. The glycolic acid pool size remained constant or possibly increased slightly from 500 \(\mu\)l \(1^{-1}\) to the \(\mathrm{CO}_2\) compensation point and decreased thereafter. At 1\% \(\mathrm{O}_2\), \(\mathrm{CO}_2\) concentration had no consistent effect on the glycolic acid pool size.

Steady-State Glycine and Serine Levels During Photosynthesis

The glycine pool size during steady-state photosynthesis at 300 \(\mu\)l \(1^{-1}\) \(\mathrm{CO}_2\) increased greatly and in a linear manner with increasing \(\mathrm{O}_2\) concentration in the atmosphere as shown in Figure 10. Serine pool size, also shown in Figure 10, was independent of \(\mathrm{O}_2\) concentration.

The effects of \(\mathrm{CO}_2\) concentration on glycine and serine levels at 1, 20, and 60\% \(\mathrm{O}_2\) are shown in Figures 11 and 12, respectively. The serine pool size showed no interaction with \(\mathrm{CO}_2\) concentration in the \(\mathrm{CO}_2\) concentration range tested at 1 and 60\% \(\mathrm{O}_2\). An increase in the serine
Figure 9. The steady-state photosynthesis level of glycolic acid vs CO₂ concentration at 1%, 20%, and 60% O₂.
Figure 10. The steady-state photosynthesis levels of glycine and serine at 300 μl l⁻¹ CO₂ vs O₂ concentration
Figure 11. The steady-state photosynthesis level of glycine vs CO₂ concentration at 1%, 20%, and 60% O₂.
Figure 12. The steady-state photosynthesis level of serine vs CO₂ concentration at 1%, 20%, and 60% O₂.
pool size with decreasing $\text{CO}_2$ at 20% $\text{O}_2$ is indicated by the regression line of serine level vs $\text{CO}_2$ concentration. The effects of $\text{CO}_2$ concentration on the glycine pool were similar to those observed in the serine pool.

The level of both the glycine and serine pools during photosynthesis was quite variable. While some of this variation may arise from the analytical method, the problem of variable pool size was also encountered by Canvin et al. (1975) in determining the specific activity of glycine and serine in $^{14}\text{CO}_2$ fed sunflower leaves. A major part of this variability is apparently due to plant to plant differences and is inherent in single leaf determinations.

Approximately equal levels of glycine and serine were measured at air levels of $\text{CO}_2$ and $\text{O}_2$ in this study. The relative amount of glycine and serine present in photosynthesizing leaves seems to vary with species. Mahon et al. (1974) found that the serine pool size was almost twice that of glycine in sunflower, as did Noguchi and Tamaki (1962) in tobacco leaves. Bauer et al. (1977) and Platt et al. (1977), however, found nearly equal pool sizes in pea and alfalfa leaves, respectively.
DISCUSSION

Even though RuBP in green leaves apparently exists only in the chloroplast (Bassham et al. 1968, Heber 1974), the observed RuBP levels are below levels calculated to saturate the RuBP binding sites present in the chloroplast. Jensen and Bahr (1977) have calculated the intrastroma concentration of RuBP binding sites as about 3.5 mM. The measured RuBP levels may similarly be converted to concentrations using a value of 25 μl stroma volume per mg chlorophyll (Heldt and Sauer 1971). The measured RuBP level at 300 μl 1⁻¹ CO₂, when corrected for yield, represents a concentration of 0.8 mM. Other workers have arrived at similar values, approximately one-fourth of the binding site concentration, using spinach chloroplast preparations (Sicher and Jensen 1978) and spinach leaf cell preparations (Collatz 1978).

If the calculated RuBP concentration represents a reasonable steady-state value, the observed rate of CO₂ exchange must be supported by these substrate and enzyme concentrations. The rate of CO₂ fixation is described by:

\[ v = \frac{\kappa_{cat}}{[E \cdot RuBP \cdot CO₂]} \]

where \( v \) is the reaction velocity, \( \kappa_{cat} \) the catalytic constant, and [\( E \cdot RuBP \cdot CO₂ \)] the carboxylase-bisubstrate complex. The rate of apparent photosynthesis in 1% O₂ approximates true photosynthesis (Ludwig and Canvin 1971) and, therefore, \( v \). The net CO₂ exchange rate in 1% CO₂ at 330 μl 1⁻¹ averaged 40 nmoles sec⁻¹ (mg Chl)⁻¹. This may be expressed as the instantaneous steady-state CO₂ concentration change if divided
by the stroma volume per mg chlorophyll:

\[ v = \frac{40 \text{ nmoles sec}^{-1} \text{(mg Chl)}^{-1}}{25 \mu l \text{(mg Chl)}^{-1}} = 1.6 \text{ mM sec}^{-1}. \]

Solving the velocity equation for \([E \cdot RuBP \cdot CO_2]\) and substituting the above value for \(v\) and 25 sec\(^{-1}\) (Badger and Collatz 1977) for \(k_{cat}\) gives a steady-state concentration of \(E \cdot RuBP \cdot CO_2\) of 0.064 mM required to give the observed rate of CO\(_2\) fixation.

