Characterization of photorespiration and photosynthesis in soybean cotyledons during seedling development, cotyledon senescence and rejuvenation

Laura Fredrick Marek

Iowa State University

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

Part of the Botany Commons

Recommended Citation

https://lib.dr.iastate.edu/rtd/9697
INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the original text directly from the copy submitted. Thus, some dissertation copies are in typewriter face, while others may be from a computer printer.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyrighted material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each oversize page is available as one exposure on a standard 35 mm slide or as a 17" × 23" black and white photographic print for an additional charge.

Photographs included in the original manuscript have been reproduced xerographically in this copy. 35 mm slides or 6" × 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
Accessing the World’s Information since 1938
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
Characterization of photorespiration and photosynthesis in soybean cotyledons during seedling development, cotyledon senescence and rejuvenation

Marek, Laura Fredrick, Ph.D.
Iowa State University, 1988
PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark ✓.

1. Glossy photographs or pages
2. Colored illustrations, paper or print
3. Photographs with dark background
4. Illustrations are poor copy
5. Pages with black marks, not original copy ✓
6. Print shows through as there is text on both sides of page
7. Indistinct, broken or small print on several pages
8. Print exceeds margin requirements
9. Tightly bound copy with print lost in spine
10. Computer printout pages with indistinct print
11. Page(s) lacking when material received, and not available from school or author.
12. Page(s) seem to be missing in numbering only as text follows.
13. Two pages numbered. Text follows.
14. Curling and wrinkled pages
15. Dissertation contains pages with print at a slant, filmed as received
16. Other

________________________________________
________________________________________

UMI
Characterization of photorespiration and photosynthesis in soybean cotyledons during seedling development, cotyledon senescence and rejuvenation

by

Laura Fredrick Marek

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Botany
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
1988
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Soybean Cotyledons as a Model System to Study Senescence</td>
<td>1</td>
</tr>
<tr>
<td>Senescence in Leaves</td>
<td>2</td>
</tr>
<tr>
<td>Uses of the Soybean Cotyledon Model System</td>
<td>4</td>
</tr>
<tr>
<td>Problems With the Soybean Model System</td>
<td>5</td>
</tr>
<tr>
<td>Physiological characterization</td>
<td>5</td>
</tr>
<tr>
<td>Storage function to photosynthetic function transition</td>
<td>6</td>
</tr>
<tr>
<td>Photosynthesis and Photorespiration in Soybean Cotyledons</td>
<td>11</td>
</tr>
<tr>
<td><strong>PART I. PHOTOSYNTHESIS, PHOTORESPIRATION AND RESPIRATION IN</strong></td>
<td>15</td>
</tr>
<tr>
<td>PRESENESCENT, SENESCING, AND REJUVENATING SOYBEAN COTYLEDONS: PHYSIOLOGICAL MEASUREMENTS</td>
<td></td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>16</td>
</tr>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td>17</td>
</tr>
<tr>
<td><strong>MATERIALS AND METHODS</strong></td>
<td>20</td>
</tr>
<tr>
<td>Plant Material</td>
<td>20</td>
</tr>
<tr>
<td>Gas Exchange</td>
<td>21</td>
</tr>
<tr>
<td>Chlorophyll Determinations</td>
<td>22</td>
</tr>
<tr>
<td><strong>RESULTS</strong></td>
<td>24</td>
</tr>
<tr>
<td>Growth Parameters</td>
<td>24</td>
</tr>
<tr>
<td>Gas Exchange</td>
<td>27</td>
</tr>
<tr>
<td><strong>DISCUSSION</strong></td>
<td>32</td>
</tr>
<tr>
<td>Growth Parameters</td>
<td>32</td>
</tr>
<tr>
<td><strong>PART II. PHOTOSYNTHESIS, PHOTORESPIRATION, AND RESPIRATION IN</strong></td>
<td>38</td>
</tr>
<tr>
<td>PRESENESCENT, SENESCING, AND REJUVENATING SOYBEAN COTYLEDONS: BIOCHEMICAL CHARACTERIZATION</td>
<td></td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>39</td>
</tr>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td>40</td>
</tr>
</tbody>
</table>
### MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant Material</td>
<td>42</td>
</tr>
<tr>
<td>Gas Exchange</td>
<td>42</td>
</tr>
<tr>
<td>(^{14})CO(_{2}) Incorporation</td>
<td>44</td>
</tr>
<tr>
<td>Metabolite Extraction and Determination</td>
<td>45</td>
</tr>
<tr>
<td>Purification of Rubisco</td>
<td>46</td>
</tr>
<tr>
<td>Quantification of Rubisco</td>
<td>47</td>
</tr>
<tr>
<td>Chlorophyll</td>
<td>49</td>
</tr>
</tbody>
</table>

### RESULTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth Parameters</td>
<td>50</td>
</tr>
<tr>
<td>Rubisco</td>
<td>50</td>
</tr>
<tr>
<td>Gas Exchange</td>
<td>53</td>
</tr>
<tr>
<td>(^{14})CO(_{2}) Incorporation</td>
<td>60</td>
</tr>
</tbody>
</table>

### DISCUSSION

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas Exchange</td>
<td>68</td>
</tr>
</tbody>
</table>

### SUMMARY

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>78</td>
</tr>
</tbody>
</table>

### BIBLIOGRAPHY

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>80</td>
</tr>
</tbody>
</table>
INTRODUCTION

Soybean Cotyledons as a Model System to Study Senescence

Soybean (Glycine max [L.] Merr.) is unusual among the legumes because it possesses cotyledons that become green and emerge during germination. The cotyledons develop leaf-like characteristics, including photosynthetic capability, and function for a time as both storage and photosynthetic organs (Smith, 1983). Data from Brown and Huber (1987a, 1987b) suggest that metabolism associated with the storage function virtually disappears before there is much if any loss of photosynthetic capacity. The cotyledons then begin to yellow and senesce as the seedling develops. If the epicotyl is removed before the cotyledons become fully yellow and abscise, they will remain attached to the stem and regreen. Krul (1974) showed that soybean cotyledons could lose up to 90% of their nucleic acids and up to 80% of their protein before senescence became irreversible. During a 15-day rejuvenation period, the cotyledons recovered a significant proportion of the lost components. The change in nucleic acid content was due almost entirely to changes in RNA. The increase during rejuvenation was greatest in the heavy ribosomal and messenger RNA fraction. Krul suggested that comparisons among presenescent, senescing, and rejuvenated cotyledons would provide a good model system with which to investigate senescence.

Senescence is commonly induced by detachment, which probably initiates a wounding effect, and darkness (for example, Thimann's
extensive work with oat (*Avena sativa* cv. Victory) leaves, reviewed in Thimann, 1987). Early evidence indicated that there were differences in senescence in attached and detached organs (Lewington et al., 1967). Lewington et al. (1967) found different patterns of change in RNA, DNA, ribonuclease, several other enzymes, chlorophyll, fresh weight, and the extent of bacterial contamination in aging attached and detached cucumber (*Cucumis sativus* var. Long Green Trailing) cotyledons. Ultrastructural evidence from the same system (Butler, 1967) showed only differences in timing and in the extent of changes seen, not in the overall pattern of senescence in attached and detached cotyledons. Hurkman (1979), however, found major differences in the pattern of changes in chloroplasts in senescing attached or detached wheat (*Triticum aestivum* L. em. Thell.) leaves. More recently, Miller and Huffaker (1985) have reported differential induction of endoproteinases during senescence of attached and detached barley (*Hordeum vulgare* L. var. Numar) leaves. The soybean cotyledon model system could provide a means for investigating changes associated with senescence without the possible artifacts caused by detachment or other artificial induction of senescence.

**Senescence in Leaves**

Senescence in leaves is an ill-defined syndrome, the symptoms of which include loss of chlorophyll, loss of protein (although some protein synthesis occurs), changes in nucleic acids and ultrastructural alterations that include membrane disruptions (Thimann, 1980). Changes in chlorophyll provide a convenient visual
index of senescence, although it is now clear that chlorophyll
degradation can be uncoupled from other processes associated with
senescence (Thomas, 1987). Thomas and his colleagues have isolated a
nonyellowing mutant of Festuca pratensis, which retains pigment
proteolipid complexes during senescence but which does not retain
functional integrity of electron transport. Loss of total protein and
ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) in the
mutant is similar to the wild type. The recessive mutation is
ascribed to a single gene for which no product has been discovered.
The mutation is apparently for a factor responsible for the
dissociation of the pigment-proteolipid complexes, not an effect on
enzymes of chlorophyll or carotenoid degradation (Gut et al., 1987).

Photosynthetic capacity declines during senescence and as such it
is part of the overall syndrome. In barley leaves, over 95% of the
protein lost during senescence was due to the loss of RubisCO (Thayer
et al., 1987). It is not surprising, therefore, that the loss of
photosynthetic carbon assimilation capacity during senescence
correlates closely with the loss of RubisCO (Friedrich and Huffaker,
1980; Mae et al., 1987). In spite of the massive loss of RubisCO,
ultrastructural studies show that there is not a significant decline
in chloroplast number per cell during senescence (Mae et al., 1984;
Wardley et al., 1984) and that the chloroplast envelope remains intact
until the very final stages of senescence (Tuquet and Newman, 1980;
Huber and Newman, 1976). Woolhouse (1987) feels that there must be a
signal from the nucleus, most probably a protein message, that
initiates senescence within the chloroplast.

Uses of the Soybean Cotyledon Model System

Comparisons among presenescent, senescing, and regreening soybean cotyledons could delineate factors unique to each state, and as such screen for a possible senescence signal and for a factor(s) induced by that signal. Such comparisons between developing and germinating seeds have been successful in identifying proteins unique to each state (Simon, 1984).

Bricker and Newman (1980) isolated thylakoid proteins from presenescent, senescing, and 5-day rejuvenated soybean cotyledons and fractionated the proteins using SDS-PAGE. They found most polypeptides present in all cotyledons, although in different amounts. They did find one protein that increased significantly during senescence but was absent in the rejuvenated cotyledons. Skadsen and Cherry (1983) isolated poly A mRNA from presenescent, senescing, and 20-day rejuvenated soybean cotyledons. They compared protein profiles derived from in vitro translation products of the mRNA. They found no product unique for a particular cotyledon age, although there were clear quantitative differences between different proteins. They did find five proteins that were prominent only in rejuvenated cotyledons. Skadsen and Cherry did not identify any of the proteins. One explanation for the difference between Bricker and Newman's results (1980) and Skadsen and Cherry's results (1983) could be that the unique polypeptide noted by Bricker and Newman was synthesized in the chloroplasts. Chloroplast mRNA is not polyadenylated and would not
have been isolated by Skadsen and Cherry's method. Another possible explanation could be related to sensitivity of detection between the two systems, since Skadsen and Cherry do mention that some of the bands they obtained were very faint even after prolonged exposure.

