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LIGHT INDUCED OXYGEN UPTAKE IN WHEAT: ROLE OF ETIOPLASTS AND MITOCHONDRIA

by

Thomas Eugene Redlinger

A Dissertation Submitted to the Faculty of the COMMITTEE ON GENETICS (GRADUATE) In Partial Fulfillment of the Requirements For the Degree of DOCTOR OF PHILOSOPHY In the Graduate College THE UNIVERSITY OF ARIZONA 1976
I hereby recommend that this dissertation prepared under my
direction by Thomas Eugene Redlinger
entitled Light Induced Oxygen Uptake in Wheat: Role
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ABSTRACT

Photoinduced oxygen uptake has been studied in vitro with etioplasts and mitochondria extracted from dark-grown *Triticum aestivum*. The present study traces the biological origin of this light mediated phenomenon to etioplasts and also presents evidence that mitochondria enhance oxygen consumption in etioplasts under light conditions. The first part of the study demonstrates that etioplasts are responsible for increased oxygen uptake when illuminated. Since etioplasts could not be completely separated from mitochondria, this conclusion was deduced on the basis of the following experiments. The photoinduced oxygen response was observed only when organelles were isolated from dark-grown plant tissue that normally turns green, e.g., primary leaves or cotyledons. These selective tissue experiments indicated that etioplasts and not mitochondria were the sites of the measured light induced metabolic behavior. Inhibitor studies utilizing potassium cyanide (KCN) confirmed this. KCN effectively inhibited mitochondrial respiration, but had little or no effect on light induced oxygen uptake.

Further investigation into the nature of the light phenomenon revealed the involvement of a receptor pigment, the absorption qualities of which resulted in maximum oxygen utilization between 640 and 680 nm. Results of this action
spectrum narrowed pigment possibilities to two putative candidates, phytochrome and protochlorophyllide-650 (PChle-650). Negative results from experiments designed to test the participation of phytochrome suggested that the photoinduced oxygen response was associated with PChle-650. To confirm the involvement of PChle-650 additional experiments were performed analyzing photoconversion of PChle-650 to Chle-678 as well as PChle-650 regeneration from PChle-630. A comparison of results from spectrophotometric or pigment measurements on PChle-650 with those obtained under similar conditions from the photoinduced oxygen assay led to the conclusion that PChle-650 was the light receptor pigment molecule.

Mitochondria by themselves do not show a light mediated respiration response but their presence has an indirect effect on the light induced oxygen uptake in etioplasts. Mixing experiments showed that a scutellar mitochondrial fraction, which alone does not increase oxygen uptake in light, could cause increased oxygen consumption when mixed with an etioplast-containing fraction. In addition, it was demonstrated that pure ATP could substitute for the mitochondrial fraction with the same result. Inhibitor studies with atractyloside confirmed that ATP was the effective molecule in the organelle interaction.

A mechanism was delineated which can explain the effect of added ATP on PChle-650 photoconversion. Mitochondria supply ATP through oxidative phosphorylation which is used in
etioplasts for PChle-650 regeneration. In dark-grown leaves, red light stimulation of PChle-650 photoconversion reduces the PChle-650 pool. ATP, which is required for PChle-650 regeneration, when maintained at a high level insures a rapid turnover of PChle-650. This in turn is responsible for the observed high oxygen uptake associated with PChle-650 photo-reduction.
INTRODUCTION

Light is a fundamental physical factor in the life of the plant. A unique association exists between plant pigments and light which is the basis of the photosynthetic process. As a result of this association, light energy is transformed into chemical energy and conserved in the bonds of organic compounds. Plants and animals use this stored chemical energy directly through the process of oxidation to drive essential life processes.

Light and plant pigments also cooperate in regulating certain chemical reactions in plants. One example is the light-mediated reduction of protochlorophyll to form chlorophyll a. Here light energy serves as a control mechanism in the chlorophyll biosynthetic pathway. In this reaction protochlorophyllide, an intermediate pigment in the chlorophyll pathway, is the light receptor for its own conversion. Another example is the light receptor pigment phytochrome which exerts control over chemical reactions as a separate agent. Because phytochrome is not an intermediate compound of a pathway, it is capable of regulating diverse reactions.

The present work focuses on light-mediated chloroplast development emphasizing early biochemical events involved in the greening process. The respective roles of light and pigments controlling chlorophyll biosynthesis are
investigated. Metabolic behavior of isolated etioplasts and mitochondria is measured under various experimental conditions. Evidence for organelle interaction is also presented. How light regulates oxygen uptake in organelles of dark grown plants is the central theme in this dissertation.
LITERATURE REVIEW

The phenomenon of the light-induced oxygen uptake in isolated etioplasts has recently been under observation but the underlying biochemical metabolism has remained enigmatic. Boardman (1976) purified etioplasts by sucrose gradient centrifugation and found that etioplasts when illuminated consume oxygen. However, he offered no explanation for the oxygen uptake. Mohamed-Osman (1973), working with soybean cotyledons, also noted this phenomenon and attributed it to photoinduced mitochondrial respiratory activity. His conclusion was based on electronmicrographs which showed the organelle fraction composed only of mitochondria. It was noted that he did not include any supportive mitochondrial respiratory inhibitor studies. Due to the above conflicting reports as well as the lack of any reported in-depth study on this subject, the present investigation was undertaken in an attempt to discover the unknown origin and nature of the light controlled oxygen uptake.

The investigation of a light mediated reaction must logically begin with a consideration of photoreceptor

1. Etioplasts are yellow chromoplasts found in leaves of dark grown plants. They can be considered immature chloroplasts which have been arrested at a stage just short of complete chlorophyll development. Etioplasts require light for further development
pigments. Since the light mediated reaction was shown to require red light (Mohamed-Osman 1973), two classes of receptor pigments are immediately suspect — phytochrome and the chlorophyll precursor chromophores. Both mitochondria and etioplasts contain phytochrome (Manabe and Furuya 1973; Evans and Smith 1976) while only etioplasts contain chlorophyll precursor pigments (Bogorad 1965). Pertinent literature regarding these two pigment classes are presented in this review under a broader background to include plastogenesis, protochlorophyllide photoconversion, and phytochrome systems.

**Plastogenesis**

The structural changes occurring during the light stimulated development of etioplasts have been studied with the electron and light microscopes and some correlations have been made between subcellular structure and pigment formation. The lamellar structures or grana of mature chloroplasts normally seen in electron micrographs are absent from etioplasts. Instead one observes proplastids containing dense vesicular centers or prolamellar bodies1 (Hodge, McLean and Mercer 1956). More recently it has been shown that conversion of protochlorophyllide-650 to chlorophyllide-684

1. A prolamellar body (PLB) results from the accumulation of membrane products due to the blockage of the light reactions in the normal greening process. The PLB basic structural unit has been shown to consist of a six-pointed star with four tubules uniting at each of the nodes (Weier and Brown 1970).
(see Figure 1) causes no structural change in PLB (Henning-
sen, Thorne and Boardman 1973). However, a further conver-
sion of chlorophyllide to chlorophyll is associated with a
dispersal of PLB membranes (Horton and Leech 1974). Although
chlorophyll cannot be detected in electromicroscopy, it can
be detected in light microscopy by a red fluorescence under
ultraviolet light.

Boardman and Anderson (1964) used phase-contrast
microscopy to study structural changes in developing bean
plastids. They found that upon illumination of dark-grown
plants there was a lag period of about 3 hours before chloro-
phyll could be detected in the plastids. The grana-like
structures were not observed until 6 hours after illumination.
When the development of photochemical activity was investi-
gated (Anderson and Boardman 1964), it was shown that even
after 16 hours of illumination, plastids could reduce NADP
at only half the rate of mature chloroplasts.

At the molecular level it has been demonstrated that
light-induced development of chlorophyll in proplastids re-
quires DNA-dependent RNA synthesis. Bogorad and Jacobson
(1964) used etiolated beans and maize plastids and treated
them with actinomycin-D to show that chlorophyll synthesis
is inhibited. These data imply that the greening process is
dependent upon the light activated synthesis of mRNA. Board-
man (1966a) has shown by a series of experiments that the
location of 70 S ribosomes was in the proplastids and that
Figure 1. The final steps in the chlorophyll biosynthetic pathway: from the light requiring protochlorophyllide conversion to chlorophyll a and b.
the number of 70 S ribosomes per proplastid increased only slightly during the greening process. This observation indicated that the synthesis of 70 S ribosomes was not a light dependent process. Furthermore, together with the findings of Bogorad and Jacobson (1964), viz., the synthesis of mRNA occurred in light-induced etioplasts, the suggestion was that the resulting mRNA was translated by the existing 70 S ribosomes of the proplastid. In summary, this evidence indicated that an increase in mRNA and not rRNA would be expected to occur in etioplasts as a result of light stimulus.

Pigment studies have shown that in etioplasts chlorophyll biosynthesis is arrested at the protochlorophyllide-650 (PChle-650) stage. Shibata (1957) has demonstrated spectrophotometrically that PChle-650 of dark-grown bean seedlings upon illumination is transformed into chlorophyllide-684 (Chle-684). This change was observed after a one minute illumination of 1,000 foot candles. Thus the transformation of PChle-650 to Chle-684 was both mediated by light and occurred rapidly, taking not more than one minute. This rapid shift was followed by a slower dark conversion involving a change in Chle-684 to chlorophyll a (Chl-672). The latter pigment change occurs within 10 to 40 minutes following illumination and is referred to as the "Shibata Shift" (Thorne 1971). Figure 1 outlines the final steps in the biosynthesis of chlorophyll.
Protochlorophyllide and Photoconversion

Spectroscopic studies of pigments have shown that protochlorophyllide\(^1\), i.e., PChle-650, upon illumination is transformed into chlorophyllide-684 (Shibata 1957). Furthermore, Smith and Ahrne (1955) have shown that the protochlorophyllide in dark-grown bean seedlings are of two forms, PChle-635 and PChle-650. More recently this has been investigated in numerous plant material and evidence for two forms of protochlorophyllide, PChle-635 and PChle-650, is now accepted (Boardman 1966b; Bogorad 1965; Granick 1967; Kirk 1970). However, only the PChle-650 type is photoconvertible. The nature of this photoactivity and pigment absorption difference has been the subject of much speculation.