Calculation of the RuBP concentration required to give this concentration of \(E \cdot RuBP \cdot CO_2\) is made difficult by lack of knowledge of the CO\(_2\) concentration within the chloroplast and the binding order of the carboxylation reaction. Hatch (1975) calculated the CO\(_2\) concentration surrounding photosynthesizing mesophyll cells to be about 5 \(\mu M\). However, this value should be greater than the free CO\(_2\) concentration near the active sites of RuBP carboxylase due to resistance to transfer within the cell.

Using the calculated RuBP concentration and the kinetic constants for RuBP carboxylase, the free CO\(_2\) concentration required within the chloroplast to give \([E \cdot RuBP \cdot CO_2]\) of 0.064 mM can be calculated for each of the possible substrate binding orders, RuBP first, CO\(_2\) first, and random.

Taking the random mechanism first, the Michaelis-Menton constant, \(K_m\), for RuBP is:

\[ K_{m_{RuBP}} = \frac{[RuBP] ([E] - [E \cdot RuBP])}{[E \cdot RuBP]} \]

where \([E]\) is the RuBP carboxylase active site concentration (3.5 mM), \([RuBP]\) is the concentration of free RuBP, and \([E \cdot RuBP]\) is the
concentration of $E \cdot \text{RuBP}$ complex. Free RuBP is equal to the total, minus bound so that:

$$[\text{RuBP}] = [\text{RuBP}_{\text{total}}] - [E \cdot \text{RuBP}]$$

or

$$[E \cdot \text{RuBP}] = [\text{RuBP}_{\text{total}}] - [\text{RuBP}].$$

Substituting this into the equation defining $K_m_{\text{RuBP}}$ gives:

$$K_m_{\text{RuBP}} = \frac{[\text{RuBP}][E] - ([\text{RuBP}_{\text{total}}] - [\text{RuBP}])}{[\text{RuBP}_{\text{total}}] - [\text{RuBP}]}.$$

This may be rearranged to the general form of the quadratic equation:

$$0 = [\text{RuBP}]^2 + ([E] + K_m_{\text{RuBP}} - [\text{RuBP}_{\text{total}}])[\text{RuBP}] - K_m_{\text{RuBP}}[\text{RuBP}_{\text{total}}]$$

and solved for $[\text{RuBP}]$ using the above mentioned values and 0.035 mM for $K_m_{\text{RuBP}}$ (Badger and Collatz 1977). The solution gives $[\text{RuBP}] = 0.01$ mM and $[E \cdot \text{RuBP}] = 0.79$ mM.

If binding is completely random, the probability of any binding site containing RuBP or $\text{CO}_2$ is:

$$p(E \cdot \text{CO}_2) = \frac{[E \cdot \text{CO}_2]}{[E]}, \quad p(E \cdot \text{RuBP}) = \frac{[E \cdot \text{RuBP}]}{[E]}.$$

The probability of $E \cdot \text{CO}_2 \cdot \text{RuBP}$ occurring should then be the product of the single substrate binding probabilities:

$$p(E \cdot \text{CO}_2 \cdot \text{RuBP}) = p(E \cdot \text{CO}_2) \times p(E \cdot \text{RuBP}).$$
The concentration of \( E \cdot CO_2 \cdot RuBP \) is given by:

\[
[E \cdot CO_2 \cdot RuBP] = p(E \cdot CO_2 \cdot RuBP) \times [E].
\]

Solving for \( p(E \cdot CO_2 \cdot RuBP) \) and using the values given previously for 
\[ [E \cdot CO_2 \cdot RuBP] \] and \([E]\) gives \( p(E \cdot CO_2 \cdot RuBP) = 0.018 \). Using this 
value for \( p(E \cdot CO_2 \cdot RuBP) \) and 0.226 for \( p(E \cdot RuBP) \), \( p(E \cdot CO_2) \) is 
found to be 0.08.

The concentration of \( E \cdot CO_2 \) given this probability is 0.28 mM.

The concentration of free \( CO_2 \) in the chloroplast required to give 0.28 
mM \( E \cdot CO_2 \) may be calculated in a manner similar to that used for 
\( E \cdot RuBP \), however, solving for \([CO_2]\), the equation defining \( Km \) becomes:

\[
[CO_2] = \frac{Km \times [E \cdot CO_2]}{[E] - [E \cdot CO_2]}.
\]

Using 0.02 mM for \( Km \) (Badger and Collatz 1977) and evaluating gives
1.8 \( \mu \)M as the intra chloroplast \( CO_2 \) concentration required to support
the observed rate of \( CO_2 \) fixation.

It may be reasoned from the known discrimination of RuBP carboxylase
against \( ^{13}CO_2 \) in \( C_3 \) plants, and has been shown experimentally (Berry
and Troughton 1974), that the pool of free \( CO_2 \) within the chloroplast
of \( C_3 \) plants is in equilibrium with atmospheric \( CO_2 \). In such a situation
the activity and concentration of \( CO_2 \) in solution should be maintained
constant at any given atmospheric \( CO_2 \) partial pressure, regardless of
the concentration of ionized or bound forms of \( CO_2 \) in solution. It may
be algebraically shown in this case that ordered binding with RuBP
binding first will not affect the internal \( CO_2 \) concentration required
to give 0.064 mM \( CO_2 \). Thus a free \( CO_2 \) concentration of 1.8 \( \mu \)M would
still be required to maintain the observed CO₂ fixation rate.