Loss of membrane integrity is a symptom of senescence (Thimann, 1980). Pauls and Thompson (1984) suggested that lipoxygenase mediated lipid peroxidation was an important factor in causing membrane damage. Peterman and Siedow (1985) have used the soybean cotyledon model system to follow the presence and activity of lipoxygenase during germination and senescence and in 13-day rejuvenated cotyledons. They found the highest amount and activity of lipoxygenase in the youngest cotyledons they examined (3-day old cotyledons, which in this study were pre-emergent). Total lipoxygenase activity decreased during senescence and was detected at low levels in rejuvenated cotyledons.

Problems With the Soybean Model System

Physiological characterization

In spite of the various studies using the soybean model system to characterize senescence, the system itself is not well characterized physiologically. When we initiated our work with soybean cotyledons, there was only one report in the literature dealing with photosynthesis and respiration in soybean cotyledons (Abrahamsen and Mayer, 1967), which used out-dated methodologies. Since we began our work, two labs have reported measurements of cotyledon photosynthesis and respiration (Brown and Huber, 1987a, 1987b; Harris et al., 1986a).
Neither study measured photosynthesis or respiration in rejuvenated cotyledons. As part of a study to better evaluate the use of presenescent, senescing, and rejuvenated soybean cotyledons as a model system for studying senescence, we measured light and dark carbon dioxide exchange rates (light and dark CERs) and various growth parameters. This work is reported in the first paper of this dissertation. We compared the work of Brown and Huber (1987a, 1987b) and Harris et al. (1986a, 1986b) with our results in that paper.

**Storage function to photosynthetic function transition**

The use of comparisons between presenescent, senescing, and rejuvenated cotyledons as screens for detecting factors unique to senescence or essential for survival, requires a stable presenescent baseline. One problem with using the soybean model system to study senescence is the complication introduced by the transition of the cotyledons from storage organs to photosynthetic, leaf-like structures because these two functions overlap in time.

Soybean seeds are high in oil and protein and are commercially important sources of both compounds (Trelease and Doman, 1984). During seed germination, these components are mobilized to support the growing seedling. Most of the mobilization supplies substrate for gluconeogenesis. The pathways leading to and including gluconeogenesis have been best described in castor bean, Ricinus communis, (Huang et al., 1983; Stewart and Beevers, 1967). The storage tissue in a castor bean seed, however, is the endosperm. It is completely digested during germination and never becomes green (Beevers, 1979).
There are species from plant families other than the Leguminosae that have cotyledons which undergo a storage to photosynthesis transition. The storage to photosynthesis transition has been best characterized in cucumber, primarily by examination of microbodies and enzymes characteristic of each functional state.

Initial breakdown of storage lipid in germinating seeds takes place in a microbody unique to storage tissues, the glyoxysome. The enzymes responsible for β-oxidation of fatty acids to succinate are present in the glyoxysome. As part of the overall process of gluconeogenesis, succinate is oxidized in the mitochondria to malate. Malate is oxidized to oxaloacetate (OAA), and the OAA is decarboxylated to phosphoenolpyruvate (PEP) in the cytoplasm. Carbohydrate is then synthesized from PEP by a reversal of the processes of glycolysis. This overall pathway is illustrated in Fig. 1. Enzymes unique to this process are the glyoxysomal enzymes malate synthase and isocitrate lyase.

In photosynthetically competent tissues, peroxisomes are microbodies that perform an essential function in the recycling of photorespiratory metabolites. The overall pathway of carbon metabolism during photorespiration, the glycolate pathway, is illustrated in Fig. 2. The conversion of glycolate to glycine and serine to glycerate takes place in the peroxisome, catalyzed by glycolate oxidase, glutamate:glyoxylate aminotransferase, and serine:glyoxylate aminotransferase.

In germinating cucumber cotyledons, the activities of enzymes
Fig. 1. Schematic representation of pathway of carbohydrate synthesis from stored lipid in a germinating seed. Glyoxysomal enzymes unique to the pathway: malate synthase (1); isocitrate lyase (2). Adapted from Huang et al., 1983.
Fig. 2. Photorespiration: the glycolate pathway. Peroxisomal enzymes characteristic of the pathway: glycolate oxidase (1); glutamate-glyoxylate aminotransferase (2); serine:glyoxylate amino transferase (3). Adapted from Tolbert, 1981.
from the two classes of microbodies overlap (Trelease et al., 1971; Becker et al., 1978). Titus and Becker (1985) have used double-label immunoelectron microscopy to demonstrate that the glyoxylate cycle enzyme isocitrate lyase and the glycolate pathway enzyme serine:glyoxylate aminotransferase exist within the same microbodies. These data demonstrate that during the transition from storage to photosynthetic organ there is an actual conversion of glyoxysomes to peroxisomes. During the overlap it would be necessary to differentiate between factors specific to reserve mobilization and factors specific to photosynthetic function in comparisons between senescing and rejuvenated tissues. Because the enzymes of the glyoxylate cycle and their corresponding mRNAs disappear after lipid mobilization is completed (Smith and Leaver, 1986) but before RubisCO degradation begins (Becker et al., 1978), at least in cucumber, a better baseline for comparison would be after the transition is completed. In cucumber, the metabolism of storage lipid is largely completed by day 5 and the related enzyme activity is undetectable by day 7 under the growth conditions used. The decrease in lipid content corresponds with decreases in total protein content and dry weight.

The cucumber system may not be entirely analogous with that of a germinating soybean. Cucumber cotyledons expand much more and become thinner during greening than soybean cotyledons do. By 7 days after planting the cucumber cotyledons pictured by Becker et al. (1978) had increased approximately 15x in area. In our experience (data presented in Parts I and II of this dissertation), soybean cotyledons
never enlarge more than 5x during expansion. In addition, we never observe an increase in dry weight or in total protein content in presenescent or senescing soybean cotyledons, in contrast with cucumber cotyledons, in which dry weight and total protein increased after lipid mobilization was completed.

The enzyme changes seen in expanding cucumber cotyledons suggest that Skadsen and Cherry (1983) should have seen some qualitative differences in their protein profiles in addition to the quantitative changes they observed (discussed earlier in this Introduction).

**Photosynthesis and Photorespiration in Soybean Cotyledons**

We are aware of no studies with cucumber cotyledons in which photosynthesis was measured during germination and during the storage to photosynthesis transition. In the course of our work (results reported in Part I), we found that soybean cotyledons were capable of photosynthetic rates in the range of those observed for field grown soybean leaves, and that in rejuvenated cotyledons CO₂-exchange rates (CERs) recovered to levels approximately equal to those seen in presenescence cotyledons. In addition to attaining leaf-like levels of light CER, soybean cotyledons exhibited the typical C₃ photosynthetic leaf response to a sudden light to dark transition: the post-illumination outburst of CO₂ (PIB). Decker (1955, 1959) first reported the existence of the PIB and its dependence on O₂ concentration and on the prior light intensity. After some controversy, it is now generally accepted that the PIB represents a remnant of photorespiration (Canvin, 1979) that results from the
metabolism of pools of photorespiratory metabolites after CO₂ uptake no longer occurs.

The biochemical pathway of photorespiration, the glycolate pathway, was elucidated by Tolbert (see Tolbert (1981) and Ogren (1984) for recent reviews) and is shown in Fig. 2. Carbon flow into the glycolate pathway is initiated by the oxygenase activity of Rubisco. The CO₂ evolution that results in the PIB arises during the synthesis of serine from two molecules of glycine. The PIB is one of several external manifestations of photorespiration (Zelitch, 1979; Ogren, 1984). CO₂ evolution in the light into CO₂-free air that is 1.4 to 4 or more times the rate of CO₂ evolution observed in the dark (Tolbert, 1981) is another physiological indication of photorespiration. The stimulation of photosynthesis by decreasing levels of O₂ is a result of reduced photorespiration. Because photorespiration results in CO₂ evolution in the light, when a photorespiring plant is placed into a closed container in the light, it will reduce the CO₂ concentration to a point where photosynthetic uptake and photorespiratory evolution are equal, the CO₂ compensation concentration (Ogren, 1984). All of these external indicators of photorespiration are assumed to underestimate the true rate of photorespiration because of the probable refixation of some of the photorespired CO₂ (Canvin, 1979; Tolbert, 1981), and because of the unknown contribution of dark respiration in the light. An absolutely reliable method for the measurement of the rate of photorespiration does not exist.
Various attempts have been made to devise a means of quantifying photorespiration from the PIB. Whereas rate calculations from steady-state processes are straightforward, the PIB represents a non-steady-state rate. Marynick and Marynick (1975) developed a non-steady-state equation that was used by Hitz (1979) and Doehlert et al. (1979) to calculate photorespiration rates. A problem with the non-steady-state equation is that it requires instantaneous mixing throughout the monitoring system, not just in the sample chamber. Although it is generally possible to arrange gas exchange systems so that instantaneous mixing in the sample chamber can be assumed, this assumption is seldom also valid for the monitoring system because of the high flow rates that would be required (Hitz, 1979).

Peterson and Ferrandino (1984) have described a numerical simulation model based on the physics of solute dispersion in linear flow systems to correct the non-steady state mathematical formulation for the effect of mixing in the infra-red gas analyzer (IRGA) sample cell. Peterson has found that in his system the model accurately predicts observed IRGA responses (Peterson, 1983, 1987). Use of this model requires a computer to facilitate calculations and for determination of variables. The values of the variables must be ascertained for each IRGA system because the timing and shape of the PIB response are dependent on the resolution (i.e., volume, flow rate) of each system and its measuring cell (Canvin, 1979; Peterson, 1983). Nevertheless, within a particular measuring system, rates of photorespiration calculated from the PIB should provide a relative
estimate of photorespiration (Canvin, 1979).