Dujardin and Sironval (1970), working with in situ and in vitro PChle holochromes\(^2\) of Phaseolus seedlings, obtained data suggesting that the various forms of protochlorophyllide resulted from aggregated complexes involving

\[1. \text{The major pheoporphyrin pigment in etiolated leaves is protochlorophyllide (PChle). Protochlorophyll (PChl) is also present but in smaller amounts. The ratio of protochlorophyllide to protochlorophyll depends on the age of the seedling (Sironval, Michel-Wolwertz and Madsen 1965; Lancer, Cohen and Schiff 1975). PChl is formed from PChle by the esterification with phytol of the propionic acid side chain at position 7 (Bogorad 1965). In the present study, protochlorophyllide will be used with the understanding that smaller amounts of protochlorophyll are also present.} \]

\[2. \text{In the natural state protochlorophyllide is complexed with protein. When it is extracted with organic solvents it assumes a different chemical state. The term holochrome designates the pigment in its natural state, i.e., a chromophore complexed with protein.} \]
pigment-protein and pigment-pigment interaction. The exact nature of associations comprising the holochrome-protein complex is, however, speculative. One of the more popular notions is to visualize the holochrome protein to which the protochlorophyllide chromophore has been attached as a shuttling photoenzyme (Gassman and Bogorad 1967; Granick and Gassman 1970; Süzer and Sauer 1971). The attachment of the photoenzyme is viewed as necessary for phototransformation and, once converted, the photoenzyme is freed and can attach to another protochromophore.

The phototransformation of the protochlorophyllide holochrome can be defined spectrophotometrically as a shift in absorbance from 650 to 684 nm upon illumination. This is the now classic finding demonstrated by Shibata (1957). In addition, phototransformation can be described in chemical terms by a reduction step involving the addition of two hydrogen atoms at positions 7 and 9 of ring IV of magnesium 2-vinyl pheoporphyrin a5, i.e., protochlorophyllide. This chemical relationship that was established by the analytical and synthetic labors of Fischer (1940) and Fischer and Oestreich (1940) is presented in Figure 2.

The source of the hydrogen atoms involved in the above photoreduction has not yet been elucidated. Rudolph and Bukatsch (1966) have suggested the concomitant photoxidation of ascorbic acid as a reductant serving as the hydrogen donor for PChle-650 photoreduction. In support of
Figure 2. The chemical relationship of protochlorophyllide to chlorophyllide.
this, Kransnovski, Bystorova and Lang (1970) demonstrated the chemical photoreduction of PChl to Chl in solutions of ascorbic acid (0.02 M). An additional possibility is that the holochrome protein, now considered to be a photo reductase (Gassman and Bogorad 1967), is itself the reductant, i.e., the source of the hydrogen atoms. This was deduced from the observation that the PChle complex was self-contained and did not depend on a collision process for reduction (Boardman 1962). However, whatever the source of hydrogen atoms, it must be in agreement with the findings that photoconversion obeys first order kinetics (Thorne and Boardman 1972).

Experimentally, photoreduction of PChle-650 is accomplished by a light exposure of the appropriate duration and intensity. This can be monitored by a shift from 650 to 678 nm. Unfortunately no standardized procedures have been reported in the literature. Granick and Gassman (1970) employed a Zeiss Ukatron microflash Xenon arc tube that delivers 60 w sec of energy per 0.5 millisecond flash. No report of the distance between the flash unit and the sample was given. Other experiments have used less intense light for longer periods. Akoyunoglou and Siegelman (1968) used a 150 w reflector lamp at 30 cm from the sample for 1 min. Lancer, Cohen and Schiff (1976) employed a 30 v locomotive head lamp to produce up to 340 w/m^2 for 30 sec.

In addition to the light factor, a temperature response in the phototransformation of PChle-650 has also been
observed. Smith and Benitez (1953) showed 90% PChl transformation at 20.2 C but only 65% PChle transformation at -40 C. The light intensity in both cases was set at 2000 foot candles for 1 minute. A series of experiments conducted at -70 C inhibited photoconversion to an even greater degree. Thus, the dependency on temperature suggested that PChle-650 phototransformation was not purely a photochemical process.

Another often studied characteristic of PChle has been its recovery rate and conditions necessary for its reappearance after complete photoconversion. A mechanism for PChle-650 regeneration has been put forth by Sundquist (1973) and will be discussed later. Protochlorophyllide-650 reappearance can be detected within only a few minutes after saturating photoconversion. Sequential studies of PChle regeneration have been made by Akoyunoglou and Siegelman (1968), Granick and Gassman (1970) and Lancer et al. (1976). In general, these researchers discovered that in 5-day old etiolated leaves of Phaseolus vulgaris there was a rapid regeneration of protochlorophyllide. On the other hand, by day 6 a lag phase in PChle reappearance was observed which increased with age. By day 15, the lag phase was 180 min and the rate of resynthesis greatly diminished. The underlying mechanism responsible for the lag period is not yet known.
**Phytochrome System**

The pigment phytochrome is a bluish chromoprotein and consists of two forms that are interconvertible by light, $P_r$ (absorption max. 660 nm) and $P_{fr}$ (absorption max. 730 nm). The effector molecule in the phytochrome system is the $P_{fr}$ form which is produced from $P_r$ by a complex reaction when irradiated with red light (Butler, Hendricks and Siegelman 1965).

In dark grown tissue, only the $P_r$ (inactive) form of phytochrome is present. Many photomorphogenetic responses such as plastid development, seed germination, stem and leaf growth, flavonoid synthesis and the appearance of many enzymes must await the transformation of phytochrome into $P_{fr}$, the active form. In addition to the above mentioned $P_{fr}$ photomorphogenic responses, a $P_r$ response is also evident. This can be seen by exposing dark grown seedlings to 24 hours of far-red light and observing histologically the increased degradation of storage protein in mustard cotyledons (Hacker 1967). Furthermore, using histochemical techniques, Mohr (1972) has detected in mustard cotyledons a concomitant de novo synthesis of structural proteins with far-red light treatment. These far-red treated plastids appear similar to normal chloroplasts in size, number and shape. Mohr cited this as further evidence that portions of plastid development were under the control of phytochrome.
Other investigators have concentrated on the biosynthetic pathway of the chlorophylls. It was found that etiolated seedlings when fed δ-amino-levulinic acid (ALA) produce large amounts of protochlorophyllide as well as other chlorophyll precursors (Granick 1959). This discovery led to the notion that the enzymes that converted ALA to protochlorophyllide were rate limiting. Nadler and Granick (1970) investigated the control of chlorophyll synthesis in barley by the use of inhibitors and by feeding seedlings with ALA. Their data suggested a rate limiting enzyme, viz., ALA-synthetase, in the chlorophyll pathway. They also postulated a light mediated activation of stored mRNA's coding for the ALA-synthetase.

Sisler and Klein (1963) showed increases in protochlorophyllide-628 as high as tenfold by incubating dark grown bean leaves in ALA solution. However, the 628 nm form of protochlorophyllide was not photoconverted to chlorophyllide and, as Mohr (1970 and 1972) has shown, the rate of chlorophyll formation was not a function of increasing the protochlorophyllide-628 pool. In a related experiment, Sisler and Klein (1963) obtained data that suggested that chlorophyll synthesized in the presence of ALA undergoes a photodestruction. This reduction in chlorophyll in ALA-treated tissue was also confirmed by Mohr (1972) who maintained that, rather than resulting from photodestruction, the ALA presence induced plastid destruction resulting in loss of chlorophyll.
Investigations aimed at determining if phytochrome was involved in synthesis of protochlorophyll after total photoconversion of PChl to Chl have been conducted by Spruit and Raven (1970). They subjected dark grown maize seedlings to 5 min of red light to photoconvert all PChle to Chle. Subsequent exposures to "red + far red" light indicated that Pfr, the active phytochrome, controlled the rate of synthesis and pool size of photoconvertible PChle. This conclusion has been supported by other investigators using 48 hr dark grown mustard seedlings (Masoner, Unser and Mohr 1972).

A recent review by Zucher (1972) cited several works that indicated light regulation of the activities of a number of enzymes. Stobart and Pinfield (1970) demonstrated a photo-induced increase in enzymatic activity of succinyl coenzyme A synthetase by a fourfold maximum after a 10 hr tissue illumination. Succinyl-CoA is an initial component in porphyrin biosynthesis (see Figure 3). In addition, succinyl-CoA is the active form of succinic acid and an energy rich compound synthesized by reactions involving the substrates of the tricarboxylic acid cycle.

1. In photosynthetic bacteria, α-aminolevulinate (ALA) is formed by the condensation of one molecule each of succinyl-CoA and glycine followed by the loss of CO₂ (Rebeiz and Castelfranco 1973). Two molecules of ALA condense to form the pyrrole, porphobilinogen, which in turn join forming cyclic tetrapyrroles. A number of cyclic tetrapyrroles are intermediates between porphobilinogen and protochlorophyllide. The latter accumulate in etiolated leaves.
Figure 3. Early steps in the biosynthesis of chlorophyll: from the condensation of succinyl-CoA and glycine to the formation of the tetrapyrrole, uroporphyrinogen III.
Other experimenters have suggested that phytochrome regulates mitochondrial respiration. Gordon and Surrey (1958 and 1960) reported red and far-red light effects upon mitochondrial oxidative phosphorylation. Their data on rat liver mitochondria under red light conditions showed increased rates of phosphorylation when compared with dark controls. Furthermore, in agreement with phytochrome regulation, a far-red light inhibited ATP synthesis. However, these findings have been generally discredited. In related light studies, Mohamed-Osman (1973) investigated light effects on respiration in soybean cotyledonary mitochondria. Using dark grown mitochondria preparations, he found that oxygen uptake increased in red light and decreased in far-red light. Although a phytochrome mediated mitochondrial respiratory system was suggested, he did not include any supportive respiratory inhibitor studies.