If, on the other hand, CO₂ is the first binding substrate in an ordered reaction, the equation defining \( K_m \) for RuBP becomes:

\[
K_m^{RuBP} = \frac{[RuBP] \left( [E \cdot CO_2] - [E \cdot CO_2 \cdot RuBP] \right)}{[E \cdot CO_2 \cdot RuBP]} .
\]

The ordered binding with CO₂ binding first changes the active site concentration available for RuBP to \([E \cdot CO_2]\) and the only (active site \cdot RuBP) species present would be \(E \cdot CO_2 \cdot RuBP\). From the equation defining \(K_m^{CO_2}\), \([E \cdot CO_2]\) may be expressed as:

\[
[E \cdot CO_2] = \frac{[CO_2][E]}{K_m^{CO_2} + [CO_2]} .
\]

Substituting this expression into the equation for \(K_m^{RuBP}\) gives:

\[
K_m^{RuBP} = \frac{[RuBP] \left( \frac{[CO_2][E]}{K_m^{CO_2} + [CO_2]} - [E \cdot CO_2 \cdot RuBP] \right)}{[E \cdot CO_2 \cdot RuBP]} .
\]

Solving for CO₂, we get:

\[
[CO_2] = \frac{K_m^{CO_2}[K_m^{RuBP}[E \cdot CO_2 \cdot RuBP] + [RuBP][E \cdot CO_2 \cdot RuBP]]}{[RuBP][E] - K_m^{RuBP}[E \cdot CO_2 \cdot RuBP] - [RuBP][E \cdot CO_2 \cdot RuBP]} .
\]

Since the measured RuBP concentration represents the total RuBP present:

\[
[RuBP] = [RuBP_{total}] - [E \cdot CO_2 \cdot RuBP] .
\]

Substituting this expression for RuBP and evaluating using the values 0.035 mM for \(K_m^{RuBP}\), 0.02 mM for \(K_m^{CO_2}\), 0.8 mM for RuBP_{total}, and 0.064 mM for \(E \cdot CO_2 \cdot RuBP\) gives 0.39 µM as the free CO₂ concentration required to give 40 nmoles sec⁻¹ (mg Chl)⁻¹ fixation.
These estimated CO\textsubscript{2} concentrations required to give the observed photosynthesis rate with the measured RuBP level are well below estimates of intra-cellular CO\textsubscript{2} concentration, and may be possible concentrations within the chloroplast.

While the measured RuBP level at air levels of O\textsubscript{2} and CO\textsubscript{2} may be sufficient to provide the observed photosynthesis rate, for either the random or ordered-RuBP first mechanism, they are far below levels which would be required to saturate the available binding sites. The total RuBP concentration required to occupy 98\% of the available binding sites may be calculated, again using the equation defining $K_m^{RuBP}$. The calculated concentration using the $K_m$ and total active site concentrations as before is 5.1 mM or a level of about 130 nmoles (mg Chl)\textsuperscript{-1}.

An ordered reaction mechanism with CO\textsubscript{2} binding first may reduce the total binding sites available to RuBP sufficiently to allow the observed total concentration to saturate the active species for binding. As calculated above, the total bound RuBP to give the observed photosynthesis rate, assuming this mechanism, need be only 0.06 to 0.07 mM, leaving about 0.7 mM free RuBP.

At present the binding order for the enzyme is not known. Laing and Christeller (1976) have suggested an ordered reaction with RuBP binding first. Badger and Collatz (1977) have disagreed with this interpretation, however, and have suggested that at present there is insufficient evidence to choose between the possible mechanisms.

As shown in Figure 7, removal of both CO\textsubscript{2} and O\textsubscript{2} from the atmosphere and thus reducing the rates of both the carboxylation and oxygenation reactions causes an increase in the RuBP level. Allowing oxygenation
to continue while decreasing carboxylation stops the RuBP buildup, and a nearly constant pool size is maintained even at CO$_2$ concentrations below the CO$_2$ compensation point.

The effect of CO$_2$ concentration on the PGA pool size indicates that low CO$_2$ concentration in the presence of O$_2$ does deplete PCR cycle intermediates. The total PGA pool in photosynthesizing leaves has been shown to be available for photosynthetic metabolism (Galmiche 1973) and to be in rapid equilibrium with the chloroplast PGA pool (Heber 1974). In the presence of 1% O$_2$ decreasing the atmospheric CO$_2$ concentration from 300 µl l$^{-1}$ to 50 µl l$^{-1}$ decreased the PGA pool by 10% (Figure 8). Over the same CO$_2$ concentration range, CO$_2$ fixation rate decreased by 85%. Increasing O$_2$ in the atmosphere magnified the drop in the PGA pool size with decreasing CO$_2$ concentration. In 20% O$_2$, the total PGA pool size decreased by 32% as CO$_2$ decreased from 300 to 50 µl l$^{-1}$, while in 60% O$_2$ a 69% decrease in the PGA pool size occurred over the same concentration range.