The interest in photorespiration centers around the observation that net carbon fixation is stimulated by as much as 45% by decreasing the \( \text{O}_2 \) concentration from 21% to 2% (see, for example, Creach and Stewart, 1982) presumably because photorespiration is eliminated. There is no clear physiological justification for the existence of the glycolate pathway (Ogren, 1984). The characterization of *Arabidopsis* mutants for different enzymes of the glycolate pathway demonstrates that the pathway is only necessary to recycle glycolate produced due to the oxygenase activity of RubisCO. The pathway seems dispensable in the absence of oxygenase activity (Ogren, 1984). It was of interest, therefore, to further investigate the observations of our earlier study (see Part I), that photorespiration, as estimated from the PIB, appeared to have a different relationship to light CER in rejuvenated and senescing soybean cotyledons. Our investigations of these observations are reported in the second paper of this dissertation. As part of this study, we quantified the amount of RubisCO present in the soybean cotyledons using the sensitive and specific method of rocket immunoelectrophoresis (Burrin, 1986). The antibodies used for the immunoelectrophoresis were prepared by Regina Diethelm.
PART I. PHOTOSYNTHESIS, PHOTORESPIRATION AND RESPIRATION IN PRESENECSENT, SENESCING, AND REJUVENATING SOYBEAN COTYLEDONS: PHYSIOLOGICAL MEASUREMENTS
ABSTRACT

We measured light and dark carbon dioxide exchange rates (CER) in germinating, presenescent, senescing and rejuvenating soybean cotyledons. We observed net photosynthetic rates equivalent to rates reported for field-grown soybean leaves. Net photosynthesis in presenescent cotyledons was greatest 8 days after planting, after which time the rate began to decrease. Total chlorophyll in presenescent cotyledons was also maximal 8 days after planting. Cotyledons were rejuvenated by epicotyl removal on different days after planting. Net photosynthesis in rejuvenated cotyledons recovered to levels equivalent to those measured in presenescent cotyledons. Total chlorophyll levels in rejuvenated cotyledons were as much as 1.5x greater than maximal levels measured in presenescent cotyledons. Light CER was a function of total chlorophyll except in rejuvenated cotyledons after excess chlorophyll was accumulated. Dark CER was greatest in the youngest cotyledons and decreased to a nearly constant rate by 12 days after planting. Dark CER did not increase in rejuvenated cotyledons to presenescent rates. Analysis of the post-illumination burst of CO₂, which appeared after rapid light to dark transitions, indicated that photorespiration was changing relative to light CER throughout the developmental sequence studied.
INTRODUCTION

Soybean is unusual among the legumes because it has cotyledons that initially function as storage organs which develop leaf-like characteristics, including photosynthetic capability, as the cotyledons green and emerge during germination (Smith, 1983). The cotyledons function for a time as both storage mobilization and photosynthetic organs before becoming primarily leaf-like, and then they senesce and abscise as the seedling develops. Senescence can be arrested if the epicotyl is removed before the cotyledons abscise. The cotyledons will regreen and remain attached to the stem if axillary buds are removed as the buds grow out.

Krul (1974) showed that soybean cotyledons could lose up to 90% of their nucleic acids, primarily RNAs, and up to 80% of their protein before senescence became irreversible. A significant portion of the lost components was recovered during a fifteen-day rejuvenation period. Krul suggested that comparisons of the presenescent, senescing and rejuvenated states would offer a good model system in which to study leaf senescence without the possible artifacts induced by using detached organs and artificially induced senescence. Because there was a time after which the cotyledons could no longer be induced to rejuvenate (Krul's "point of no return"), Krul concluded that the cotyledons had lost some specific factor(s) that could not be replaced upon epicotyl removal. In search of some specific senescence factor(s), Skadsen and Cherry (1983) used this model system to generate protein profiles from *in vitro* translated poly A mRNA
isolated from different aged soybean cotyledons. They found that no translation products were unique to presenescent, senescing or rejuvenated cotyledons, although there were clear quantitative differences in different proteins. This model system is not well-characterized physiologically, and Skadsen and Cherry (1983) suggest that the rejuvenated state may be unrelated to that of the presenescent cotyledon because their data showed proteins that became very prominent only in the rejuvenated cotyledons.

In an early study investigating the photosynthetic capacity of soybean cotyledons, Abrahamsen and Mayer (1967) concluded that carbon fixed by the cotyledons was never more than 80% of the carbon lost by dark respiration, and for over half of the life of the cotyledon, carbon fixation provided no more than 10% of the carbon respired. The data raised doubts about how leaf-like the cotyledons really became and what, if any, contribution cotyledonary photosynthesis made to the seedling. More recently, reports have been published from two labs better characterizing soybean cotyledon photosynthesis, although the data from Brown and Huber (1987a, 1987b) do not indicate that net photosynthesis is ever much greater than dark respiration. Harris et al. (1986a) show net photosynthesis greater than dark respiration only after the primary leaves become photosynthetic, although they included the whole seedling in their dark respiration measurements. Neither of these studies measured photosynthesis in rejuvenated cotyledons, although an additional report from Harris et al. (1986b) showed an increase in Rubisco activity during a twelve-day rejuvenation period.
to about 50% of the maximal presenescent activity from a level at decapitation of 21% of the presenescent maximum. This would still indicate that the cotyledons are never capable of photosynthesis in excess of their own dark respiration. It seems unlikely that soybean cotyledons could recover from senescence and maintain themselves and their roots for extended periods without ever being capable of photosynthesis in excess of dark respiration.

Therefore, we initiated this work to provide a more complete physiological characterization of the model system of presencescent/senescing/rejuvenated soybean cotyledons through measurements of light and dark carbon dioxide exchange rates (CER) and various growth parameters.
MATERIALS AND METHODS

Plant Material

Soybean (Glycine max. [L.] Merr. cv. Corsoy 79) was grown in 28 cm x 40 cm flats in sterilized soil (1:1:1 soil:peat:perlite) in a greenhouse. Seeds were screened to uniform size before planting and were planted 35 to a flat. Planting was always completed before 10:30 a.m. Cotyledon age was measured as number of days after planting. Seedlings were thinned daily to eliminate any plants not at the same developmental stage, as determined by visual morphological characteristics. Each replication used cotyledons from one or occasionally two flats. Flats were moved daily to provide uniform lighting. Temperature and irradiance varied by season. Minimum temperature was always greater than 18°C. Maximum temperature was normally 22-24°C, although during July and August maximum temperature at times ranged from 29-32°C. Supplemental lighting was provided for 16 hours during the day by high pressure sodium lamps which supplied, at plant height, 56 μmol photons m⁻² s⁻¹. Maximum daily irradiance was never less than 1200 μmol photons m⁻² s⁻¹. Plants were watered twice weekly with modified Hoagland solution (Johnson et al., 1957) in which iron was supplied as the chelate Sequestrene 138 Fe at the rate of 16.7 mg L⁻¹, and on other days with tap water. For decapitation treatments, the stem, which included unifoliate leaves, trifoliate and apical bud (hereafter referred to as the epicotyl), was removed one or two cm above the cotyledonary node on various days after planting. Axillary buds were carefully removed as they expanded. On
the day that plants were to be used experimentally, flats were removed from the greenhouse approximately 30 minutes before the first series of measurements and taken to a growth chamber. Irradiance at plant height in the growth chamber was 340 μmol photons m⁻² s⁻¹. Gas exchange measurements were made on cotyledons from 4-day through 18-day old plants and from plants decapitated at 11 through 18 days at 1, 2, 4, 6, 10, and 20 days after epicotyl removal. Representative data from three decapitation dates are reported.

Gas Exchange

Gas exchange was measured with an Anarad infra-red gas analyzer (IRGA) Model AR500R (Anarad Inc., Santa Barbara, California) in an open system with a flow-through reference cell as described by Creach and Stewart (1982). Gas was mixed from pressurized cylinders of O₂, N₂, and 2% (v/v) CO₂ in N₂ to a final concentration of 350 μL L⁻¹ CO₂, 21+2% (v/v), O₂ and balance nitrogen. Flow rate was measured before the sample chamber; it ranged from 1.8 to 2 L min⁻¹. Sample and reference gas lines were each passed through a simple condenser to remove water vapor before the gas entered the IRGA. A flow meter with a bleed-out valve was inserted in each line between the condenser and the IRGA so that the gas entered the IRGA at 0.6 L min⁻¹ to reduce calibration fluctuations. Experiments were conducted at 22±1°C. Irradiance in the leaf chamber was 720 μmol photons m⁻² s⁻¹.

Cotyledons were removed from plants in the growth chamber and were weighed just prior to beginning gas exchange measurements. Usually 10 cotyledons were used for each set of measurements.
determinations with 4-day old cotyledons, 12 cotyledons were used. Gas exchange measurements were always made between 11 a.m. and 4 p.m. Cotyledons were allowed to reach steady-state photosynthesis (8-10 min.), and then the lights were turned off and the chamber covered with a black cloth to allow measurement of the post-illumination burst of CO$_2$ (PIB) and dark respiration. After 5-8 min the cloth was removed, the lights were turned on, and the light/dark cycle was repeated. The cotyledons were then removed from the chamber, weighed, and dried in an 80°C oven for dry weight measurements. Transpiration during the course of gas exchange measurements was determined gravimetrically from the change in cotyledon fresh weights. Cotyledon area was measured with a LI-COR 3000 portable leaf area meter (LI-COR, Lincoln, NE) and conveyor belt assembly.

**Chlorophyll Determinations**

Chlorophyll was extracted into cold N,N-dimethylformamide as described by Moran (1982). Individual cotyledons were weighed and leaf area was determined. The cotyledon was then sliced into 4 or 5 slices and placed in test tubes containing 3-10 ml of solvent. Appropriate volume was determined empirically because it was dependent on chlorophyll concentration which varied considerably over the ages studied. Minimum fresh weight to volume ratio was 1:20. The tubes were tightly capped and were kept cold and dark for at least 2 days before determining $A_{647}$ and $A_{664}$. Total chlorophyll, chlorophyll a, and chlorophyll b were calculated from the equations derived by Moran (1982). Acetone extractions, reading $A_{652}$ and calculating total
chlorophyll according to Arnon (1949), gave comparable values as long as the cotyledons were sliced into the N,N-dimethylformamide (data not shown). The chlorophyll was much more stable in the N,N-dimethylformamide (98% of original values after 2 weeks, data not shown) than in acetone and the procedure was much simpler.
RESULTS

Growth Parameters

During this study, seedlings emerged by the fourth day after planting at which time the cotyledons were green and still enlarging. Maximum fresh weight and maximum area were attained by day 6 (Fig. 1a,c). Cotyledon area remained relatively constant throughout the remainder of the experiments. Fresh weight decreased rapidly after day 8 and less rapidly after day 12. Dry weight decreased from the first measurement, rapidly at first and more slowly after day 8 to a relatively constant dry weight by day 10 (Fig. 1b). In the rejuvenated tissues, fresh weight either remained relatively constant at the value it had at the time of decapitation or increased slightly. Dry weight increased slowly during rejuvenation (Fig. 1a,b).