**Mitochondrial-etioplast Interaction**

The study of compartmentalization of metabolic activity in cells must necessarily precede the study of interactions between organelles within the cell. Investigations aimed at locating metabolic reactions in the cell have depended mainly on techniques developed for organelle isolation and inhibitor or labeling of selected metabolic reactions. Up to the present time, this work has been slow and tedious. Heber (1974) has recently compiled a review of this topic.
Probing exchanges of metabolites between organelles has posed an even greater obstacle. As a result of vicissitudes in experimentation and interpretation of the few results obtained, there has been a dearth of published works in this area.

Bourque and Naylor (1972) studying respiring mitochondria in jack bean (*C. ensiformis*) leaves noted morphological and chemical changes associated with greening. When etiolated leaves were exposed to light to induce chloroplast development, they noticed a concomitant condensation in the ultrastructure of the inner mitochondrial membranes. In addition, a rapid increase was observed in respiratory control for up to 4 hours after the initial light exposure. They viewed the condensed state of the mitochondria as a mechanism for increasing coupling. Although this phenomenon was caused by light, the problem still remained whether light was acting directly on the mitochondria or whether it was being mediated through the etioplast.

A more direct assessment of interaction between mitochondria and chloroplasts in cells has resulted from studies of Chevallier and Douce (1976). They investigated the effects of cyanide and 3,4-dichlorophenyl-1,1-dimethylurea (DCMU) on photosynthesis and respiration of spores of the chlorophyllic moss, *Funaria hydrometrica*. Their results showed that cyanide strongly inhibited dark respiration without affecting photosynthesis at high light intensities (above
saturation plateau value) but stimulated photosynthesis at low light intensities (below saturation plateau level). On the other hand, DCMU inhibited photosynthesis and showed no effect on dark respiration even under light conditions. From these results, they reached the conclusion that the cytochrome oxidase pathway is not functioning under high light intensity unless photosynthesis is inhibited by DCMU. Their work implies a shuttle mechanism across the chloroplast envelope controlling the cytoplasmic ratio of ATP to ADP. Such a system has been discussed by Helld, Sauer and Rapley (1971) in terms of a hypothetical membrane-bound molecule termed the adenylate translocator.

The effects of ATP on the photoconversion of protochlorophyllide in isolated maize etioplasts have been investigated by Horton and Leech (1975). Their evidence indicated that ATP both enabled the holochrome protein to bind to PChle-630 chromophore as well as facilitated additional association (dimer, etc.) of the pigment-protein complex to form PChle-650. They also noted that after primary photoconversion, the addition of ATP stimulated resynthesis of PChle-650. They concluded that maintaining a high ATP concentration is important to prevent the degradation of photoconvertible PChle as well as to stimulate the rapid resynthesis of PChle-650 after photoconversion.
MATERIALS AND METHODS

Source of Biological Material

The majority of experiments performed in the present study employed a soft red Spring wheat (*Triticum aestivum* L. cv. 'Cajeme') as biological material. Wheat seed was of certified quality and obtained from the Arizona Crop Improvement Association, The University of Arizona. A few experiments used maize (*Zea mays*, L.) and soybeans (*Glycine max* L. Merr. cv. 'Kino') both of which were obtained from The University of Arizona collection.

Preparation of Material

Seeds were surface treated with 0.5 % sodium hypochlorite solution for one min, washed with running water for 15 min, and germinated in the dark in shallow pans of moist vermiculite. Germination temperature alternated between 30 C and 20 C on a 12 hr schedule.

At the appropriate age (see Table 1), the etiolated seedlings were harvested under dim green safelight conditions. The safelight was improvised by covering a fluorescent "view box" with eight sheets of green cellophane. Primary leaves were harvested by cutting them above the coleoptile sheath. Root tissue was obtained by first pulling up the entire seedling, washing the roots free from vermiculite and finally
Table 1. The amount and age of various plant tissues employed in organelle extraction.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>g fresh weight</th>
<th>age (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary leaf</td>
<td>3</td>
<td>6-16</td>
</tr>
<tr>
<td>Coleoptile sheath</td>
<td>5</td>
<td>4-6</td>
</tr>
<tr>
<td>Root</td>
<td>4</td>
<td>4-6</td>
</tr>
<tr>
<td>Scutellum</td>
<td>1</td>
<td>1.5</td>
</tr>
</tbody>
</table>
severing the roots from the seedling. The coleoptile sheath
was collected by manually separating it from around the
primary leaf material. Embryonic axes (scutella) were
squeezed out of 36 hr imbibed seeds. All plant parts were
weighed (fresh weight) and immediately chilled in the ap­
propriate extraction medium.

**Organelle Isolation: Mitochondria**

The harvested plant tissue (see Table 1) was cut into
small segments (1 to 2 cm) and placed in a chilled porcelain
mortar along with extraction medium (6 ml/g tissue) and bo­
vine serum albumin (BSA) 0.2% in total volume. The tissue
was gently disrupted for 30 sec and the resulting slurry was
filtered through a single layer of monofilament cloth (4,624
apertures/cm²). All operations were carried out under dim
green safelight and the material was kept at 0 to 4 C.

Mitochondrial isolation was accomplished by differ­
ential centrifugation modified after Bonner (1967). The
method is outlined in the flow diagram in Figure 4. A Sor­
vall RC-2B centrifuge equipped with a SS-34 rotor which ac­
comodated eight 40 ml nylon tubes was used for all prepara­
tions. The extraction medium consisted of 4 mM EDTA, 30 mM
potassium phosphate, 0.5 M sucrose at pH 7.2. The wash medi­
um contained 0.3 M mannitol, 10 mM potassium phosphate at pH
7.2. During the grinding procedure of leaf tissue, the pH
Figure 4. Flow diagram for mitochondrial isolation (after Bonner 1967).
was monitored with narrow range indicator paper and NaOH was used to maintain the pH.

**Organelle Isolation: Etioplasts**

Dark grown primary leaf tissue was used for etioplast isolation since other plant tissue contained few or no etioplasts. The method of cell disruption was the same as that used in the mitochondrial procedure except for the extraction medium which consisted of 0.5 M sucrose, 1 mM MgCl$_2$, BSA 0.2% (w/v) and 0.5 M Tris HCl at pH 8.0. Etioplasts were isolated in their purest form after a procedure modified from Jacobson (1968) outlined in Figure 5.

Although Jacobson's method yields relatively pure etioplast preparations, there is only a 6% recovery of etioplasts as estimated by protochlorophyll analysis. In studies where several hundred samples were required, a more efficient method was needed to avoid using an unnecessarily large quantity of tissue. In an attempt to increase etioplast recovery, Jacobson's procedure was further modified as shown in the flow diagram of Figure 6. This method was found to increase etioplast yields by as much as 50%. Etioplast estimates were based on a β-carotene assay which is described in the final section of Materials and Methods.

**Polarographic Oxygen Assay**

Isolated organelle samples (0.4 to 0.8 mg/ml protein) were suspended in 2 ml of reaction buffer which consisted of
Figure 5. Flow diagram for etioplast isolation (after Jacobson 1968).
Figure 6. Flow diagram for high yielding etioplast isolation.
0.3 M manitol, 10 mM potassium phosphate, 5 mM MgCl₂, 1 mM thiamine diphosphate, 10 mM Tris HCl at pH 7.4 (McDaniel 1969). This suspension was kept on ice until introduced into a reaction vial where it was then magnetically stirred and kept at constant temperature (25°C) by a water jacket. A Clark type electrode was inserted into the reaction vial and the direct current, which is directly proportional to O₂ concentration of the solution, was monitored on an oxygen polarograph (Yellow Springs Instrument Co.).

Respiratory control and ADP:O ratios were determined as outlined by Chance and Williams (1956) and Hagihara (1961). Isolated mitochondria naturally have a low rate of respiration which is referred to here as State 1. If exogenous substrate is added to the system, an increased rate of respiration results and is known as State 2. In the present study, α-ketoglutarate (5 mM) was employed as the exogenous substrate. Subsequent additions of 1 µl adenosine-5’-diphosphate (to make 150 mM in 2 ml of organelle suspension) were used to induce State 3 respiration. State 3 respiration occurs in the presence of excess substrate and ADP. It is characterized by maximum oxygen uptake. When the ADP level is lowered due to phosphorylation to ATP, respiration decreases and mitochondria assume a State 4 rate. Inhibitory studies of mitochondrial respiration employed the following inhibitors and molarities: potassium cyanide (270 µM) and atractyloside (82.5 µM).
Phototransformation Study

Light induced metabolic reaction of organelle isolates was monitored by an assemblage of instruments consisting of an oxygraph, a reaction vial chamber mounted atop a magnetic stirrer, a circulating water bath, a Sargent recorder and a light source (Figure 7A). The light source was a modified 500 watt projector from which light was collimated to pass first through a small hole in a screen, then through a filter set, after which it passed through a water filter before it was allowed to strike the reaction vial (Figure 8). The reaction vial was heat-shielded by 10 cm of circulating water from a 10 liter temperature controlled bath. A pin silicone photo-diode (pin 3D, United Detector Technology, Inc.) was inserted directly into the reaction vial to measure impinging light. Light energy was read in milliamperes and converted to the radiometric unit of ergs/cm²/sec. Desired light intensity was adjusted by means of a rheostat (variac 500, Staco, Inc.) connected to the light source. Figure 7B shows the actual experimental light conditions used to stimulate organelle oxygen uptake.

An action spectrum for light-induced oxygen uptake in isolated organelles was determined by utilizing a set of narrow range interference filters (380 nm to 740 nm) spaced at 20 ± 5 nm (Barnes Engineering Co.) to provide discrete visible spectral wave lengths. Filter transmittance spectra which produced maximum response are given in Figure 9.
Figure 7. Assembly of instruments used in the photoinduced oxygen assay, (A) under room light conditions and (B) under light conditions of assay; see text for description.
Figure 8. Top view of light and filter system used to illuminate sample cuvette.
Figure 9. Narrow range interference filter transmission spectra for those wavelengths crucial to delineating the action spectrum.
Subsequent light experiments employed actinic light of 650 ± 30 nm transmitted by a series of three red plexiglass filters (R-650 Carolina Biological Supply).