Some decrease in the PGA pool at lower rates of photosynthesis might be expected, however, the pronounced O$_2$ effect at low CO$_2$ indicates an oxygen requiring reaction which causes a drain on the PGA pool. The oxygenation of RuBP to glycolate represents such a reaction, however, the CO$_2$ effect on the PGA pool size does not necessarily indicate increased glycolate production at CO$_2$ concentrations near the compensation point. The decrease in the PGA pool size is not reflected in an increased pool size of glycolate, glycine, or serine (Figures 9, 11, and 12) and thus may indicate a constant or slightly decreased rate of glycolate formation and a greatly decreased rate of CO$_2$ fixation.
The pool size of glycolate follows to some extent the PGA pool size with changing CO$_2$. At 300 μl l$^{-1}$ CO$_2$, increasing O$_2$ concentration from 20% to 60% slightly increased the glycolate pool size. At both 20% and 60% O$_2$, the glycolate pool size decreased at CO$_2$ concentrations below the CO$_2$ compensation point. As indicated by the increase in the CO$_2$ compensation point, increased CO$_2$ evolution into CO$_2$ free air (Forrester et al. 1966) and increased CO$_2$ evolution during photosynthesis (D'Aoust and Canvin 1973), carbon flow through the PCO cycle must increase with increasing O$_2$. This increased flow could occur even at low CO$_2$ if glycolate oxidase functions at maximum velocity and glycolate saturation at any O$_2$ concentration (Zelitch 1971). The Km for O$_2$ for glycolate oxidase is 174 μM O$_2$ (Kerr and Groves 1975), while O$_2$ concentration in solution at 0.2 atmosphere O$_2$ is 240 μM. At 20% O$_2$ then, glycolate oxidase is substantially below saturation and the reaction velocity may increase at 60% O$_2$ without increasing glycolate level. The significant glycolate pool at 1% O$_2$ may be explained by the same reasoning. While glycolate synthesis may be greatly reduced at 1% O$_2$, its oxidation to glyoxylate is also greatly reduced.

Taken by itself, the PGA and glycolate data indicate a decreased level of intermediates in the PCR and PCO cycles, however, neither RuBP nor glycine show the net drop in pool size that occurs at very low CO$_2$ in PGA or glycolate. The uncertainty in the measurement of glycine and RuBP is much greater than in PGA or glycolate and may be sufficient to cover any small change in pool size. Also, two nonequilibrium reactions occur between PGA and RuBP in the PCR cycle (Bassham and Krause 1969) and between glycolate and glycine in the PCO cycle (Tolbert and Ryan...
1975). These irreversible steps may prevent the glycine and RuBP pools from directly reflecting the glycolate and PGA pools, respectively.

In 20% O₂, the regression line of the glycine pool size vs CO₂ concentration does show a slight increase with decreasing CO₂. The glycine pool increased from 106 nmoles (mg Chl)^-1 at 500 µl l^-1 CO₂ to 160 µl l^-1 at 0 CO₂. The correlation between the glycine pool size and CO₂ concentration was very poor, however (r = -0.04). Data points below the CO₂ compensation point fell below the regression line. Since the PGA and glycolate pools decreased at CO₂ concentrations below compensation point, a second regression, excluding data below the CO₂ compensation point (55 µl l^-1) was run for the glycine pool at 20% O₂. While the slope remained essentially unchanged, the absolute value of the correlation coefficient increased somewhat (r = -0.14). If data points below the CO₂ compensation point are considered in this way, the pattern of the glycine data at 20% O₂ is somewhat similar to the glycolate data at 20% O₂. The glycine pool at 60% O₂ did not show such an increase, however, and showed no measurable decrease at CO₂ concentrations below the compensation point. The variability and the inconsistency between oxygen treatments does not allow judgment as to increasing or decreasing pool size to be made.

The serine pool is apparently much more complex than the glycine pool although the evidence is conflicting. Canvin et al. (1975) determined, from specific activity changes during flushing of ^14CO₂ fed sunflower leaves with ^12CO₂, that the entire serine pool was available for metabolism in the PGO cycle. Other studies (Platt et al. 1977, Hess and Tolbert 1966), however, indicate that at least two pathways to serine
synthesis occur in photosynthesizing tissue. Whether or not the entire serine pool in soybean leaves is available for participation in the PCO cycle cannot be ascertained from this work. However during the light to dark transition, no measurable decrease in the serine pool size was observed (PART B). If the total serine pool present in the light is partitioned such that only a small sub-pool is immediately available for metabolism through the PCO cycle and if multiple pathways to serine synthesis are functioning, changes in the CO$_2$ and O$_2$ responsive pool could easily be obscured by the variability in the data.