Unifoliolate leaves were fully expanded by day 10, and the first trifoliolate was unfolding by day 12. By 16 days after planting, the third trifoliolate was beginning to unfold and the cotyledons were yellowing. In our system, the failure of cotyledons to respond to epicotyl removal coincided with the early expansion of the third trifoliolate, regardless of seedling age. And, although plants were culled daily, variation was still observed in the onset and degree of yellowing. In this study, 20% of the cotyledons survived after epicotyl removal at 18 days; 60% survived after decapitation at 16 days. We considered 16 days to be the "point of no return." The relative timing of senescence and abscission was markedly influenced by water stress, necessitating careful and frequent summertime watering.
Fig. 1. Fresh weight (A), dry weight (B), and area (C) of presenescent, senescing and rejuvenated soybean cotyledons. Presenescent and senescing cotyledons (●); rejuvenated cotyledons, decapitation at 11 days (○); rejuvenated cotyledons, decapitation at 14 days (△); rejuvenated cotyledons, decapitation at 16 days (□). When measurements from senescing cotyledons coincide with measurements from rejuvenating cotyledons, the open symbol characteristic of rejuvenating cotyledons contains a small solid black circle.
Fig. 2. Total chlorophyll (A) and chlorophyll a to b ratio (B) in presenescent, senescing, and rejuvenated soybean cotyledons. Symbols are as described in Fig. 1.
When expressed on a per cotyledon basis, total chlorophyll in the cotyledons increased until day 5 and then remained relatively constant until day 8 (Fig. 2a). After that, it decreased rapidly until it was not detectable in cotyledons that appeared fully yellow. When chlorophyll was expressed per unit area, it decreased after day 5 because the cotyledons did not attain maximum area until day 6 (Fig. 1c). In rejuvenated tissue, chlorophyll accumulated to as much as 1.5x the maximum observed in presenescent tissue (Fig. 2a). The younger the cotyledons were at the time of epicotyl removal, the higher the level of chlorophyll accumulated at equivalent rejuvenation times. Differences in chlorophyll present at the different decapitation dates accounts for the most of the differences in chlorophyll accumulation. The cotyledons from plants decapitated at 16 days showed the longest delay in chlorophyll resynthesis. The chlorophyll a to b ratio declined from a high value of 4 in the 4-day old tissue to a low value of 3 in the senescing cotyledons. The a/b ratio then increased during rejuvenation to 3.5, the ratio in 8-day to 10-day old cotyledons (Fig. 2b).

Gas Exchange

Because of the precipitous changes observed in the chlorophyll levels and the relative constancy of cotyledon area, CERs were expressed on a per unit area or a per cotyledon basis. Light CER was detectable by 4 days after planting, the youngest tissue used in this study. The exchange rate increased rapidly to a maximum by day 8 (Fig. 3a), after which it began to decrease until CER was not
Fig. 3. Light CER (A), dark CER min (B), PIB (C) and transpiration (D), in presenescent, senescing, and rejuvenated soybean cotyledons. Symbols are as described in Fig. 1
detectable in fully yellow cotyledons. In contrast with the pattern observed in chlorophyll accumulation, light CER in rejuvenated tissue never exceeded the presenescent maximum by more than 10%. In most cases, presenescent and rejuvenated light CER maxima were not significantly different. In all cases, light CER increased by the first day after epicotyl removal although the increase was slower at the later decapitation dates. Light CER was only less than dark CER in the 4-day old tissue. It exceeded dark CER by day 5.

Two calculations were made from the dark gas exchange measurements. The first, dark CER\textsubscript{max}, was calculated from the maximum CO\textsubscript{2} differential observed in our system in the dark, a non-steady state value which occurred approximately 30 seconds after darkening the tissue. The second calculation, dark CER\textsubscript{min}, was made using the CO\textsubscript{2} differential after approximately 4 minutes of darkness, a near steady state value. We used dark CER\textsubscript{max} - dark CER\textsubscript{min} as an approximation of the PIB (and hence of photorespiration). Dark CER\textsubscript{min} (dark respiration) was highest in the 5-day old cotyledons (Fig. 3b). The rate decreased rapidly until day 10 and then remained relatively constant for the remainder of the experiment. The PIB was greatest in 6-day old cotyledons. It decreased to an undetectable level by day 15 and then increased during rejuvenation to levels greater than that seen in presenescent tissue. During rejuvenation, the PIB seemed to recover more slowly to its presenescent maximum than did light CER. Equivalent light CER in senescing and rejuvenating tissue resulted in a smaller PIB in rejuvenating tissue (Fig. 3a,c).
Estimates of transpiration made by gravimetric determinations indicated that transpiration recovered to approximately the presenescent maximum at least one sampling period before light CER recovered to its presenescent maximum, suggesting that stomatal aperture was not the limiting factor early in CER recovery (Fig. 3a,d). During senescence, transpiration and light CER showed a similar pattern of change.

Light CER appeared to be highly correlated with chlorophyll content except in the youngest tissue and during the later stages of rejuvenation when chlorophyll accumulation exceeded the presenescent maximum. In the oldest rejuvenated cotyledons, chlorophyll clearly was not limiting light CER (Fig. 4a). If a correction is made to light CER to account for the higher dark CER seen in the youngest tissue, then the data from the younger cotyledons fit more closely into the linear portion of the curve (Fig. 4b).
Fig. 4. Light CER as a function of total chlorophyll concentration in presenescent, senescing, and rejuvenated soybean cotyledons (A). Inset (B) illustrates light CER that has been corrected for dark CER$_{max}$ - dark CER$_{min}$, plotted as a function of total chlorophyll. Symbols are as described in Fig. 1 with the addition that data from 4-day and 6-day old cotyledons are enclosed by a larger, open circle.
DISCUSSION

Growth Parameters

Patterns of change in growth parameters observed in this study are qualitatively similar to those observed by Brown and Huber (1987b), Harris et al. (1986a, 1986c), and Peterman and Siedow (1985) although differences in timing are seen. We have observed variation over the year in the age of cotyledon senescence and, therefore, in the age after which the cotyledons would no longer respond to epicotyl removal. Cotyledons on plants grown in early spring did not abscise until 24 days after planting and could be rejuvenated through 22 days (data not shown), in contrast with the 16-day point of no return determined in this study. In the early spring-grown plants, chlorophyll accumulation did not show such a dramatic peak as it did in the current study (data not shown), a pattern similar to that observed by Abrahamsen and Mayer (1967) and Harris et al. (1986a, 1986c). Variations in the senescence patterns are probably related to differences in the amount of light supporting different growth rates, since in our studies the third trifoliolate was in its early stages of expansion when the cotyledons senesced and abscised, regardless of the season. Harris et al. (1986c) and Peterman and Siedow (1985) report similar timing between cotyledon abscission and third trifoliolate unfolding, although there may be varietal differences in this characteristic. Other studies (Krul, 1974; Skadsen and Cherry, 1983; Brown and Huber, 1987a, 1987b) do not report the data necessary to allow a comparison.
Cotyledon light CERs measured in this study are much greater than those reported by Abrahamsen and Mayer (1967) who reported maximal rates of approximately 0.33 $\mu$mol CO$_2$ cm$^{-2}$ h$^{-1}$, and those reported by Harris et al. (1986a), who observed maximal rates of approximately 0.9 $\mu$mol CO$_2$ cm$^{-2}$ h$^{-1}$. Brown and Huber (1987a, 1987b) reported a maximal rate of 1.6 $\mu$mol CO$_2$ cm$^{-2}$ h$^{-1}$. Our maximal light CERs of 4.7 $\mu$mol CO$_2$ cm$^{-2}$ h$^{-1}$ fall in the range reported for field-grown soybean leaves (for example, Secor et al., 1984). These rates are clearly in excess of dark respiration even in young cotyledons which did show high dark respiration rates during reserve mobilization. The low rates reported by Abrahamsen and Mayer (1967) are most likely a result of methodological limitations and of their use of low light intensities, which did not allow for maximal development of the photosynthetic apparatus. A decrease in the respiration rate was the primary cause of the large increase they saw in cotyledon capacity to replace carbon lost by respiration (from 10% at 10 days to 80% at 14 days).

In agreement with the data of Harris et al. (1986a), the CER of cotyledons in our experiments increased and then decreased during seedling development (although the time scale was more compressed in our experiments). This pattern contrasted with the data reported by Brown and Huber (1987a, 1987b) which showed an early peak in light CER (at 6 days), a relatively constant rate from 7 to 10 days, and then an increase to a maximal rate at 14 days. After 14 days, their light CER decreased rapidly to a very low level by 16 days. They do not indicate at what age their cotyledons abscised. Dry
weight and chlorophyll data that they report would suggest that abscission was around 19 days, comparable with this study. We cannot explain why they would have observed such a different pattern in CER, their lowest values coming at times when our highest CERs were occurring and their peak value occurring when our CER had decreased to 40% of maximum. They used lower irradiance for growth (450 μmol m⁻² s⁻¹) and a shorter daylength than used in this study. Harris et al. (1986a, 1986c), however, used even lower irradiances (230 μmol m⁻² s⁻¹) and obtained a CER pattern similar to the one we observed. The differences in irradiances probably do account for the absolute differences in CER observed among the studies.

There was less difference among these studies in the maximum level of chlorophyll found in presenescent cotyledons than would be expected from the differences in light intensity during growth. Total chlorophyll in our study peaked early (6 or 8 days) and then began a rapid decline that was reversed during rejuvenation. Only Peterman and Siedow (1985) also observed an excess accumulation of chlorophyll in rejuvenated cotyledons, although such a phenomenon has been reported in other systems (for example, Chang and Beevers, 1973).

The decrease in the chlorophyll a/b ratio that we observed during senescence resulted from a faster decrease in chlorophyll a. The increase in the ratio upon rejuvenation corresponded with a faster accumulation of chlorophyll a than chlorophyll b. A decreasing chlorophyll a/b ratio is a commonly reported characteristic of senescence (Kura-Hotta et al., 1987) and was also observed in soybean
cotyledons by Harris et al. (1986c) and Huber and Newman (1976). The preferential degradation of chlorophyll a may reflect the fact that chlorophyll b is primarily an accessory pigment for photosystem II, and as such, is embedded in the grana thylakoids. Ultrastructural observations made by Huber and Newman (1976) in senescing and regreening soybean cotyledons showed a preferential destruction of stroma thylakoids as plastids degraded during senescence. They also saw membrane reconstruction, particularly of the stroma lamellae, during rejuvenation. Treffry et al. (1967) also showed preferential maintenance of grana thylakoids in senescent soybean cotyledons. They did not examine rejuvenated tissue.