In phototransformation experiments no substrate or ADP was added, keeping mitochondria in State 1 respiration. Oxygen uptake was monitored as described above. Figure 10 graphically illustrates the increased oxygen consumption in the presence of light. Initially, three minutes of darkness was given to allow the sample to equilibrate to the reaction temperature (25°C). The dark rate of respiration stabilized within one to two minutes after which recording of O$_2$ concentration began. Three minutes after introduction of the sample, a one minute light exposure (5 x 10$^5$ ergs/cm$^2$/sec) was given. Following a 10 to 15 sec lag, the rate of oxygen utilization roughly doubled. During the one minute period of darkness which followed the light exposure, a 10 to 15 sec carryover of the light reaction rate was observed before a lower dark reaction rate ensued. Two more light/dark cycles were repeated for each sample. Each cycle gave progressively lower oxygen uptake rates.

A method of quantifying the light-induced rate must also include provisions for the dark rate since the light rate is the sum of the O$_2$ consumed in the dark plus the additional increment due to the light effect. Because the dark rate change is not necessarily proportional to the light-induced change, any difference could be compensated for by
Figure 10. Typical polarographic trace recording oxygen depletion as the sample is alternately subjected to one minute of light and darkness.

The sample consists of $0.8 \pm 0.04$ mg organelle protein in 2 ml reaction buffer. An initial three minutes of darkness allows the sample, once it is introduced into the reaction vial, to equilibrate to reaction temperature. A lag period of approximately 10 sec occurs after light perturbation before increased rate of $O_2$ uptake is observed. Likewise, there is a carry-over as the stimulated $O_2$ rate continues for about 10 sec into the dark period. The average L/D value for three cycles is recorded for the sample.
Figure 10. Typical polarographic trace recording oxygen depletion as the sample is alternately subjected to one minute of light and darkness.
dividing the light rate by the dark rate. In the present study these differential rates are expressed as the quotient of light over dark (L/D). Thus L/D values represent a relative increment in O₂ uptake due to light.

\[
\text{L/D value} = \frac{\text{Oxygen consumption in the light/min}}{\text{Av. oxygen consumption in the dark/min (before and after light period)}}
\]

The actual calculation of the dark rate was taken as the mean of the oxygen utilization rates on either side of the light rate. It was assumed that the initial dark rate represented a low rate of respiration due to the presence of mitochondria in State 1 as defined by Chance and Williams (1955). Samples consisted of three light-dark cycles for which separate L/D values could be calculated. The L/D value of each sample was an average of L/D values calculated for each of its three light-dark cycles. For any particular experimental condition at least three samples were each run through three cycles giving a total of nine cycles from which to calculate the final mean L/D value.

**Red and Far-red Light Studies**

Red and far-red light studies required a different arrangement of light equipment from that used for the phototransformation experiments. Two light systems were juxtaposed so that one red, 650 ± 30 nm, and one far-red, 750 ± 30 nm could simultaneously illuminate the sample cuvette. Figure 11 diagrams the paths of light produced by the dual
Figure 11. Top view of dual light and filter systems used to simultaneously illuminate sample cuvette.
system. Standard red and far-red light intensities were both adjusted to a flux of $6 \times 10^5$ ergs/cm$^2$/sec. The dual light source provided a method for investigating sample response to: (1) continuous red light with alternating far-red light and darkness at one min intervals, and (2) continuous far-red light with alternating red light and darkness at one min intervals.

**Mitochondria-etioplast Fraction Purification and Interaction**

Separate preparations of mitochondria and etioplasts were isolated and then combined to determine any interaction that might be present. Mitochondria were isolated from root, scutellar and coleoptile sheath tissue as described earlier. These mitochondrial preparations were tested for respiratory control and for the presence of etioplasts by assaying for the presence of $\beta$-carotene. The $\beta$-carotene assay consisted of homogenizing the final mitochondrial pellet in 10 ml distilled water using a glass homogenizer fitted with a Teflon pestle to lyse the organelle membranes. After centrifugation at 30,000g for 15 min, the clear supernatant was removed and the pellet homogenized in chloroform/methanol (2:1, v/v). Following centrifugation at 30,000g for 15 min the supernatant was analyzed in a Beckman DB-G double beam spectrophotometer for absorbancy at 466 nm.

Of the mitochondrial samples analyzed only the coleoptile sheath contained any $\beta$-carotene. This may have
resulted from the presence of adhering leaf cells after tissue separation. All mitochondrial preparations showed good respiratory control with RC values ranging from 1.5 to 2.5 for 0.40 \pm 0.15 mg protein in 2 ml reaction medium. However, the same preparations did not respond to the light-induced oxygen assay, i.e., they had an L/D value of 1.0. These analyses indicated that the mitochondrial preparations were free of etioplasts (except the coleoptile sheath preparation) and in good respiratory condition.

By selecting plant tissue devoid of etioplasts it was not difficult to obtain relatively pure mitochondrial preparations. In contrast, it is not possible to obtain plant tissue which contain etioplasts and not mitochondria. Leaf, stem, and cotyledonous tissue, which are the primary sources of etioplasts, also contain mitochondria. The problem, then, becomes how to separate the two classes of organelles. The techniques of differential velocity centrifugation either alone or in conjunction with sucrose density gradient (SDG) centrifugation have been used with some success. Purity of the resulting fractions can be determined by electronmicroscopy.

1. Respiratory control (RC) ratios are calculated as the ratio of State 3 mitochondrial respiration to that of State 4. The degree of respiratory control observed indicates the functional capacity of the mitochondria based on the degree of coupling of electron transport and oxidative phosphorylation (McDaniel 1969).
Differential velocity centrifugation for etioplast separation has already been outlined in Figures 5 and 6. The resulting pellets were immediately fixed overnight in a chilled fixing formula consisting of 3% glutaraldehyde and 0.1 M cacodylic acid, pH 7.6. To facilitate sample transfer the washed and fixed samples were suspended in melted agar (1.4%) containing 1.3 M sucrose and 0.1 M potassium phosphate buffer at pH 7.2. The agar was allowed to harden and then was cut into small pieces. The agar cubes were dehydrated stepwise in ethanol and embedded in Epon 812. Sections were post-stained with lead citrate stain for 15 min and examined with a Phillips 200 electronmicroscope. Etioplasts prepared in this manner appear in Figure 12.

The combined techniques of differential velocity centrifugation followed by SDG centrifugation were also used in an attempt to further purify the etioplast fraction. After initial differential velocity centrifugation, the next step was to estimate the density ranges of different classes of organelles in order to apply the SDG centrifugation technique. This was accomplished by using a continuous sucrose gradient from 0.6 M to 1.8 M. The gradients were at pH 7.8 and contained 0.2% BSA (w/v). Centrifugation was carried out in a Sorvall OTD-2 ultracentrifuge using a Beckman SW-27 rotor at 35,000g for 45 min. Fractions were recovered by suspending them in 20 ml extraction medium and centrifuging for 15 min at 20,000g. After approximating the density ranges, a
Figure 12. Electron micrograph of an etioplast fraction.

Etioplasts were isolated from 6-day old primary wheat leaves as outlined in Figure 5. See text for explanation (X 30,100).
discontinuous gradient consisting of 15 small steps from 0.9 M to 1.6 M was set up. Finally, this was reduced to a 5-step gradient where the upper shelf collected material at the 1.02/1.27 M interface and the lower shelf gathered particles at the other end of the range, the 1.54/2.0 M interface. Intermediate density organelles collected at the three middle interfaces. The upper and lower shelf material was collected into fractions and immediately fixed for electron-microscopy. The gradient and resulting fractions are presented in Figure 13.

The technique of differential velocity centrifugation was employed to create enriched fractions. After an initial 5 min spin at 100g, a series of differential centrifugations were conducted and the resulting low and high-spin fractions were collected (see Figure 14). The relative amount of etioplasts recovered in each fraction was estimated by the β-carotene analysis described earlier. In addition, organelle protein concentrations were measured for each fraction by the method of Lowry et al. (1951).

The enriched fractions were used in two series of experiments. The first involved adding adenosine-5'-triphosphate (ATP) at a final concentration of 150 μM to the enriched fractions and analyzing the effect. The second was a mixing experiment designed to detect the presence of organelle interaction. Mixing experiments were conducted according to the scheme shown in Figure 15. The low-spin
Figure 13. Separation of the etioplast fraction by SDG technique showing organelle distribution in electronmicrographs of upper and lower bands.

The techniques are described in the text (X 14,600).
Figure 14. Flow diagram for organelle enrichment experiments.
Figure 15. The scheme for mixing the low-spin fraction with a scutellar mitochondrial fraction and the corresponding analyses.
fraction was mixed with mitochondria and L/D values of this combined fraction were compared with those of a separate but similar low-spin fraction. The high-spin fractions served as controls.
RESULTS

Receptor Pigment Location

Photomorphogenesis is the process of light controlled growth and differentiation. In general, the mechanism involves two components: (a) a receptor pigment that perceives an external light stimulus, and (b) a linked biochemical response culminating in an observed change. In an attempt to determine the location of the receptor pigment responsible for the light induced phenomenon, organelle samples from root, scutella, coleotile and primary leaf tissues were assayed. Organelles were extracted from plant tissues as outlined in Figures 4 and 6. The resulting samples were then assayed for photoinduced O₂ uptake as well as for organelle protein. Table 2 shows that only preparations extracted from dark-grown primary leaf tissue responded to the O₂ assay even though all samples contained approximately the same quantity of organelle protein. This indicated that the etiolated leaf tissue must possess some unique property. An examination of the tissues shown in Table 2 reveals that only leaf tissue turns green when exposed to light. This is due to the presence of etioplasts in leaf tissue. Furthermore, this suggested that the light receptor pigment was located in the etioplast.
Table 2. L/D values and protein concentrations for various dark-grown wheat tissues.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>L/D value(^b)</th>
<th>Organellar protein (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary leaf</td>
<td>3.60 ± 0.15</td>
<td>0.40 ± 0.04</td>
</tr>
<tr>
<td>Coleoptile sheath</td>
<td>1.00 ± 0.10</td>
<td>0.38 ± 0.04</td>
</tr>
<tr>
<td>Scutellum</td>
<td>1.00 ± 0.00</td>
<td>0.41 ± 0.03</td>
</tr>
<tr>
<td>Root</td>
<td>1.00 ± 0.00</td>
<td>0.36 ± 0.03</td>
</tr>
</tbody>
</table>

a. Age of tissue: 6 days except scutellum (36 hrs).

b. Each data value represents the mean of three replicates.
Additional assays were made using two other plant species to verify that the light phenomenon was not peculiar to wheat. L/D values comparable to those of wheat were found for both maize leaves and soybean cotyledons. L/D values of these three plants ranged from 2.5 to 3.8 for organelle protein concentrations of from 0.4 to 0.5 mg/ml. This finding indicated that the light induced \( \text{O}_2 \) uptake occurs both among monocots and dicots as well as in \( \text{C}_3 \) and \( \text{C}_4 \) type plants.