The flux of carbon through the glycine to serine transformation in the PCO cycle should be proportional to the glycine level. Activity of the glycine decarboxylase-serine hydroxymethyl transferase system has been estimated at a minimum of twice that necessary for the release of photorespiratory CO$_2$ at 21% O$_2$ (Woo and Osmond 1976). In this study, the steady-state CO$_2$ release into CO$_2$ free air increased from 4.8 nmoles sec$^{-1}$ (mg Chl)$^{-1}$ at 20% O$_2$ to 10.2 nmoles sec$^{-1}$ (mg Chl)$^{-1}$ at 60% O$_2$ (data not shown). As the rate of CO$_2$ release increased by 2.1 fold, over the same O$_2$ range, the glycine level increased by 2.5 fold. Apparently most of the glycine is in a single, rapidly turning over metabolic pool available to reactions in the PCO cycle. Canvin et al. (1975) reached a similar conclusion from studies of the specific activity of glycine in $^{14}$CO$_2$ fed sunflower leaves. The large increase in glycine pool size with increasing O$_2$ concentration may indicate that the steady-state level of glycine is an indicator of relative flow through the PCO cycle.

If this is the case, the small glycine response to CO$_2$ indicates at most a slight increase in carbon flow through the PCO cycle at very
low concentrations of CO$_2$. This result and the glycine levels measured
at high and low CO$_2$ by others (Mahon et al. 1974, Snyder and Tolbert
1974) agree with the observation (Ludwig and Canvin 1971) that little
or no increase in the evolution of photorespiratory CO$_2$ occurs with
decreasing CO$_2$.

The reason for this constant flow of carbon through the PCO cycle
as the inhibitor (CO$_2$) of the rate-limiting reaction in the cycle is
removed is apparently not a decreasing level of RuBP available for oxy­
genation. While the levels of RuBP present may be less than that
required to saturate the catalytic capacity of the enzyme, they are no
more limiting at 50 μl 1$^{-1}$ CO$_2$ than at 500 μl 1$^{-1}$ CO$_2$. The loss of
photorespiratory carbon at these very low CO$_2$ concentrations does reduce
the level of PGA, however, control of nonequilibrium reactions within
the cycle may serve to maintain a relatively constant pool of RuBP. As
mentioned earlier, Bassham and Krause (1969) have determined that both
the Ru5P kinase reactions and the RuBP carboxylation reactions have
physiological standard free energy changes great enough to indicate
nonequilibrium reactions. As well, two other nonequilibrium reactions
occur in the RuBP regeneration pathway between PGA and Ru5P. Thus the
RuBP pool is substantially removed from equilibrium with the PGA pool
and may not reflect the level of other sugar phosphates in the photo-
synthetic pool.

Alternative reasons for the constant rate of photorespiration are
not immediately evident. It should be noted, however, that the predic­
tions of a competitive interaction of CO$_2$ and O$_2$ at the oxygenation
reaction are based upon the kinetics of RuBP carboxylase/oxygenase at
saturating levels of RuBP and solution concentrations of $CO_2$ much greater than those likely to be present in the chloroplast. Reaction kinetics determined with this system differ in at least two ways from the situation present in the chloroplast. First, the pattern of inhibition of $CO_2$ or $O_2$ vs RuBP concentration may be expressed since the level of RuBP present is below the saturation level. The pattern of inhibition of $CO_2$ or $O_2$ vs RuBP concentration is a noncompetitive one (Badger and Collatz 1977) and may alter the expression of the $O_2$-$CO_2$ competition. Secondly and possibly more importantly, the oxygenase reaction of RuBP of carboxylase/oxygenase has been shown to undergo a $CO_2$ activation in a manner similar to the $CO_2$ activation for the carboxylase reaction (Badger and Lorimer 1976, Marsho and Kung 1976). Badger and Lorimer (1976) showed that the oxygenase activity of the purified spinach enzyme was increased by pre-incubation with Mg$^{++}$ and $CO_2$ prior to addition of RuBP into the reaction mixture. At optimal Mg$^{++}$ concentration and pH, the enzyme incubated without $CO_2$ showed only 18% of the oxygenase activity of the enzyme pre-incubated with optimal Mg$^{++}$ concentration, pH, and $CO_2$ concentration. Thus, while decreasing the atmospheric $CO_2$ concentration may remove a competitor for RuBP at the active site for oxygenation, it also removes an apparent allosteric activator of the oxygenase reaction. Calculation of the theoretical rate of $CO_2$ evolution into $CO_2$ free air, assuming all RuBP binding sites present in the chloroplast are fully active, overestimates the observed steady-state $CO_2$ evolution rate.