Senescence is an ill-defined syndrome that includes protein degradation, changes in nucleic acids and changes in membrane structure (Thimann, 1980). Chlorophyll loss is most often used as an index of senescence because it provides a general visual index and because no other parameter is so easily measured. Photosynthetic capacity decays during senescence and as such it is part of the overall syndrome. Some studies in the past have found a close relationship between chlorophyll loss and loss of photosynthetic capacity (for example, Secor et al., 1984; Camp et al., 1982) while others have not (for example, Makino et al., 1983; Friedrich and Huffaker, 1980). One factor in the differences between the studies may be methodological, both in how photosynthetic capacity is determined and in the method for chlorophyll determination. In the current study, light CER and chlorophyll are highly correlated during
senescence and during rejuvenation. These results indicate that in some aspects at least, rejuvenation is the reversal of senescence, although the relationship becomes uncoupled in the later stages of rejuvenation.

The relationship between chlorophyll and light CER is also different in the youngest cotyledons, which appear to have excess chlorophyll for the CERs observed. Because the CER measurements are net measurements, and because it is still unclear how much dark respiration continues in the light (Dry and Wiskich, 1987), it may be difficult to compare light CER rates over our entire developmental sequence when we observe significant changes in dark CER as well. The youngest cotyledons had the highest dark CER$_{\text{min}}$, which decreased to a minimal rate in concert with a rapid decrease in dry weight, presumably as reserve mobilization was completed. This conclusion is supported by data reported by Brown and Huber (1987a), showing that reserve mobilization in soybean cotyledons was completed by 8 days and was coincidental with the major loss of dry weight. If reserve mobilization occurs in the light, then light CER would be more of an underestimation of true photosynthesis in the youngest cotyledons. If the dark CER$_{\text{min}}$ observed in the rejuvenated cotyledons is more representative of a basal, true, dark respiration rate and the excess observed in the youngest cotyledons is made as a correction to light CER, then CER appears as a linear function of chlorophyll in the youngest cotyledons as well.

We did not observe any characteristics unique to the CER pattern
in presenescent, senescing or rejuvenating cotyledons that would
delineate the point of no return. Differences were observed in
patterns of chlorophyll accumulation and in the relationship between
chlorophyll and light CER. Dark CER_{min} and the PIB also varied over
the developmental sequence studied. Our data suggest that the
rejuvenated cotyledon is a very different structure from the young,
presenescent organ. In spite of the close relationship between light
CER and chlorophyll in senescing and rejuvenating cotyledons, the
apparently different relationship between the PIB and light CER
suggests that the process of rejuvenation is not an exact reversal of
senescence. If comparisons of presenescent, senescing, and
rejuvenating soybean cotyledons are to be used as a model system to
investigate factors unique to senescence, there must first be a clear
baseline for comparison. It may be difficult to isolate factors
unique to senescence against such a changing background.
PART II. PHOTOSYNTHESIS, PHOTORESPIRATION, AND RESPIRATION IN PRESENECENT, SENESCENT, AND REJUVENATING SOYBEAN COTYLEDONS: BIOCHEMICAL CHARACTERIZATION
We measured the amount of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) present in germinating, presenescent, senescing, and rejuvenating soybean cotyledons using rocket immunoelectrophoresis. We also measured light and dark carbon dioxide exchange rates (CER) in the cotyledons. Cotyledons were rejuvenated by epicotyl removal at 18 days after planting. Light CER was a function of the amount of Rubisco detected except in 4-day and 6-day old cotyledons and in rejuvenating cotyledons immediately after epicotyl removal. The increase in CER in rejuvenating cotyledons that occurred without a concurrent change in the Rubisco content coincided with a rapid increase in the transpiration rate. Light CER in 4-day and 6-day old cotyledons was much lower than expected based on the amount of Rubisco present in the cotyledons. Light CER was a function of Rubisco amount in rejuvenated cotyledons that showed chlorophyll accumulations in excess of those observed in presenescent cotyledons. The post-illumination outburst of CO₂ (PIB) appeared to recover more slowly than light CER in rejuvenating cotyledons when compared with equivalent presenescent light CER. The percentage of total $^{14}$C incorporated which accumulated in glycine indicated, however, that equivalent light CER resulted in equivalent photorespiration in senescing and rejuvenating cotyledons. Late rejuvenated cotyledons appeared to have a higher relative photorespiration rate than did presenescent cotyledons.
Soybean cotyledons emerge during germination, become green, and develop leaf-like characteristics. The cotyledons then senesce as the seedling develops. If the epicotyl is removed sometime before the cotyledons abscise, the cotyledons will regreen. Krul (1974) and others (Peterman and Siedow, 1985; Skadsen and Cherry, 1983) have suggested using the presenescent/senescent/rejuvenating soybean cotyledon progression as a model system with which to study leaf senescence. As part of a study of the physiological characteristics of this model system (see Part I), we found several indications that rejuvenation results in a state that is quite different from the senescing or presenescent states, a possibility acknowledged by Skadsen and Cherry (1983). One of the unusual characteristics of the rejuvenated cotyledons is their apparent accumulation of chlorophyll in excess of the maximal amount seen in presenescent cotyledons. Peterman and Siedow (1985) also observed more chlorophyll in rejuvenated cotyledons than in presenescent cotyledons. Other studies of rejuvenated cotyledons probably were not carried out long enough to see this chlorophyll effect (Krul, 1974; Harris et al., 1986b; Huber and Newman, 1976). We found that the excess chlorophyll did not result in a corresponding increase in light CER. Because we expected to see a better correlation between light CER and the amount of RubisCO present in the cotyledons than between light CER and total chlorophyll, in the present study we determined the levels of RubisCO in presenescent, senescing, and rejuvenating soybean
cotyledons. We quantified RubisCO using rocket immunoelectrophoresis. Because they become photosynthetic, soybean cotyledons also exhibit evidence of photorespiration. One external manifestation of photorespiration is the PIB (Canvin, 1979), which is a pulse of CO$_2$ evolution seen immediately after a rapid light to dark transition. As another anomalous characteristic of rejuvenated cotyledons, we observed that during recovery of light CER, PIB recovery appeared delayed when compared with an equivalent light CER in presenescent or senescing cotyledons (see Part I). These data suggested a possible differential effect of photorespiratory metabolism in senescing and rejuvenating cotyledons. To determine whether or not the PIB was actually reflecting changes in photorespiration, we labelled cotyledons with $^{14}$CO$_2$ during photosynthesis. Because glycine is the photorespiratory intermediate from which most, if not all, CO$_2$ lost during photorespiration originates (Tolbert, 1981), we looked for differences in its labeling pattern that would correspond with the changes seen in the PIB.
MATERIALS AND METHODS

Plant Material

Soybean (Glycine max. [L.] Merr. cv. Corsoy 79) was grown as described previously (see Part I) except that maximum irradiance was decreased by one-third during the summer months because the greenhouse roof was white-washed. Maximum daily irradiance was still always at least 1200 μmol photons m$^{-2}$ s$^{-1}$. The decreased summer irradiance permitted rejuvenation of cotyledons at 18 days from May through October. For decapitation treatments, the stem, which included unifoliolate leaves, trifoliolates, and the apical bud (hereafter referred to as the epicotyl), was removed one or two cm above the cotyledonary node on various days after planting. Because there were still variations between experiments in the timing of various growth parameters, the data reported are from one representative experiment. Measurements were made on two sets of 8 cotyledons from 2-day through 18-day old plants and from plants decapitated at 18 days, 1, 2, 4, 6, 10, 14, 20, 26, and 32 days after epicotyl removal.

Gas Exchange

Carbon dioxide gas exchange rates (CER) were measured with an Anarad infra-red gas analyzer (IRGA) Model AR500R (Anarad, Inc., Santa Barbara, CA) in the system described previously (see Part I). We mixed gas from pressurized cylinders of O$_2$, N$_2$, and 2% (v/v) CO$_2$ in N$_2$ to a final concentration of 350 l l$^{-1}$ CO$_2$, 21±2% (v/v) O$_2$ and balance nitrogen. Usually eight cotyledons were used for each set of
measurements. More cotyledons were used for 2-day and 4-day CER
determinations to compensate for lesser area per cotyledon. Gas
exchange measurements were always made between 10:30 a.m. and 4:30
p.m. On the day that plants were to be used experimentally, flats
were removed from the greenhouse approximately 30 minutes before the
first series of measurements and taken to a growth chamber.
Cotyledons were removed from plants in the growth chamber and weighed
immediately before beginning CER measurements. The three cotyledons
that were to be used for $^{14}$CO$_2$ labelling were removed and traced for
later area determination. The weight of the remaining five cotyledons
was recorded. All cotyledons were then put into the sample chamber.
After the cotyledons reached steady-state photosynthesis (10-15 min),
the lights were turned off and the chamber was covered with a black
cloth to allow for measurement of the PIB and dark respiration. After
8-12 min the cloth was removed and the lights were turned on. The
light/dark cycle was repeated. Five cotyledons were removed, the
sample chamber was resealed, and the lights were turned back on. The
five cotyledons were weighed, and their areas were determined with a
Li-COR Li 3000 portable leaf area meter (Li-COR, Lincoln, NE) and
conveyor belt assembly. The cotyledons were frozen in liquid N$_2$ and
stored in a liquid N$_2$ refrigerator in a glass vial covered with
cheesecloth until they were used for RubisCO determinations.
Transpiration was estimated from fresh weight losses.
Incorporation

A 20.2 L closed system consisting of a 20 L glass carboy, a 125 ml side-arm flask, a peristaltic pump (Varistaltic pump, S Series, Manostat, New York, NY), and Tygon connecting tubing was used for $^{14}$CO$_2$ incorporation. After IRGA calibration but before beginning gas exchange measurements, the closed system was flushed for at least 20 min with the mixed gas. The system was then closed and $^{14}$CO$_2$ generated from NaH $^{14}$CO$_3$ (New England Nuclear, s.a. 57 mCi mmol$^{-1}$) in the side-arm flask by the addition of 10 ml of 8.5 N phosphoric acid. Thirty $\mu$Ci $^{14}$C were used for each day's experiments, which consisted of four or fewer sets of gas exchange measurements. Air was circulated within the closed system by the peristaltic pump at 1.5 L min$^{-1}$ for at least 15 min while gas exchange measurements were started. At the conclusion of the gas exchange measurements and after removal of the five cotyledons used for RubisCO determination, the sample chamber was disconnected from the flowing sample gas line and quickly connected to the closed system. The pump was restarted and air was circulated through the closed system plus sample chamber at 1.7 L min$^{-1}$. After a 15 min photosynthetic period, the pump was turned off, and the closed system was reconnected to itself. The sample chamber outlet was connected to a soda-lime column. The flowing sample gas line was reconnected to the sample chamber inlet. The sample chamber was flushed for 3 s, and the cotyledons were removed and frozen in liquid N$_2$. The sample chamber outlet was reconnected to the system. Disconnecting the closed system and
freezing the cotyledons usually took less than 10 s. These cotyledons were stored in liquid $N_2$ until extracted for metabolite analysis.