**Experimental Parameters Affecting the Light Reaction**

Age studies on 6-day to 32-day old wheat primary leaf tissue demonstrated that when samples are adjusted on a mg/ml protein concentration basis similar L/D values are obtained. Figure 16 relates differences in age observed in L/D value versus gram fresh weight plots. This relationship was linear under light intensity of \( 5 \times 10^5 \) erg/cm\(^2\)/sec for all aged tissue tested; however, there was a disproportionate relationship between the two variables when old tissue (32-days) was contrasted with young tissue (16-days). Six-day tissue gave a similar response to that of 16-day tissue. Figure 17 contrasts the variables, gram fresh weight and organelle protein concentration. This data suggested that the differences between young and older tissue was due to a reduction in quantity of organelle protein in older tissue. Finally, Figure 18 shows that age of tissue between 6 and 32
Figure 16. Effect of initial harvested tissue in gram fresh weight on L/D values.

Three ages of tissue were tested: 32, 16, and 6 day old. Each data point (• △ • ) represents the mean of three sample replicates.
Figure 17. Effect of initial tissue in gram fresh weight on organelle protein concentration (mg/ml).

Data for two tissue ages (32- and 16-day) are shown. Six day tissue was not significantly different from 16-day tissue (data not shown). Each data point (● ●) represents the mean of three sample replicates.
Figure 18. Effect of organelle protein (concentration) on L/D values.

Data points are from both 16- and 32-day old tissue. Each data point (•) represents the mean of three sample replicates.
days is not a factor in determining L/D values if samples are adjusted on a mg/ml protein basis.

In addition to the above mentioned quantitative organelle effect, other parameters affecting L/D values were examined. The first of these was the observation that light intensity markedly affected the induced oxygen consumption rate. The light intensity studies utilized effective electromagnetic radiation ranging from $10^5$ to $6 \times 10^6$ ergs/cm\(^2\)/sec. The sample material for the light intensity curve study consisted of 3 grams fresh weight cuttings of 7 to 9-day old primary wheat leaves. The in vitro suspension of organelle protein was $0.8 \pm 0.04$ mg in 2 ml of reaction medium. Findings from these experiments are presented in Figure 19 and indicated that as a consequence of increased light flux there was a commensurate increase in L/D values. The method of least squares was used to approximate a line that best fit the data. It was noteworthy that the reaction was not saturated by light even at very high flux ($6 \times 10^6$ ergs/cm\(^2\)/sec). On the other hand, if light intensity was below $10^5$ ergs/cm\(^2\)/sec, no light response was observed. This indicated that either the oxygen assay was not sensitive enough to measure the reduced photoinduced effect or the light effect was absent.

Light quality studies also decisively affected the calculated L/D values. The capability of different wave lengths to induce increased $O_2$ uptake was tested over the visible
Figure 19. The effect of light intensity on L/D values of organelle extracts from 7- to 9-day old primary leaf tissue.

Samples (0.8 ± 0.04 mg organelle protein) suspended in 2 ml reaction medium were exposed to red light, 650 ± 20 nm, of varying intensities. Light flux was measured as described in the text. Each data point (●) represents the mean of three sample replicates.
Figure 19. The effect of light intensity on L/D values of organelle extracts from 7- to 9-day old primary leaf tissue.
range. A set of narrow range interference filters was used to produce essentially monochromatic light in 20 ± 5 nm steps covering a range from 400 to 720 nm. Samples used in determining the absorption spectrum were of the same age and quantity as those used in the light intensity experiment. Light flux was set at $3 \times 10^5$ ergs/cm$^2$/sec. Figure 20 illustrates the observed differential response in the oxygen assay to wave lengths over the visible spectrum. Wave lengths between 640 and 680 nm resulted in maximum response, whereas wave lengths above 720 nm and below 580 nm elicited the same response as complete darkness.

In Vivo Preharvest Light Exposure

The effect of light on 7 to 9-day old wheat seedlings prior to tissue harvest was also investigated. Experiments were designed in which dark grown seedlings were to receive light exposures of $5 \times 10^5$ ergs/cm$^2$/sec for 2, 5, 12, 20, and 30 minutes. Immediately after the exposures, 3 gram fresh weight samples were taken and promptly chilled in extraction medium at 4 C. Organelle extraction was carried out as described above and consisted of $0.8 \pm 0.04$ mg organelle protein suspended in 2 ml reaction medium. The light induced oxygen assay was simultaneously executed with red light perturbation of $9.5 \times 10^5$ ergs/cm$^2$/sec as previously described. The decrease in calculated L/D values associated with increased length of preharvest light exposure is
Figure 20. Action spectrum for 7- to 9-day old primary leaf organelle extract.

Samples were prepared the same as those used in the light intensity experiment. Light intensity was $3 \times 10^5$ ergs/cm$^2$/sec. A set of narrow range interference filters provided essentially monochromatic light over the visible range at spacings of $20 \pm 5$ nm. Each data point ($\Delta$) represents the mean of four sample replicates.
Figure 20. Action spectrum for 7- to 9-day old primary leaf organelle extract.
illustrated in Figure 21. These results graphically resemble an asymptotic curve. As can be seen by this distribution, there was a rapid half reduction\(^1\) in the light inducible reaction which occurred within 2 to 3 min. On the other hand, further reduction of the numerical L/D values required progressively longer preharvest light exposure. Finally, after 30 min of light, essentially all photoinducible reaction had disappeared, i.e., dark respiration rates were constant and unaffected by light perturbations.

**Dark Restoration of Light Induced Phenomenon**

The fact that 30 min of light at \(5 \times 10^5\) ergs/cm\(^2\)/sec was sufficient to destroy the light reaction was the basis for a subsequent series of experiments. In these studies, in vivo seedlings were first exposed to 30 min of white light after which they were subjected to dark periods of various duration before harvesting and assaying. In this manner the effect of a dark period on restoration of the photoinducible reaction could be assessed. Samples for these experiments were prepared from wheat seedlings of two different ages. The young tissue (5-day) had the primary leaf still enclosed in the coleoptile sheath, whereas the old tissue (14 to 16-day) contained two 10 to 15 cm leaves. Organelles were

\(^1\) Half reduction is used to designate the time required for the decrease of one half the control L/D value. For example, a half reduction of an L/D = 5 is the time needed to achieve an L/D = 3.
Figure 21. Kinetics of preharvest light exposure (5 x 10^5 ergs/cm^2/sec) on L/D value.

Samples were prepared the same as those used in the light intensity experiment. A 30 min in vivo white light exposure reduced the in vitro light induced O_2 uptake rate to L/D = 1. Each data point (•) represents the mean of three sample replicates.
extracted under conditions described in the previous experiment. In vitro samples contained 0.8 ± 0.04 mg organelle protein in 2 ml reaction medium.

Distinct recovery rates as measured by L/D values were observed for young and old tissue. The data are presented in Figure 22. Young tissue quickly recovered with a half restoration\(^1\) of 10 to 15 min at 25 C. Further recovery required progressively longer preharvest dark exposures until the L/D values approximated that of the dark control. For young tissue, this occurred after 2 to 3 hrs at 25 C. When older tissue was used, a much slower recovery was observed. In the latter case, half restoration of the photo-induced \(O_2\) assay now required from 2 to 3 hrs at 25 C. Recovery approaching that of the dark control level was achieved only after 45 to 48 hrs at 25 C.

**Low Temperature Studies**

An attempt was also made to determine the effect of temperature on the rate of dark recovery after a 30 min light exposure. The experimental procedure was similar to that described above for dark restoration except the temperature during the dark period was lowered from 25 C to 5 C. This was accomplished by transferring seedlings to a cold room where temperature was maintained at 5 ± 2 C. Only older

---

1. Half restoration is used to designate the time required for the increase from L/D = 1 to one half the control L/D value.
Figure 22. Kinetics of the photoinduced O₂ assay during dark restoration.

Samples prepared from 5-day tissue and from 14- to 16-day tissue both consisted of 0.4 ± 0.04 mg organelle protein/ml. Thirty min of light (Figure 21) was applied in vivo and the time course of dark recovery was assayed in vitro periodically. Seedlings (14- to 16-day) were given dark recovery periods under two temperature regimes: 25 C and 4 ± 2 C. Five-day seedlings were tested only at 25 C. Each data point (o • Δ) represents the mean of two to three sample replicates.
Figure 22. Kinetics of the photoinduced O$_2$ assay during dark restoration.
leaf tissue (14 to 16-day) was utilized in the cold temperature experiments. Young tissue (5-day) was not used because the very rapid half restoration rates make the detection of temperature differences extremely difficult.

Figure 22 shows that recovery is indeed temperature dependent. There was a marked increase in duration of the dark period at 5 C required for half restoration over that needed at 25 C. The lowered recovery rate at low temperature conditions indicated that preharvest dark restoration was due to an enzyme-limited biosynthesis of a light reaction substrate or a change in the state of the enzyme or enzyme-substrate complex.