If the concentration of $O_2$ within the chloroplast at zero external $CO_2$ is assumed to be equal to the external $O_2$ concentration, the concentration of $E \cdot RuBP \cdot O_2$ present in the chloroplast may be calculated.
by the methods described for CO₂. Using \( K_{\text{m,CO}_2} = 0.4 \text{ mM} \) (Badger and Collatz, 1977), at 20% \( \text{O}_2 \) (0.24 mM in solution), the concentration of 
\( E \cdot \text{RuBP} \cdot \text{O}_2 \) may be calculated as 0.3 mM for either a random or ordered RuBP binding first mechanism. The \( k_{\text{cat}} \) for the oxygenase reaction is 4.1 sec\(^{-1}\) (Badger and Collatz 1977), therefore, the oxygenase reaction velocity expressed as the instantaneous \( \text{O}_2 \) or glycolate concentration change is 1.2 mM sec\(^{-1}\). Expressed as nmoles sec\(^{-1}\) (mg Chl\(^{-1}\)), the rate is 30.5. The stoichiometry of the PCD cycle requires 0.5 moles of CO₂ release for each mole of glycolate entering the cycle. The estimated rate of true CO₂ efflux under these atmospheric conditions is then approximately 15 nmoles sec\(^{-1}\) (mg Chl\(^{-1}\)), as compared to an average measured rate of 4.8 nmoles sec\(^{-1}\) (mg Chl\(^{-1}\)). A part of this difference should be due to refixation of photorespired CO₂, however, gas flow conditions through the leaf chamber were such that an average external \( \text{CO}_2 \) concentration of less than 4 μl l\(^{-1}\) of \( \text{CO}_2 \) was present, thus refixation should have been much less than the observed difference in measured and calculated rates.

These possible mechanisms for explanation of the constant rate of photorespiration at \( \text{CO}_2 \) concentrations below atmospheric \( \text{CO}_2 \) may not completely explain the observations, however, control of the process is apparently more complex than can be explained by direct enzyme-substrate concentration interaction.

Further knowledge of the intermediate levels within the PCR cycle may help identify rate controlling and limiting reactions within the cycle as it operates under physiological conditions. From the studies of these few intermediates, it can be seen that both the RuBP
carboxylase/oxygenase and the glycine decarboxylation systems operate at substrate levels well below saturation during photosynthesis at near atmospheric \( O_2 \) and \( CO_2 \) concentrations. It has been noted by others (e.g., Goldstein et al. 1976) that the level of RuBP carboxylase per unit leaf area is more than one hundred fold greater when assayed at substrate saturating conditions, than the measured photosynthesis rate per unit leaf area of the same plant. While enzyme activity has been used as an indication of maximal rates of metabolite flow through various metabolic pathways, it appears that the effective substrate concentrations at the enzyme may be equally important in assessing control of these processes under physiological conditions. In many cases, determination of substrate concentration effective to a specific enzyme may be made difficult or impossible by lack of absolute knowledge of metabolite compartmentation. The chloroplast represents an ideal model for study in this respect. Considerable information has been gathered as to metabolite flow into and out of the chloroplast. While much is left to be determined concerning the exchange of carbon between the chloroplast and the cytoplasm, several key intermediates and the enzymes which catalyze their metabolism are limited to the chloroplast. Determination of enzyme and substrate concentration in the intact organ is then equivalent to the determination of enzyme and substrate concentration in the chloroplast if the concentrations are expressed on a common basis unique to the chloroplast. Expansion of this technique may lead to increased understanding of the operation of the metabolic pathways within the chloroplast both for its own sake and as an example of an organelle functioning within a cell.
PART B. RATE OF CARBON DIOXIDE EVOLUTION AND POOL SIZES OF SOME PHOTOSYNTHETIC AND PHOTOESPIRATORY INTERMEDIATES DURING THE POST-ILLUMINATION PERIOD IN GLYCINE MAX
INTRODUCTION

Since its discovery by Decker (1955), the rapid rate of CO$_2$ evolution which occurs upon darkening a photosynthesizing leaf has been attributed to a transient remainder of the steady rate of photorespiration.

Since its original discovery, the rapid post-illumination burst of CO$_2$ has been used in various ways to help characterize the process of photorespiration. The relative magnitude of the burst has been shown to be a function of the O$_2$ concentration of the atmosphere (Krotkov 1963, Egle and Fock 1967), of the previous illumination of the photosynthesizing tissue (Decker 1959, Egle and Fock 1967), and of the CO$_2$ concentration of the atmosphere (Decker 1959, Egle and Fock 1967, D'Aoust and Canvin 1972). The specific activity of the $^{14}$CO$_2$ in the post-illumination burst has also been used to characterize the source of substrates for the photorespired CO$_2$ (Krotkov 1963, D'Aoust and Canvin 1972).

Although the relative magnitude of the post-illumination CO$_2$ burst has been used frequently, absolute determination of the rate of CO$_2$ evolution during the first seconds after darkening of a photosynthesizing leaf have been attempted only recently (Doehlert et al. 1978). If absolute exchange rates measured during the course of the post-illumination burst are to be of use in determination of the rate of photorespiration immediately preceding darkening, some knowledge of the level of photorespiratory intermediates present in the light and in the first seconds after darkening should be gained. Since the immediate substrate
for the photorespiratory CO$_2$ has been shown to be glycine (Kisaki and Tolbert 1970) and since competing CO$_2$ uptake might come from RuBP remaining after the first seconds of darkening, the levels of these two intermediates and of glycolic acid were determined in soybean leaf discs at various times during the course of the post-illumination CO$_2$ burst. The effect of gas flow rate on the maximum rate and time of the post-illumination CO$_2$ burst corrected for nonsteady-state conditions was studied also.
MATERIALS AND METHODS

Plant materials were grown as described in PART A. Leaf discs were used for the metabolite level determinations instead of intact leaves to minimize the variation between sampling times. A leaf disc gas exchange chamber was constructed and placed in the open CO$_2$ exchange monitoring system in place of the whole leaf chamber.