**Metabolite Extraction and Determination**

The $^{14}$CO$_2$ labelled cotyledons were ground to a fine powder in liquid $N_2$ with a small amount of acid-washed sand in a chilled mortar and pestle. Three ml of 2 N HCl were added and the frozen mass that resulted was ground until it thawed. The grindate was decanted into a centrifuge tube. The mortar was rinsed with 1 ml of the acid, and the rinse was added to the centrifuge tube. The grindate was centrifuged at 12000 xg in an SS34 or SM24 Sorvall rotor in a Sorvall model RC2-B centrifuge for 20 min. An aliquot of the supernatant was counted to determine total soluble acid-stable $^{14}$C incorporation. The scintillation cocktail was toluene based (toluene: Triton X-100:PPO; 100:50:0.4; v:v:w). Counting was done in a Beckman LS 3801 Liquid Scintillation Counter.

The remaining supernatant was filtered through a Millipore prefiltre (Millipore AP20 13 mm) and was added to 5 ml of chloroform. The chloroform mixture was vortexed and then centrifuged (International Centrifuge, Universal Model UV) to insure phase separation. Lipid material gathered at the interface. The aqueous phase was applied to a Dowex 50-H$^+$ ion exchange column (5 cm x 0.5 cm). Acidic and neutral compounds were washed through the column with 7 ml of water. The basic fraction was eluted with 11 ml of 2 N NH$_4$OH. The basic fraction was dried under air at room temperature, resuspended in a minimal volume of water, and aliquots spotted onto thin-layer
cellulose plates (Kodak 13255 Cellulose). The plates were chromatographed in non-equilibrated glass tanks in butanol:acetone:wate:diethylamine (20:10:10:3, Spalding et al., 1983). The diethylamine was added just before the plates were put into the tanks. Separation was complete within 2 hours. The plates were air-dried overnight before being used for autoradiography. The autoradiograms were developed for 14 days. The film (Kodak XAR 5) was developed by standard processing methods. The print was placed on the original TLC plate and the radioactive amino acid spots located and outlined. The spots were cut out and solubilized in scintillation cocktail before being counted as described above. Counting efficiency was 91%. Standard $^{14}$C labelled amino acids were run on each chromatogram.

**Purification of Rubisco**

Rubisco was purified from the youngest, expanded trifoliolates of greenhouse-grown Corsoy 79 soybeans by the method of Paech and Dybing (1986). Of the three options they described for the final centrifugation step, we used a VTi 65 rotor. Fractions containing Rubisco were pooled, and sucrose was removed by dilution with gradient buffer (50 mM Bicine/KOH, pH 8.0, 0.2 mM EDTA, 1 mM DTT) and ultrafiltration (Amicon 8050). The Rubisco was repurified on 10-30% sucrose gradients centrifuged at 26000 rpm (95000 xg) for 28 h at 2°C in a Beckman SW28 rotor in a Beckman Model L8-70 preparative ultracentrifuge. The purity of the Rubisco was evaluated by SDS-PAGE in 7.5 to 15% gradient gels. The gels were silver-stained (Heukeshoven and Dernick, 1985). The Rubisco was stored at 4°C as
Quantification of RubisCO

RubisCO was quantified using the method of rocket immunoelectrophoresis, which is generally described by Weeke (1973). Antisera were raised against soybean RubisCO in New Zealand White rabbits as described by Diethelm and Shibles (personal communication, Agronomy Department, ISU, Ames, IA). The specificity of the antisera was verified using the Ouchterlony double diffusion method (Diethelm and Shibles, personal communication, Agronomy Department, ISU, Ames, IA).

Samples were prepared by grinding 5 cotyledons to a fine powder in liquid N\textsubscript{2} with a small amount of acid-washed sand in a chilled mortar and pestle. Six ml of cold 50 mM Bicine (pH 8) were added, and the resulting frozen mass ground until it thawed. The grindate was decanted into a centrifuge tube. The mortar was rinsed with 4 ml of buffer. The combined solutions were centrifuged in an SM24 Sorvall rotor at 8000 rpm (7900 xg) for 10 min. An aliquot of the supernatant was used to measure soluble protein by the method of Bradford (1976). Fraction V BSA (Sigma) in Bicine was used to determine a standard curve. An aliquot of the supernatant was also used for RubisCO quantification. Assuming that in a mature, leaf-like cotyledon, RubisCO would comprise approximately 50% of the soluble protein, appropriate dilutions were made so that the amount of RubisCO added to the gel was between 0.3 and 0.6 \textmu g in a 5 \mu l sample.

Standards were prepared from the RubisCO purified as described
above. The aliquots were centrifuged in a microfuge (Fisher Model 59A) at 90% full power for 10 min to collect the protein. The supernatant was aspirated. The pellet was resuspended in 50 mM Bicine (pH 8.0). Protein was determined using the Bradford assay. Dilutions were made to 0.10, 0.050, and 0.025 μg/μl for the standards. Protein was measured in the standards using the micro Bradford assay (Bradford, 1976).

Gels were cast on Gel Bond (10 cm x 10 cm, FMC) from 15 ml aliquots of 0.9% agarose (Bio-Rad Standard Low Electroendosmosis) mixed in Gelman High Resolution Buffer (0.03 M ionic strength) that contained 1.5% antiserum. Nineteen wells 2.5 mm in diameter were punched into the gel in two offset rows. The wells were 8 mm apart, center to center, and at least 1.5 cm from the sides and 2 cm from the bottom of the gel. The samples and the standards were each run twice on a gel. The gels were electrophoresed on an LKB Multiphor Horizontal Bed apparatus (LKB Bromma, Sweden) for 16 h at 2 to 2.5 V cm⁻¹. The bed was cooled to 5 or 10°C by a circulating, refrigerated water bath. Gelman High Resolution Buffer was used for the running buffer. Ultrawicks from Bio-Rad were used to connect the gels to the running buffer. Gels were washed and stained with Coomassie brilliant blue G-250 (Sigma) as described by Weeke (1973). Rubisco was quantified from peak heights as measured from the top of the application well to the tip of the peak compared to the standard peak heights.
Chlorophyll

Chlorophyll was extracted in N,N-dimethylformamide as described previously (see Part I). Concentrations of total chlorophyll and chlorophylls a and b were calculated from the equations of Moran (1982).
RESULTS

Growth Parameters

Patterns of change observed in most growth parameters of soybean cotyledons in this study were qualitatively similar to those observed in our earlier study (see Part I). Fresh weight increased until day 8 and remained relatively constant until day 12. It then decreased until decapitation at 18 days, after which time it remained relatively constant (Fig. 1a). Maximal leaf area was attained by day 8 (Fig. 1b). Area remained relatively constant throughout senescence and rejuvenation.

Total chlorophyll increased until day 8 when expressed per cotyledon (Fig. 2a). During senescence, total chlorophyll decreased. It increased during rejuvenation. Maximum chlorophyll accumulated in rejuvenated cotyledons was 1.25 x greater than the maximum observed in presenescent cotyledons. During senescence, the chlorophyll a to b ratio slowly decreased (Fig. 2b), as a result of a relatively more rapid decrease in chlorophyll a. During rejuvenation, the chlorophyll a to b ratio initially increased slowly due to a more rapid increase in chlorophyll a. The rate of increase in chlorophyll a was relatively constant throughout rejuvenation. In contrast, the rate of accumulation of chlorophyll b slowly increased during rejuvenation. As a result, the chlorophyll a to b ratio began to decrease again after approximately 10 days rejuvenation.
Fig. 1. Fresh weight (A) and area (B) of presenescence, senescing, and rejuvenated soybean cotyledons. Presenescence and senescing cotyledons (●); rejuvenated cotyledons decapitated at 18 days (○)
Fig. 2. Total chlorophyll (A) and chlorophyll a to b ratio (B) in presenescent, senescing, and rejuvenated soybean cotyledons. Symbols are as described in Fig. 1.
**RubisCO**

RubisCO was detectable in 2-day old, pre-emergent cotyledons, before there was detectable chlorophyll (Figs. 2a and 3). The enzyme increased to a maximum level at day 6 when expressed on a per cotyledon basis. Then the amount of RubisCO decreased steadily until decapitation at day 18. After a two-day lag, RubisCO increased during rejuvenation. Maximal levels of RubisCO in rejuvenated tissue were comparable to levels in 10 and 12-day old cotyledons, about 75% of the presenescent maximum. Total soluble protein was highest in the 2-day old cotyledons (Fig. 3). Protein decreased very rapidly until day 8, after which time the decrease was much slower. After epicotyl removal, total soluble protein increased slowly. The amount of total soluble protein remained fairly constant after 20 days rejuvenation at a level intermediate between that found in 10-day and 12-day old cotyledons. RubisCO represented an equivalent percentage of the total protein in 12-day old cotyledons and in rejuvenated cotyledons beyond 6 days after epicotyl removal (Fig. 4).

**Gas Exchange**

Light and dark CER patterns were similar to those observed in our earlier study (see Part I). We observed equivalent maximal rates. In the current study, light CER increased until day 8, remained relatively constant, and decreased after day 12 (Fig. 5). In the earlier study, light CER began to decline soon after reaching a peak at day 8. The earlier decline fits with an over-all speeded-up growth pattern in the first study, which resulted in an earlier decapitation
Fig. 3. Rubisco (●, ○) and total soluble protein (■, □) in presenescent, senescing, and rejuvenated soybean cotyledons. Solid symbols refer to presenescent and senescing cotyledons; open symbols refer to rejuvenated cotyledons decapitated at 18 days.
Fig. 4. Rubisco expressed as the percent of total soluble protein in presenescent, senescing, and rejuvenated soybean cotyledons. Symbols are as described in Fig. 1.
Fig. 5. Light CER (●, ○); dark CER_{min} (■, □); dark CER_{max} (▲, △); and transpiration (●, ○) in presenescent, senescing, and rejuvenated soybean cotyledons. Solid symbols refer to presenescent and senescing cotyledons; open symbols refer to rejuvenated cotyledons decapitated at 18 days.
age (16 day, in contrast with 18 days in the current study). Light CER showed a clear relationship to amount of total chlorophyll except perhaps in the youngest cotyledons and during the later stages of rejuvenation (Fig. 6). The data from the youngest cotyledons fit into the curve better when a correction is made for dark CER (Fig. 6b). The data from the rejuvenated cotyledons remain aberrant. Based on chlorophyll content, one would expect higher rates of light CER in the rejuvenated cotyledons. This relationship was also observed in our earlier study (see Part I).