**Red and Far-red Light Studies**

The action spectrum in Figure 20 demonstrates that the quality of light affecting the photochemical response is red light, 660 ± 20 nm. The question then arises concerning the role of far-red light in the above response. Is the reaction photoreversible? In an attempt to answer this question samples were illuminated alternately with red and far-red light with one minute perturbations. Sample preparation was the same as for organelle suspensions used in the action spectrum experiments. Light flux was $6 \times 10^5$ ergs/cm$^2$/sec. Results indicated that the effect of far-red light was not different from that of darkness or light below 600 nm. This was in agreement with the action spectrum data.
Further investigations into the possibility of a photoreversibility phenomenon utilized the dual light source described in Materials and Methods (Figure 11). Samples for this experiment were the same as those described above. In the first of two experiments, the sample cuvette was illuminated with continuous red light while a second far-red light source provided one minute far-red light exposures alternating with one minute of darkness. A second experiment was the reverse of the first one: continuous far-red light exposed the sample cuvette with alternating one minute red light-dark exposure.

The results of the above experiments were similar. In the first case, continuous red light resulted in a high rate of oxygen uptake in the samples. However, simultaneous far-red and dark perturbation showed no effect on the red light influenced rate of \( O_2 \) utilization. In the second situation, continuous far-red light initially gave a low \( O_2 \) utilization rate. When red followed by dark perturbations began, the L/D values increased and decreased in the predicted manner. Both experiments indicated that the effect of far-red light does not differ from that of darkness on \( O_2 \) uptake of organelle suspensions.

**Respiratory Inhibitor Studies**

Inhibitors of mitochondrial respiration were used to minimize the effect of mitochondria present in the sample
extract. Experiments were devised to analyze the effect of different inhibitors with regard to changes in \( \text{O}_2 \) uptake during both light and dark exposures. Inhibitors used were of two types: (1) inhibitor of cytochrome \( c \) oxidase, potassium cyanide (KCN), and (2) inhibitor of phosphorylation of added ADP, potassium atractylate. Mitochondria prepared from 36 hour wheat scutella were utilized in calculating effective inhibitor concentration as well as the percent of inhibition. Inhibitors, maximum effective concentrations, and percent inhibition are presented in Table 3.

Samples for the light studies were prepared in the usual manner and consisted of \( 0.8 \pm 0.04 \) mg organelle protein in 2 ml reaction medium. Light exposures were set at \( 6 \times 10^5 \) ergs/cm\(^2\)/sec. In all cases, an excess of alpha-ketoglutarate was first added followed by a small quantity of adenosine diphosphate (100 \( \mu \)M) to check for respiratory control. After this initial cycle, the mitochondria had returned to State 4 respiration and were now ready to be used in testing for different combinations of inhibitor and light conditions.

The effect of KCN concentration on a mitochondrial preparation is shown in Figure 23(a). Maximum inhibition of State 3 respiration (91.2\%) was achieved by the addition of 3 \( \mu l \) KCN to make 270 \( \mu \text{M} \) (in 2 ml). Figure 23(b and c) illustrate the effect of KCN when applied to a preparation containing both mitochondria and etioplasts. KCN was applied
Table 3. The effect of metabolic inhibitors on State 3 respiration of wheat scutellar mitochondria.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor conc. (µM)</th>
<th>nM O₂/mg N/min. - inhibitor</th>
<th>nM O₂/mg N/min. + inhibitor</th>
<th>percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCN</td>
<td>270</td>
<td>64.0</td>
<td>5.6</td>
<td>91.2</td>
</tr>
<tr>
<td>atracyloside</td>
<td>82.5</td>
<td>88.0</td>
<td>38.0</td>
<td>56.8</td>
</tr>
</tbody>
</table>
Figure 23. Oxygraph traces of the effect of potassium cyanide on organelle oxygen uptake.

Organelles were isolated as described in text from (a) 36 hr wheat scutella, (b and c) 16-day wheat primary leaves. One µl increments of KCN were added until maximum inhibition was achieved. Three µl KCN (270 µM) resulted in maximum inhibition.
during a dark State 3 cycle (Figure 23b) and during a light State 3 cycle (Figure 23c). When applied during the dark State 3 cycle, KCN affected $O_2$ uptake in a manner similar to that for pure mitochondrial preparations, i.e., $30/34$ or $88\%$ inhibition versus $91\%$. On the other hand, when applied during a light State 3 cycle, KCN only reduced oxygen uptake rate by approximately $34/90$ or $37\%$. Furthermore, in both cases light applied after KCN inhibition increased $O_2$ consumption by the expected amount. The above results suggested that KCN was not effective as an inhibitor of light induced $O_2$ uptake.

Figure 24(a) illustrates the effect of various concentrations of atractyloside on a pure mitochondrial sample. Maximum inhibition was attained with 15 µl atractyloside which made a $82.5 \mu M$ solution in 2 ml. Figure 24 (b and c) shows the results of administering atractyloside to a combined mitochondrial-etioplast fraction. Figure 24(c) shows that when atractyloside was applied during the State 1 dark cycle, there was approximately a $2/9$ or $22\%$ reduction in oxygen uptake. However, as shown in Figure 24(b), a $7/24$ or $29\%$ reduction in oxygen uptake occurred when the inhibitor was added during the light cycle. Thus for atractyloside, an inhibitory dose does not decrease the rate of $O_2$ uptake in light much more than would be expected to account for that portion of $O_2$ uptake loss due to mitochondrial respiratory inhibition. The small difference in inhibition between light
Figure 24. Oxygraph traces of the effect of atractyloside on organelle oxygen uptake.

Organelles were isolated as described in text from (a) 36 hr wheat scutella, (b and c) 16-day wheat primary leaves. Five µl increments of atractyloside were added until maximum inhibition was achieved. Fifteen µl atractyloside (82.5 µM) resulted in maximum inhibition.
and dark cycle additions will be referred to later under Discussion. In neither inhibitory study does the decrease of mitochondrial respiration noticeably affect the rate of light induced oxygen uptake.

**Mitochondrial-etioplast Interaction**

Interaction between two organelles could ideally be verified by first isolating each separately, then mixing the two, and finally measuring any phenomenon that was either not previously detectable in separate preparations or was altered in the combined preparations. Mitochondria can be isolated in a relatively pure form by using root or scutellar tissue which is free from etioplasts (Bonner 1967). On the other hand, etioplasts can only be isolated from cotyledonous or leaf tissue which in addition contain numerous mitochondria. Etioplasts can be separated from mitochondria by differential velocity centrifugation to yield a relatively pure fraction (see Figure 12). However, these fractions still contain some membrane-bound cytoplasmic inclusions each of which includes many mitochondria making the isolation technique inefficient. In an attempt to eliminate mitochondria from the membrane-bound cytoplasmic inclusions, the outer membranes of these as well as of etioplasts were ruptured with a Teflon pestle fitted with a glass homogenizer. Following membrane rupture, sucrose gradient centrifugation was used to separate the liberated mitochondria from the envelopeless etioplasts,
i.e., prolamellar bodies. However, because the density ranges of the liberated mitochondria and envelopeless etioplasts overlapped considerably, pure separation was virtually impossible with SDG centrifugation techniques. Electron micrographs (Figure 14) of fractions collected at the upper and lower ends of the organelle range clearly slow the continued presence of both organelles. Thus since neither centrifugation technique resulted in complete separation, it was decided to use the simpler method of differential velocity centrifugation to achieve enriched fractions.

The flow diagram for organelle enrichment centrifugation was outlined in Figure 14. The resulting fractions were characterized as to the relative content of etioplasts by the previously described β-carotene analysis. Organelle protein concentrations of each fraction were also measured. These results are presented in Table 4 and show that when a combination of 4,000/13,000 centrifugation is used, less than half of the extracted etioplasts, i.e., approximately 45% are recovered in the 4,000 rpm spin. However, approximately equal amounts of organelle protein were measured in both the high and low-spin fractions. The 4,000/13,000 rpm fractions were selected for the mixing experiments to simplify comparisons since both the high and low-spin fractions contained equal organelle protein concentrations. The mixing procedure consisted of combining the 4,000 rpm fraction with a purified mitochondrial preparation (see Figure 15) and determining
Table 4. Relative concentration of etioplasts and organelle protein in the low-spin and the subsequent high-spin fraction.

<table>
<thead>
<tr>
<th>Centrifugation fractions (rpm)</th>
<th>Etioplast ratio(^a) (β-carotene assay)</th>
<th>Organelle protein(^b) ratio (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,000/13,000</td>
<td>0.51</td>
<td>0.69</td>
</tr>
<tr>
<td>4,000/13,000</td>
<td>0.82</td>
<td>1.02</td>
</tr>
<tr>
<td>7,500/13,000</td>
<td>1.30</td>
<td>1.35</td>
</tr>
<tr>
<td>9,500/13,000</td>
<td>1.92</td>
<td>--</td>
</tr>
</tbody>
</table>

\(^a\) Carotenoid content was determined by measuring the absorption at 480 nm. The approximate etioplast ratio of the two fractions was calculated: absorption of low-spin fraction/absorption of high-spin fraction.

\(^b\) Organelle protein was determined after the method of Lowry et al. (1951). The approximate protein ratio of the two fractions was calculated: protein concentration of low-spin fraction/protein concentrations of high-spin fraction.
L/D values. These values were compared with those of a low-spin fraction without the added mitochondria. Organelle protein content of the high-spin fraction served as a control for the mixing experiments.

Table 5 shows the results of mixing the low-spin fraction with a scutellar mitochondrial fraction. A comparison of the low-spin fraction's L/D value (2.72 ± 0.15) with the mixed low-spin plus mitochondrial fraction's L/D value (3.51 ± 0.16) indicates an increased photoresponse due to the added mitochondria which alone do not respond to the photoinduced oxygen assay (L/D = 1.00).

The average oxygen uptake rates under light and dark conditions for the mixing experiments are tabulated for the various fractions in Table 6. These data also confirm that the addition of mitochondria to the etioplast fraction increased oxygen uptake in the light condition. A comparison of the low-spin fraction light total with the mixed light total revealed an increase of approximately 17% in oxygen consumption. Of additional interest was the high-spin fraction that showed a higher L/D value than that of the low-spin fraction. This can be considered the result of the relative organelle constitution of the fraction, i.e., the high-spin fraction contained relatively more etioplasts (Table 4). This was in agreement with earlier observations that higher etioplast content results in increased oxygen uptake (Figure 19).
Table 5. Protein concentrations and L/D values for individual fractions and for the mixed low-spin plus scutellar mitochondrial fraction.