The leaf disc chamber consisted of a plexiglas box with multiple gas inlet ports in the lower surface. A 0.6 cm mesh wire screen was fastened to the removable lid and remained just above the lower surface of the box when the lid was in place. Leaf discs were placed on the wire screen and maintained in a turgid condition by passing the inlet gas through a water layer in the chamber during illumination and gas exchange measurement.

Leaf discs were removed from the chamber and killed at designated times after darkening by quickly removing the lid and wire screen from the chamber and plunging the wire screen holding the leaf discs into liquid N$_2$.

Leaf discs 1.8 cm in diameter were cut from leaf margins of the third trifoliate (numbering from the primary leaves). The leaf discs were maintained by floating on aerated, distilled water in the light until use. Twelve discs (1.1 mg Chl total) were placed on the wire screen, the screen was fitted into the gas exchange chamber, and the rate of CO$_2$ exchange was monitored until a steady rate was obtained (15 to 20 min). After 5 min at steady rate, the discs were frozen as described above, freezedried, and RuBP, glycolic acid, glycine, and
serine were determined in the dried residue as described in PART A.

The rate of net CO$_2$ uptake was below average values measured in intact leaves. Similar effects were noted by Atkins and Canvin (1971) who also determined that no measurable change in the distribution of photosynthetic intermediates accompanied the lower CO$_2$ exchange rate. The levels of RuBP, glycolic acid, glycine, and serine measured in this study were all 2 to 2.5 times greater than those measured previously (PART A). No explanation for this result is readily apparent.

Whole leaves were used to determine the rate of net CO$_2$ exchange during the post-illumination burst. The open gas exchange system was used as described in PART A except that the flow rate was varied from 2 l min$^{-1}$ to 4 l min$^{-1}$, and the cold trap was removed from the outlet gas line during measurements. The volume of the gas exchange system with the cold trap removed was 160 ml.

Net exchange rates obtained during the post-illumination period were corrected for nonsteady rate conditions by the method of Marynick and Marynick (1975).
RESULTS AND DISCUSSION

In open gas exchange systems, the rate of gas exchange is calculated as

\[ R = \Delta C \times F \]

where \( R \) is the exchange rate, \( \Delta C \) the concentration change between the sample chamber inlet and outlet, and \( F \) is the gas flow rate through the chamber. It has been pointed out (Marynick and Marynick 1975) that the above equation is valid only at steady state rate conditions; that is:

\[ \frac{d(\Delta C)}{dt} = 0. \]

As this is clearly not the case during the post-illumination \( \text{CO}_2 \) burst, the instantaneous rate of \( \text{CO}_2 \) exchange should be described by the second derivative of the concentration vs time. This expression is approximated by the rate change correction given by Marynick and Marynick (1975) as follows:

\[ R_{t} = \frac{V(\Delta C_{t_3} - \Delta C_{t_1})}{\Delta t} + \Delta C_{t_2} \times F \]

where \( R_t \) is the instantaneous rate of exchange at time \( t \), \( V \) is the total volume of the exchange and monitoring system, \( \Delta C_t \) is the concentration change through the chamber at time \( t \), \( \Delta t \) is the time interval between \( \Delta C \) measurements, and \( F \) is the gas flow rate.

The corrected and uncorrected rates of net \( \text{CO}_2 \) exchange during the period of the post-illumination \( \text{CO}_2 \) burst in 20% \( \text{O}_2 \) and 350 \( \mu l \ l^{-1} \) \( \text{CO}_2 \) are shown in Figure 13 at gas flow rates of 2, 3, and 4 \( l \ min^{-1} \). At each flow rate, correction for nonsteady rate shifts the net \( \text{CO}_2 \) exchange
Figure 13. The post-illumination rate of net CO₂ exchange in air at gas flow rates of 2, 3, and 4 l min⁻¹.
curve toward t = 0. Both the exchange rate maxima and the time after darkening at which the maximum positive rate of net CO$_2$ exchange occurred were influenced by flow rate. The maximum rate of CO$_2$ evolution occurred at 9 seconds after darkening at a flow rate of 2 min$^{-1}$, 7.2 seconds at 3 min$^{-1}$, and 6.8 seconds at 4 min$^{-1}$. The maximum calculated rate of CO$_2$ exchange decreased with increasing flow rate from 24 nmoles sec$^{-1}$ (mg Chl)$^{-1}$ at 2 min$^{-1}$ to 20.5 and 14.5 nmoles sec$^{-1}$ (mg Chl)$^{-1}$ at 3 and 4 min$^{-1}$, respectively.