Light CER is also clearly a function of Rubisco content (Fig. 7), although the data from the youngest cotyledons do not fit into the general pattern. In contrast with the light CER/chlorophyll relationship (Fig. 6), correcting for dark CER does not fit the deviant data points into the general light CER/Rubisco relationship (not shown). Also in contrast with the light CER/chlorophyll relationship, data from cotyledons in the later stages of rejuvenation fit into the general light CER/Rubisco relationship (Fig. 7). Light CER increased by 60% by the first day after epicotyl removal (Fig. 5). There was no corresponding increase in dark CER (Fig. 5) or in Rubisco content (Fig. 3). The vertical portion of Fig. 6 reflects this relationship. The transpiration rate increased sharply during this period (Fig. 5) so that the initial rapid increase in CER may be due to an effect on stomatal aperture caused by epicotyl removal.

Dark CER was highest in the youngest cotyledons (Fig. 5). The exchange rate decreased to a relatively constant value by day 14, a
Fig. 6. Light CER as a function of total chlorophyll concentration in presenescent, senescing, and rejuvenated soybean cotyledons (A). Inset (B) illustrates light CER that has been corrected for dark CER max, plotted as a function of total chlorophyll. Symbols are as described in Fig. 1 with the addition that data from 4-day and 6-day old cotyledons are enclosed by a larger, open circle.
Fig. 7. Light CER as a function of RubisCO concentration in presenescent, senescing, and rejuvenated soybean cotyledons. Symbols are as described in Fig. 1 with the following additions: 4-day and 6-day old cotyledons (△); 18-day old cotyledons (■); rejuvenated cotyledons 1 and 2 days after decapitation at 18 days (□)
level that varied little throughout the remainder of the experiment. The PIB, as estimated from dark \( \text{CER}_{\text{max}} - \text{dark CER}_{\text{min}} \), was probably not significantly different in the presenescent and rejuvenated cotyledons. The shape of the IRGA traces from the light/dark transient, however, was very different (Fig. 8). In our earlier study, we observed PIBs in rejuvenated cotyledons that were much greater than those seen in presenescent tissue (see Part I). The differences between the two studies may be due to the lower dark \( \text{CER}_{\text{min}} \) observed in the current study. The lower dark \( \text{CER}_{\text{min}} \) seen in the current study are a result of using longer periods of darkness before determining an exchange rate.

\( ^{14}\text{CO}_2 \) Incorporation

In agreement with our earlier study (see Part I), we observed that during rejuvenation light CER appears to recover to the presenescent maximum more quickly than does the PIB (Fig. 9). Six days after epicotyl removal, light CER has recovered to 66% of maximum, yet no PIB is detectable. Four days later, light CER is 85% of its maximum and the PIB appears at less than 25% of its maximum. At equivalent presenescent light CER (midway between 12-day and 14-day old cotyledons), the PIB is at 45% of maximum. This change in the relationship between the PIB and light CER is reflected in the scatter of data points seen in Fig. 10.

In order to determine whether this changing relationship between the PIB and light CER resulted from changes in photorespiration rates, we looked for differences in \( ^{14}\text{CO}_2 \)
Fig. 8. Light CER as a function of time in rejuvenated soybean cotyledons 20 days after decapitation at 18 days (A) and in 8-day old cotyledons (B). The solid black bars along the time axis indicate periods of darkness.
Fig. 9. Light CER (●, ○) and the PIB (▲, △) expressed as a percentage of the maximum presenescent rates in presenescent, senescing, and rejuvenated soybean cotyledons. Solid symbols refer to presenescent and senescing cotyledons; open symbols refer to rejuvenated cotyledons decapitated at 18 days. Maximum CER was 12.9 µmol cotyledon⁻¹ hr⁻¹; maximum PIB was 2.4 µmol cotyledon⁻¹ hr⁻¹.
Fig. 10. The PIB as a function of light CER in presenescent, senescing, and rejuvenated soybean cotyledons. Symbols are as described in Fig. 1
incorporation over the sequence of presenescent, senescing and rejuvenated cotyledons that would coincide with the different PIB responses. Calculations from total $^{14}$C incorporated gave estimates of net photosynthesis that were mostly within 10% of the gas exchange measurements. Our calculations from gas exchange measurements (CER and PIB) should, therefore, relate well to the labelling data. Total label incorporated in glycine as a function of cotyledon age gave a pattern similar to that observed for light CER (data not shown). There was a general correlation among the highest values and the lowest values of light CER, total dpm incorporated in glycine, and relative PIB, although there was no distinct relationship.

The percent of the total label incorporated found in glycine (gly % total) was relatively constant from 4 days through 12 to 14 days (Fig. 11). Gly % total then decreased and did not begin to increase above the value at decapitation (18 days) until 6 days later. Gly % total increased throughout rejuvenation, reaching a new, higher steady state value by 26 days after epicotyl removal. There is nothing really striking about the labelling pattern which distinguishes cotyledons at 6 days after epicotyl removal (no PIB, Fig. 9) from those 4 days later (PIB, Fig. 9). Gly % total should be indicative of the rate of photorespiration as long as non-RubisCO carboxylation does not contribute significantly to total $^{14}$C incorporated. It is a better index of photorespiration than the PIB because the PIB decreases below detectable levels during senescence and early rejuvenation. Incorporation of $^{14}$C into glycine throughout the period
Fig. 11. The percent of total $^{14}$C incorporated found in glycine in presenescent, senescing, and rejuvenated soybean cotyledons. Symbols are as described in Fig. 1.
in which a PIB is not detectable shows that photorespiration must be occurring during this time.

Because light CER is also changing during times that the gly % total is changing (Figs. 5 and 11), we examined gly % total as a function of light CER (Fig. 12). Examination of these data shows that there is not a constant relationship between the two variables. In presenescent cotyledons, the gly % total appears to be fairly constant over greatly changing light CER. This represents a period of fairly constant photorespiration since total dpm in glycine and light CER must be changing at the same relative rate. In senescing and early rejuvenating cotyledons, the gly % of total is changing as a function of light CER, so that the rate of photorespiration is changing mostly because light CER is changing. In late rejuvenated cotyledons, the gly % total appears relatively unrelated to light CER. Similar CERs result in increasing gly % total. The relative rate of photorespiration must be increasing, perhaps to a level higher than that seen in presenescent cotyledons.
Fig. 12. The percent of total $^{14}$C incorporated found in glycine as a function of light CER in presenescent, senescing, and rejuvenated soybean cotyledons. Presenescent cotyledons (●); senescing cotyledons (▲); rejuvenated cotyledons (▲); late rejuvenated cotyledons (□)
DISCUSSION

Gas Exchange

In agreement with our earlier study (see Part I), we observed an accumulation of chlorophyll in rejuvenated cotyledons that was greater than the maximal level observed in presenescent tissue. Among other studies using rejuvenated soybean cotyledons, only Peterman and Siedow (1985) also reported excess chlorophyll accumulation in rejuvenated tissue. They did not measure CER or RubisCO content. Excess chlorophyll accumulation has also been observed in other systems of rejuvenated plant tissue (for example, Chang and Beevers, 1973), but the mechanism responsible is not known. Cytokinins are known to be involved in chloroplast development by affecting, among other things, chlorophyll a/b synthesis (Akoyunoglou and Singer, 1986). Application of endogenous cytokinins is also known to maintain chlorophyll during senescence of detached leaves and leaf discs (Thimann, 1980).

Cytokinins appear to be synthesized in roots. Heindl et al. (1982) showed that cytokinins were present in the xylem sap of field-grown and growth-chamber-grown soybeans decapitated just below the unifoliolate leaf node. Cytokinins were shown to accumulate in primary leaves of decapitated and disbudded Phaseolus vulgaris plants to levels 5x those seen in controls (Palmer et al., 1981). Based on the observations in the Phaseolus vulgaris system, we would expect the cotyledons of decapitated soybeans to accumulate cytokinins. The excess chlorophyll accumulation we observed in rejuvenated cotyledons may be controlled in some way by cytokinins.
Also in agreement with our earlier study (see Part I), we observed a close relationship between light CER and total chlorophyll in presenescent, senescing, and rejuvenating cotyledons. The excess chlorophyll accumulated in the rejuvenated cotyledons, however, did not result in a corresponding increase in CER. To further characterize this differential response in rejuvenated cotyledons, we used rocket immunoelectrophoresis to quantify RubisCO.

Makino et al. (1983) observed a close correlation between RubisCO content, as determined by rocket immunoelectrophoresis, and light CER in rice leaves from development through senescence. They also observed a constant specific activity for RubisCO throughout the same time period. Friedrich and Huffaker (1980) observed a high correlation between RubisCO content, as determined by immunoprecipitation, and light CER in senescing barley leaves. In contrast with Makino et al. (1983), Friedrich and Huffaker (1980) found an increase in the specific activity of RubisCO in late senescence. High correlation between light CER and RubisCo content was also observed in systems not used to study senescence, such as the series of tobacco mutants described by Radunz and Schmid (1987). The mutants contained very different levels of chlorophyll. Edwards et al. (1988) found that in a series of chlorophyll fluorescence mutants of maize, photosynthetic activity closely paralleled RubisCO concentration. We were not surprised, therefore, to find that over most of the developmental sequence of our system, CER was a function of RubisCO concentration. In the rejuvenated cotyledons in which
excess chlorophyll accumulation occurred, the lack of a corresponding increase in light CER can be attributed to a relatively constant RubisCO concentration.

There were two periods in this developmental sequence during which the relationship between light CER and RubisCO content was uncoupled or different. The first period included the most senescent cotyledons used during this study (18 days) and those from the first two days of rejuvenation. Equivalent amounts of RubisCO were measured in these cotyledons, yet CER increased by 60% by the first day after epicotyl removal. This rapid increase may reflect the release of some stomatal limitation imposed by senescence (Thimann, 1987), since transpiration also increased rapidly by the first day of rejuvenation. Friedrich and Huffaker (1980) found a close correlation between CER and stomatal aperture and transpiration in their system, in addition to the high correlation between CER and RubisCO content. The response time of the transpiration/stomatal aperture effect was much more rapid than that of any other parameter they measured.