<table>
<thead>
<tr>
<th>Sample:</th>
<th>Primary leaf (4g)</th>
<th>Primary leaf (4g)</th>
<th>Scutella (1g)</th>
<th>Scutella (1g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction^a:</td>
<td>high-spin</td>
<td>low-spin</td>
<td>high-spin</td>
<td>low-spin</td>
</tr>
<tr>
<td>Protein^b:</td>
<td>0.53 ± 0.06</td>
<td>0.55 ± 0.05</td>
<td>0.56 ± 0.07</td>
<td>0.92 ± 0.10</td>
</tr>
<tr>
<td>L/D value:</td>
<td>4.51 ± 0.12</td>
<td>2.72 ± 0.15</td>
<td>4.62 ± 0.14</td>
<td>3.51 ± 0.16</td>
</tr>
</tbody>
</table>

^a. High-spin and low-spin fractions are defined in the text.
^b. Protein and L/D values are the mean of three sample replicates.
Table 6. Average oxygen uptake rates under light and dark conditions of various organelle fractions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Average rate oxygen uptake (nM O₂/mg N/min) in</th>
<th>dark</th>
<th>total</th>
<th>light</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (total fraction)</td>
<td>5.0  2.3  1.3  8.6 a  36.5  21.5  14.0  72.0 a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-spin (4,000)</td>
<td>3.8  2.8  1.7  8.3 a  13.6  10.0  6.0  29.6 b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-spin (13,000)</td>
<td>3.8  1.8  0.8  6.4 b  21.7  12.5  7.8  42.0 c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mix: low-spin + mitochondria</td>
<td>4.0  3.3  2.3  9.4 c  15.0  12.0  9.0  36.0 d</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>4.0  4.0  3.9  11.9 d  0.0  0.0  0.0  0.0 e</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. Fraction preparations were outlined in Figures 6 and 14.

b. The rates of three cycles (see Figure 10) are given followed by the totals. Each one minute cycle was averaged from 3 samples.

c. The average rate of oxygen uptake is calculated: light rate — average dark rate before and after the light rate. The same letter after the totals indicates no significant difference at the 5% level according to the Least Significant Difference Test.
Table 6 further points out a difference between the light and dark oxygen uptake rates. If the light totals of low and high-spin fractions are added, they approximate the total of the control from which they were derived, i.e., $42.0 + 29.6 = 72.0$. This suggested an additive property attributable to the light induced oxygen uptake. On the other hand, the dark totals were not additive.

Additional information can be obtained from the data of Table 6 by comparing the low and high-spin fraction totals. A significant increase in light induced oxygen consumption illicits a proportional decrease in the dark oxygen uptake rate. Furthermore, it can be deduced that the decrease in dark oxygen uptake rate depends on the mitochondrial sample size, i.e., the greater the concentration of mitochondria, the less the decrease in the rate of dark oxygen uptake. This phenomenon is clearly illustrated in the situations involving the low-spin fraction and the combined low-spin plus mitochondrial fraction. In this case, dark respiration rates remained relatively high when compared with those of the low or the high-spin fractions. The above results suggested that the light induced oxygen uptake was utilizing some unknown substance that was also required in the dark respiration. One may interpret such data as a putative competition for some substrate of dual importance. There is a possibility that this substance may be succinyl-CoA.¹
To assess the effect of the added mitochondria in the mixing experiments, a set of substitution experiments was undertaken. These consisted of replacing the mitochondrial fraction in the mixing experiments with pure adenosine-5'-triphosphate (ATP). An ATP concentration of 1.5 mM was found to be saturating and was used throughout this series of experiments. Table 7 compares the results of adding ATP to the etioplast fraction with those of the control (no added ATP). The data totals indicated that the dark oxygen uptake rate was either unaffected or showed only a slight decrease due to the added ATP. On the other hand, in the light condition ATP caused an increased oxygen consumption of approximately 20%. This was in line with increases observed in the mixing experiments where added mitochondria also increased oxygen consumption in light conditions by approximately 17% (see Table 6).

1. As was mentioned earlier, succinyl-CoA along with a molecule of glycine condenses to form delta-amino-levulinic acid (ALA), a Chl precursor. In addition, succinyl-CoA is an intermediate compound in the mitochondrial tricarboxylic acid cycle. During the greening process when there is an accelerated Chl biosynthesis, the etioplasts utilize rapidly succinyl-CoA. It is conceivable that the competition between the etioplasts and mitochondria for succinyl-CoA now shifts in favor of the etioplasts. This hypothesis would offer an explanation for the observed dark respiration decreases when etioplasts are first exposed to light.
Table 7. The effect of ATP added to etioplast fractions on observed average oxygen uptake rates under light and dark conditions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Average rate oxygen uptake (nM O₂/mg N/min) in</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dark (^b)</td>
<td>total</td>
<td>light (^c)</td>
<td>total</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control(^a)</td>
<td>5.0</td>
<td>2.3</td>
<td>1.3</td>
<td>8.6 a</td>
<td>36.5</td>
<td>21.5</td>
<td>14.0</td>
</tr>
<tr>
<td>Control + ATP</td>
<td>4.5</td>
<td>2.7</td>
<td>1.9</td>
<td>8.1 a</td>
<td>45.3</td>
<td>27.3</td>
<td>16.6</td>
</tr>
</tbody>
</table>

a. The control fraction was isolated as described in Figure 6.

b. The rates of three cycles (see Figure 10) are given followed by the totals. Each one minute cycle was averaged from 3 etioplast preparations. The same letter after the totals indicates no significant difference at the 5% level according to the Least Significant Difference Test.

c. The average rate of oxygen uptake was calculated: light rate - average dark rate before and after the light rate.
DISCUSSION

Light and Photomorphogenesis

Visible light, a small portion of the electromagnetic spectrum, is the ultimate source of energy for living creatures. In nature, green plants have the role of capturing and conserving this energy. It should, therefore, not be surprising that light intimately participates in the regulation of plant development, i.e., photomorphogenesis. This light dependent development is clearly illustrated in the processes of plastogenesis where the final assemblage of the plant's light-capturing mechanism is photodependent. Such a process may be looked upon as an evolutionary device designed to channel stored seed energy into root and shoot elongation and growth. The enforced priorities of this type behavior enable the growing shoot to emerge rapidly from the ground before initiating the expenditure of energy necessary for developing its light capturing capacity. After shoot emergence, it is light that signals the plant to make ready its photosynthetic apparatus. The simplicity of this system lies in the fact that visible light is both the source of captured energy as well as the regulator of the means by which energy is captured.

The present work was an attempt to measure an early light response in organelle isolates of etiolated leaves.
This was accomplished by measuring an in vitro oxygen uptake of organelles simultaneously with light exposures and characterizing the photochemical activity. The data for this light induced metabolic behavior have been presented under Results and are now discussed in relationship to: (1) the role of mitochondria, (2) the role of etioplasts, and (3) the role of mitochondrial-etioplast interaction.

**Role of Mitochondria**

The most obvious conclusion which may be drawn from an observation of increased oxygen uptake in preparations containing mitochondria is that respiration has been stimulated. Furthermore, if light is associated with the increased oxygen uptake, then it would appear that light is responsible for observed increases in respiratory activities. This putative behavior can easily be tested. The addition of respiratory inhibitors to such a system should suppress any light induced mitochondrial respiration. The inhibitor of cytochrome c oxidase, KCN, was seen in Figure 23 to inhibit the State 3 respiratory process by 91.2%, yet had little or no effect on light induced oxygen uptake. This was strong evidence that at least the respiratory chain activities of the mitochondria were not directly involved in the light phenomenon.

In addition, an inhibitor of phosphorylation of added ADP, atractyloside, was employed to determine the effect of adenylate transport on the light induced oxygen uptake. Data
from this inhibitor study (see Figure 24) showed that atracyloside was effective in decreasing the O$_2$ uptake by only 7 percent more in the light than in darkness. This difference is small and probably insignificant when compared with the very large increases of 100 to 500 percent in rates of oxygen uptake observed in light conditions. In summary, inhibitor studies do not support mitochondrial involvement in the light phenomenon with respect to increased respiratory chain activity or increased oxidative activity due to external ADP acceptor stimulation.

Tissues such as roots, some coleoptile sheaths, and scutella, which do not turn green even under light conditions are devoid of etioplasts, yellow chromoplasts (Ledbetter and Porter 1970). If mitochondria are isolated from these preparations and assayed for increased O$_2$ uptake in light, no response is observed (Table 2). This is additional convincing evidence against the direct association of mitochondria with the photoinduced O$_2$ uptake.

Another factor worth considering is the action spectrum for the light phenomenon (Figure 20). Maximum effective wave lengths occurring between 640 and 680 nm had indicated the presence of an absorbing pigment that could conceivably be due to phytochrome participation. Furthermore, evidence for the association of phytochrome with mitochondrial membranes has been put forth by Manabe and Furuya (1973). Although the validity of this finding has been questioned by
the results of Marine et al. (1974), the possibility still re-
mained and experiments were carried out to assay for phyto-
chrome photoreversibility. These experiments (see Results)
showed a lack of a red, far-red control associated with the
light induced oxygen effect. This finding together with the
"on and off" nature of the light phenomenon suggested that
phytochrome was not involved. By implication, this is also
further evidence against mitochondrial participation in the
light phenomenon since the only known pigment with absorbance
at 640 to 680 nm and associated with mitochondria is phyto-
chrome (Manabe and Furuya 1973).

Role of Etioplasts

Plant tissues which serve as a source for etioplasts
such as leaf, stem, and cotyledons, also contain numerous
mitochondria. Even separated etioplast fractions are char-
acterized by membrane-bound cytoplasmic inclusions that con-
tain mitochondria (see Figure 12). This hinders purification
of the etioplast fraction and makes clean separation virtually
impossible either by the technique of differential velocity
or sucrose gradient centrifugation (see Figures 12 and 13).
On the other hand, organelle preparations isolated from leaf
tissue demonstrated a positive response to the light induced
oxygen assay (Table 2). This was the first piece of evidence
indicating that etioplasts may be associated with the light
phenomenon. An alternative possibility would have been a
direct interaction between etioplasts and mitochondria which synergistically produced the light phenomenon. The latter possibility was not supported by inhibitor studies.