Neither the concentration vs time nor the uncorrected exchange rate vs time plots show an appreciable dark burst of CO$_2$ at the required flow rates in this gas exchange system. Comparison of the corrected rates of net CO$_2$ exchange during the post-illumination period in 1% and 20% O$_2$ at 350 µl l$^{-1}$ CO$_2$, shown in Figure 14, indicates that an elevated rate of CO$_2$ evolution is in fact present in the 20% O$_2$ atmosphere. At 1% O$_2$, the corrected net CO$_2$ exchange rate was never greater than the steady dark rate; while the uncorrected rate in 20% O$_2$ did not show a CO$_2$ burst above the steady dark rate, the increased slope of ΔC vs time $\frac{ΔC}{Δt}$ at $t_3/t_2$ increased the exchange correction factor sufficiently to give corrected rates of exchange which indicate a substantially greater rate of CO$_2$ evolution in the first 3 to 10 seconds after darkening.

For estimations of instantaneous rates of CO$_2$ exchange to be valid, the gas exchange system used to measure the exchange rate must approximate instantaneous mixing in the leaf chamber and in the concentration monitoring device (Marynick and Marynick 1975). While the requirement for instantaneous mixing in the leaf chamber itself is likely to be approached at gas flow rates which give maximum steady state rates of
Figure 14. The post-illumination rate of net CO$_2$ exchange at 350 ul l$^{-1}$ CO$_2$ in 1% and 20% O$_2$
CO₂ exchange, the requirement for instantaneous mixing through the CO₂ concentration monitoring system is much more difficult to meet, and is a likely cause of the substantial flow rate effects observed in this study. The leaf chamber volume in the gas exchange system was 49 ml, thus the chamber volume was flushed each 1.5 seconds at 2 l min⁻¹ and 0.75 seconds at 4 l min⁻¹. The sample cell volume of the Beckman 215A IRGA used to measure CO₂ concentration is, however, 97 ml. The volume of the sample cell of the IRGA thus makes it unlikely that instantaneous sampling of the CO₂ concentration within the leaf chamber occurs.

The maximum rate of net CO₂ evolution through the course of the dark burst measured at a flow rate of 4 l min⁻¹ was 51% of the net CO₂ uptake rate during photosynthesis. Measured rates of photorespiration during photosynthesis for several species averaged 26% of the net CO₂ uptake rate (D'Aoust and Canvin 1973, Ludwig and Canvin 1971). Thus the corrected rate measured at 4 l min⁻¹ may still overestimate the true rate of CO₂ evolution immediately after darkening. Flow rates of greater than 4 l min⁻¹ caused technical problems of pressure buildup and leakage in the exchange system and thus could not be adequately studied.

The time after darkening at which the maximum concentration of the post-illumination CO₂ burst occurs has been given at times varying from 30 seconds after darkening (Decker 1955, D'Aoust and Canvin 1972) to 5 minutes after darkening (Moss 1966). The timing of this burst is greatly dependent upon the gas exchange system in which it is measured as shown in the flow rate study. To determine at what point after darkening the maximum rate of CO₂ evolution should occur, the levels
of RuBP, glycine, and glycolic acid were determined in soybean leaf discs in the light and at 2 and 3 second intervals from 2 to 20 seconds after darkening. The results, shown in Figure 15, indicate that both RuBP and glycine levels decrease very rapidly after darkening. After 7 to 10 seconds, the RuBP level approaches its darkness level (2 to 3 nmoles (mg Chl)^{-1}) but the glycine pool remains substantially above its pool size reached after 10 minutes of darkness. This remaining glycine pool could be responsible for the observed rapid rate of CO\textsubscript{2} evolution immediately after darkening. The lack of an increased rate of CO\textsubscript{2} after darkening leaves from atmospheres containing 1% O\textsubscript{2} is consistent with this reasoning as the glycine pool size in 1% O\textsubscript{2} is less than 50% of that in 20% O\textsubscript{2} (PART A, Figure 10).

The relative rates of decrease of the glycine and RuBP pools indicate that the maximum rate of net CO\textsubscript{2} evolution during the post-illumination period should occur near 3 to 6 seconds after darkening, as compared to approximately 7 seconds after darkening for the maximum rate measured at gas flow rates above 3 l min\textsuperscript{-1} in the gas exchange study. The rapid early decline of the glycine pool also indicates that this rate will slightly underestimate the maximum rate of CO\textsubscript{2} evolution during photosynthesis.

The combined metabolite level and gas exchange studies indicate that gas exchange systems which are capable of giving maximum rates of CO\textsubscript{2} evolution at near theoretically correct times after darkening are obtainable. The absolute rate of CO\textsubscript{2} evolution in these systems must be carefully evaluated. An inherent underestimation of the CO\textsubscript{2} evolution
Figure 15. The post-illumination pool sizes of RuBP, glycolic acid, and glycine in air
rate may be present due to the rapid post-illumination decline in the glycine pool, as well, overestimation is likely to occur due to mixing and sampling problems present in gas exchange rate measuring systems.


ACKNOWLEDGMENTS

I would like to thank Dr. Stewart for allowing me the opportunity to work on this problem and for his many helpful discussions and suggestions which helped me through it. I would also like to thank the Department of Botany and Plant Pathology and the Graduate College for funding the research.