The second time frame in which we saw a lack of correlation between light CER and RubisCO concentration was early development, in 4-day and 6-day old cotyledons. In contrast with cotyledons of other ages, the 4-day and 6-day old cotyledons had excess RubisCO present for the light CER which we measured. Soybeans are one of a number of commercially important oil seeds. On a dry weight basis, the cotyledons contain from 13% to 24% lipid and from 36% to 38% protein (Trelease and Doman, 1984), most of which is mobilized during
germination to support the developing seedling. We did not measure lipid in our experiments. Based on changes that we observed in total protein and dry weight in comparison with changes seen by Brown and Huber (1987a) who measured dry weight, protein, lipid, various carbohydrates, and other parameters of reserve mobilization, we would expect that our cotyledons were functioning as storage organs until day 8. Mobilization of protein and lipid and the resultant gluconeogenesis would presumably occur in the light. Brown and Huber (1987b) found that sucrose phosphate synthase activity in 7-day old soybean cotyledons decreased by only 19% during the day when compared to activity levels in the night. The carbohydrate accumulation that could result from gluconeogenesis (Brown and Huber, 1987b, found the highest levels of starch in 3-day through 7-day old cotyledons) may inhibit photosynthesis (Azcon-Bieto, 1984).

Gluconeogenesis has been best characterized in germinating castor beans (Beevers, 1979). The storage tissue in castor bean is the endosperm, which does not emerge and become green. Among plants that have cotyledons that function both as storage and photosynthetic organs, like soybean, research has been focused on the glyoxysome to peroxisome transition. Much work has been done with cucumber (Titus and Becker, 1985; Walden and Leaver, 1981; Becker et al., 1978) characterizing the transition. But no study that we are aware of has measured photosynthesis, respiration, RubisCO content, and other necessary parameters to determine the relationship between gluconeogenic metabolism and photosynthesis.
Some reserve mobilization is necessary for the establishment of soybean seedlings because a loss of the cotyledons from emergence until the primary leaves are unfolding reduces the growth rate of the seedling enough to cause yield reductions of 8 to 9% (Ritchie et al., 1985). This information does not distinguish between nutrient contributions from reserve mobilization and from photosynthesis. Rubisco (as determined by immunoprecipitation) and the peroxisomal enzymes hydroxypropiouate reductase and serine:glyoxylate aminotransferase occur at low levels in dark grown cucumber cotyledons (Becker et al., 1978; Titus and Becker, 1985). Accumulation of the enzymes is stimulated by light and coincides with an increase in fresh weight. Loss of the glyoxysomal enzymes malate synthase and isocitrate lyase is delayed in the dark. Light and the development of photosynthetic capacity shorten the storage function of the cotyledon and speed up the overall growth pattern. Previous studies on photosynthesis in soybean cotyledons concluded that cotyledon photosynthesis did not play much of a role in seedling establishment (Abrahamsen and Mayer, 1967; Harris et al., 1986a, 1986c). Because of the low light used in those studies, cotyledon photosynthesis probably did not contribute much, and the seedlings were established primarily through reserve mobilization. Those studies were not carried through to a yield determination. It would be of interest to see if a delay in seedling development (as determined by seedling age at emergence, unifoliolate leaf unfolding, cotyledon senescence, etc.) caused by lower light intensities would result in yield reduction similar to that
caused by cotyledon loss.

Our earlier study demonstrated that soybean cotyledons can have net photosynthesis rates in the range reported for true leaves (see Part 1). We also observed a typical C₃ leaf response to an abrupt light to dark transition: the PIB. Decker first reported the existence of the PIB (1955, 1959). After some controversy, it is now generally accepted that the PIB represents a remnant of photorespiration (Canvin, 1979). Attempts have been made to devise a generally applicable means of quantifying photorespiration from the PIB (Bulley and Tregunna, 1971; Doehlert et al., 1979; Peterson, 1983, 1987). Quantification is complicated by the fact that the PIB represents a non-steady-state process and because the shape and timing of the burst depend on the resolution of the system and the IRGA measuring cell (Bulley and Tregunna, 1971; Canvin, 1979). In addition, as with all measurements of photorespiration, calculations made from the PIB will underestimate photorespiration because there is no determination of the amount of photorespired CO₂ that would be refixed. Nevertheless, within a particular system, calculations from the PIB should provide a comparable estimate of photorespiration (Canvin, 1979; Sharkey, 1985).

In our system, examination of the PIB indicated that in rejuvenated tissue light CER recovered to maximal presenescent levels before the PIB did. The lack of a clear, general relationship between the PIB and light CER is shown in Fig. 10, and is in contrast to the relationship obtained by Bulley and Tregunna (1971). In their study,
they varied CER by changing the light intensity and were therefore making their PIB estimates from the same soybean leaf. In our system different light CERs came from plants in different developmental states. In addition to seeing differences in the relative magnitude of the burst (dark CER_{max} - dark CER_{min}), the shape of the PIB was different throughout this time frame, reflecting perhaps differences in pool sizes as well as differences in pool turnover rate. Dark CER_{min} was also changing throughout this time frame, making the PIB calculations in the presenescent cotyledons uncertain, because it is unclear how much dark respiration persists in the light (Dry and Wiskich, 1987).

To get another, independent estimate of photorespiration with which to compare the PIB, we labelled cotyledons with ^14CO_2 during photosynthesis. Because the CO_2 released during photorespiration is derived primarily from glycine (Tolbert, 1981), we followed the labelling pattern in glycine in germinating, presenescent, senescing, and rejuvenating cotyledons. The PIB was not detectable in 4-day old cotyledons nor in late senescent and early rejuvenating cotyledons. Incorporation of ^14C indicated, however, that photorespiration was occurring in these cotyledons. Although the appearance of a PIB is indicative of photorespiration, the lack of a PIB does not mean that photorespiration is not occurring.

There was nothing striking in the labelling data that would indicate why there appears to be a delay during rejuvenation in the recovery of the PIB as compared with light CER. A relative delay in
recovery of metabolic processing capacity for glycine could result in an accumulation of label in glycine when the PIB was not detectable. We did not observe this. We did not make determinations of intermediates in the pathway prior to glycine, so a blockage in metabolism at earlier steps would not be detected. Based on the work done with *Arabidopsis thaliana* photorespiration mutants (Somerville and Ogren, 1982) and with photorespiration mutants of barley (Hall et al., 1987), an accumulation of carbon in intermediates of the photorespiratory pathway would be expected to inhibit light CER. Light CER was recovering during the time that no PIB was detectable, so we do not feel that metabolism of carbon in the photorespiratory pathway was a limiting factor.

In an absolute sense, detection of the PIB is limited by the capacity of the plant system to refix photorespired CO₂, a function which would depend on the relative pool sizes of glycine and ribulose-1,5-bisphosphate (RuBP) and the photorespiration rate (Peterson, 1987). Practically, detection of the PIB is limited by the physical characteristics and sensitivity of the gas exchange system. The proportion of recently fixed carbon that is partitioned into glycolate pathway intermediates depends on the rate of photorespiration. The total glycine pool in soybean leaf discs is approximately 3x greater in 21% O₂ than in 2% O₂ (Creach and Stewart, 1982; Hitz and Stewart, 1980). The RuBP pool decreases by the same percentage that the glycine pool increases. In 2% O₂ there was no detectable PIB and the glycine and RuBP pools were equal (Creach and Stewart, 1982).
The glycine pool in corn, a C₄ plant without external manifestations of photorespiration, is O₂ dependent and is 3x larger than that found in soybean (Marek and Stewart, 1983); thus the absolute value of the total glycine pool cannot be used to estimate photorespiration. However, metabolism of the glycine pool in the dark was 30x faster in soybean than in corn (Hitz and Stewart, 1979; Marek and Stewart, 1983). In relatively short-term labelling experiments, therefore, we expected that changes in the labelling pattern in glycine would reflect differences in the photorespiration rate. We found that the percent of the total label incorporated by the cotyledons that was found in glycine changed significantly over the developmental series studied. Because light CER was also changing over this time span, we examined gly % total as a function of light CER. It appears that in the presenescent cotyledons, the rate of photorespiration is fairly constant, although light CER and the size of the glycine pool and the shape of the PIB change dramatically. In senescing cotyledons the rate of photorespiration is decreasing, but that is because light CER is decreasing. During rejuvenation, the rate of photorespiration increases as CER increases. During late rejuvenation the rate of photorespiration again appears to be relatively constant at a level higher than that seen in presenescent cotyledons. Our observation that the recovery of the PIB is delayed as compared to light CER depends upon the comparison with the relationship between light CER and PIB in presenescent cotyledons. The data show that photorespiration, and thus the relationship between
the PIB and light CER, is not constant. The senescing and rejuvenating cotyledons appear to represent very different states that are not directly comparable.
SUMMARY

The data presented in our first paper show that soybean cotyledons can achieve rates of net photosynthesis equivalent to rates seen for true leaves. Light CER was greater than dark CER throughout most of the developmental sequence studied, indicating that soybean cotyledon photosynthesis provides for more than replacement of carbon respired in the dark and, therefore, does contribute to seedling establishment. We followed carbon dioxide exchange rates in germinating, presenescent, senescing and rejuvenating cotyledons and determined that rejuvenated cotyledons exhibit light CER equivalent to the maximal rates seen in presenescent cotyledons.

In other aspects of cotyledon physiology, however, we did observe significant differences between rejuvenated and presenescent soybean cotyledons. One difference that we observed was an accumulation of chlorophyll in rejuvenated cotyledons greater than the maximal amount seen in presenescent cotyledons. A second difference was the observation that during rejuvenation, the PIB, a qualitative indication of photorespiration, appeared to recover more slowly than light CER when compared with presenescent but equivalent light CERs. The data presented in our second paper are the results of further investigation of these apparent anomalies in rejuvenated cotyledons.

We measured the amount of RubisCO and found that light CER was a function of RubisCO during most of the developmental sequence studied. Deviations from the general relationship between light CER and RubisCO were seen during the earliest times of rejuvenation and in 4-day and
6-day old cotyledons. Light CER increased immediately after rejuvenation, independently of the RubisCO content, probably as a result of the release of stomatal limitations to cotyledon gas exchange. In 4-day and 6-day old cotyledons, light CER was much lower than expected based on the amount of RubisCO present. Light CER may have been limited by carbohydrate build-up due to the concurrent processes of gluconeogenesis.

To investigate whether or not the differences observed in the PIB relative to light CER in senescenting and rejuvenated cotyledons reflected differences in photorespiration, we labeled cotyledons with $^{14}$CO$_2$ during photosynthesis. Assuming that the percent of the total label incorporated that accumulated in glycine represents an independent indication of photorespiration, equivalent light CERs in rejuvenating and senescing cotyledons resulted in approximately equal photorespiration rates. Later in rejuvenation, however, the cotyledons appeared to have photorespiration rates greater than those seen in presenescent cotyledons. The photorespiration rate was changing in senescing and rejuvenating cotyledons and the changing rate was responsible for the different PIB/CER relationships observed. In conclusion, use of comparisons between presenescent, senescing, and rejuvenated cotyledons as a model system to detect factors specific to the senescing state is complicated by the changing physiological background of the cotyledons.
BIBLIOGRAPHY