If etioplasts were to be implicated as participants in the light phenomenon, then a light receptor pigment had to be found that satisfied two conditions: (1) association with the etioplasts, and (2) absorption in the range of 640 to 680 nm. Previous experiments (see Results) have suggested that phytochrome was not involved both due to the lack of photoreversibility and to the "on and off" nature of the reaction. This narrowed pigment possibilities to the chlorophyll precursors. However, in etiolated tissues, chlorophyll biosynthesis is blocked near the end of the pathway and there is a build-up of one of the intermediates, viz., protochlorophyllide. At the protochlorophyllide block the pathway regulation is light mediated. This information together with the fact that the PChle holochrome absorbs at 650 nm was convincing evidence that the light receptor pigment is the protochlorophyllide holochrome (PChle-650).

Once the probable light receptor pigment, PChle-650, had been identified and located, the problem remained to determine its role in oxygen uptake. A thorough review of the literature was made to ascertain the known properties of PChle-650. An early perplexing discovery was that the light induced conversion of PChle to Chle was a reduction step. The problem then was to explain a photoreduction in terms of
an oxygen utilizing process. This, as it turned out, can easily be explained by hypothesizing a dark oxidation reaction coupled to a light reduction reaction. Such an arrangement would also explain the lag periods noted in Figure 10 as resulting from the time interval required of the dark oxidation step to catch up with the light activated reduction step. Thus, the dark oxidative step would begin after a lag period which corresponded to the time interval required for the light reduction step to assert its influence either by mass action effect or by control.

A further matter that must be dealt with is to demonstrate that the "photoinduced" oxygen uptake is indeed a measurement of the oxidation reaction that supplies the protons for the photoreduction of PChle. In brief, this requires verification of the hypothesis that the light phenomenon really is a measurement of the conversion of PChle + Chle. Investigations aimed at ascertaining this supposition consisted of experiments designed to vary (1) light quantity and quality, (2) light as well as light followed by dark, and (3) temperature. These experimental conditions are listed in Table 8 along with response measurements. The response measurements fell into two categories: spectrophotometric or pigment measurements and metabolic measurements. The former were compiled from published experimental results conducted mostly on beans, wheat, corn, and barley. The latter measurements are the results of the present study which employed
Table 8. Comparison of spectrophotometric and pigment measurements with those of metabolic activity for various experimental conditions.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Spectrophotometric or pigment measurement</th>
<th>Ref.</th>
<th>Metabolic activity: photoinduced O₂ assay</th>
<th>Ref.</th>
<th>(present study)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Variation in light intensity</td>
<td>Rate of transformation PChle→Chle is directly proportional to light intensity</td>
<td>9, 7</td>
<td>L/D value is directly proportional to light intensity</td>
<td>Fig. 19</td>
<td></td>
</tr>
<tr>
<td>2. Saturating light to achieve complete photoconversion</td>
<td>Spectral shift from 650-678 nm; high Chle and low PChle content</td>
<td>1, 4, 6</td>
<td>Rate of O₂ uptake in light is the same as that in dark: L/D = 1</td>
<td>Fig. 21</td>
<td></td>
</tr>
<tr>
<td>3. PChle dark regeneration after saturating light</td>
<td>PChle regeneration in 5-day tissue: no lag, rapid rate; in older leaves: lag increases with age, slower rate</td>
<td>1, 4, 6</td>
<td>Young leaves (5-day): rapid increase in L/D; older leaves: much slower recovery rate of L/D</td>
<td>Fig. 22</td>
<td></td>
</tr>
<tr>
<td>4. PChle dark regeneration at low temperature</td>
<td>Lower rate of PChle reappearance</td>
<td>10, 4</td>
<td>Lower rate of recovery of increased L/D</td>
<td>Fig. 22</td>
<td></td>
</tr>
</tbody>
</table>
Table 8. (continued)

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Response measured by</th>
<th>Ref. a</th>
<th>Metabolic activity:</th>
<th>Ref. (\text{present study})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spectrophotometric or pigment measurement</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>5. Light quality of photo-conversion</td>
<td>5. Photoconvertible PChle absorbs strongly at 650 nm</td>
<td>2, 3, 5, 8</td>
<td>5. Light of 640-680 nm gives max effect, i.e., highest L/D</td>
<td>Fig. 20</td>
</tr>
</tbody>
</table>

a. Reference code:
(1) Akoyunoglou and Siegelman 1968;
(2) Björn 1969;
(3) Gassman and Bogorad 1967;
(4) Granick and Gassman 1970;
(5) Koski, French and Smith 1951;
(6) Lancer et al. 1976;
(7) Madsen 1960;
(8) Shibata 1957;
(9) Smith and Benitez 1954;
(10) Virgin 1955.
wheat. The overall approach was to show that the two response measurements were compatible under similar experimental conditions.

In examining the first condition, variation in light intensity, Smith and Benitez (1954) and Madsen (1960) in kinetic studies of PChle phototransformation demonstrated by pigment analysis that the role of transformation was directly proportional to light intensity. This was in agreement with results obtained in this study utilizing the light induced O$_2$ assay. The light intensity curve (Figure 19) showed a linear relationship between L/D values and light flux.

In experiments involving the second set of conditions, photoconversion by saturating light, numerous spectro-photometric as well as pigment measurements have been made. These show that photoconversion results in a spectral shift from 650 to 678 nm as well as a pigment quantitative change from low Chle to high Chle. As noted in the Literature Review, light intensities and exposure times for complete photoconversion vary, but there is a common consensus that transformation of PChle to Chle is both rapid and light mediated. In the present study the measurement of metabolic activity expressed in terms of L/D values demonstrated that after a saturating in vivo light, the in vitro rate of O$_2$ uptake in light conditions was reduced to the same as that in dark conditions, i.e., an L/D = 1 (see Figure 21). Furthermore, the decrease in L/D value occurred rapidly taking
2 to 3 minutes for half reduction in L/D values. These results were consistent with those derived from spectrophotometric or pigment measurements.

The third set of conditions, PChle dark regeneration after saturating light, has been shown by spectrophotometric or pigment analysis to exhibit an age dependency for the rate of PChle regeneration. Using spectrophotometric measurements, Akoyunoglou and Siegelman (1968) found that the length of the lag period before PChle resynthesis in the dark was rapid (a few minutes) in young Phaseolus tissue but as much as 40 to 120 min in 13-day old tissue. On the other hand, in older tissue the rate of synthesis was much slower. These findings were consistent with the present study where 5-day old wheat leaves demonstrated a rapid increase in L/D values while older leaves (14 to 16 day) had a much slower recovery of the L/D value during dark regeneration conditions (see Figure 22).

Under the fourth set of conditions, PChle dark regeneration at low temperature, both Virgin (1955) and Granick and Gassman (1970) found a lower rate of PChle reappearance in pigment analyses. Their findings coincide with the present study results which indicated a temperature dependent recovery rate of numerically increased L/D values. These data are presented in Figure 22 and show faster recovery rates at 25 C when compared with those at 5 C.
Finally, the fifth condition, light quality for photoconversion, was investigated by numerous experimenters who found that photoconvertible PChle absorbed strongly at 650 nm. This was in accord with results of the present study where an action spectrum revealed that light of 640 to 680 nm provided maximum response, i.e., resulted in the highest L/D values (see Figure 20).

Taken as a whole, these five experimental conditions and the corresponding set of examples give firm support to the notion that the light induced oxygen uptake measurements on isolated etioplasts correspond to the rate of photoconversion of PChle to Chle.

**Role of Mitochondria-etioplast Interaction**

An organelle interaction may be considered to be the sharing of at least one molecular species common to dissimilar organelles. In a narrower sense, an interaction can be viewed as one type of organelle supplying a particular molecule that is utilized by another. With this in mind, experimental studies of Horton and Leech (1975) are relevant since they provide evidence suggesting that adenylates qualify as molecules that are shared by dissimilar organelles. They investigated the effect of ATP on the photoconversion of PChle in isolated maize etioplasts and discovered that exogenous ATP mediated two distinct events. The first concerned the fact that ATP prevented the decay of and therefore was
responsible for the preservation of the photoconvertible form of protochlorophyllide, PChle-650. The second was that following primary photoconversion, ATP induced the formation of photoconvertible PChle-650 from PChle-630. This provided evidence for a situation whereby ATP, which in etiolated tissue is produced in the mitochondria, could conceivably be shared and utilized by the developing etioplasts.

In order to test for organelle interaction by assaying oxygen uptake, experiments were designed based on the following rational. If ATP indeed were involved in the regeneration of PChle-650 from PChle-630 as suggested by Horton and Leech (1975), then increased ATP concentration should increase PChle-650 pool size. This in turn would be detectable in the oxygen assay for photoconversion. Specifically, the addition of ATP to dark grown etioplast fractions should promote alterations in the photoinduced O2 assay: (1) the L/D values may be slightly higher due to greater stabilization of the PChle-650 holochrome, and (2) as photoconversion occurs, the O2 uptake rate in the second and third cycles should be relatively higher due to ATP induced PChle-630 conversion to PChle-650, the photoinducible form. These expected results could be verified by the addition of either pure ATP or purified mitochondria (which synthesize ATP) to an etioplast fraction and then assaying for any differences when compared to a control.
In the first of two experiments devised to detect organelle interaction, a scutellar mitochondrial fraction was mixed with a low-spin etioplast fraction. Tables 5 and 6 presented comparisons of O$_2$ uptake rates of "mixed" fractions with those of individual fractions. A general agreement was observed between these data and the above expected outcomes. The mixed fractions exhibited increased O$_2$ uptake rates by the addition of mitochondria that by themselves showed no light response. The addition of mitochondria resulted in an approximately 17% increase in O$_2$ uptake under light conditions.

A second set of experiments demonstrated that when ATP was substituted for the added mitochondria, a similar increase in light-stimulated oxygen uptake occurred (see Table 7). A concentration of 150 mM ATP resulted in approximately a 20% rate increase of oxygen consumption in light conditions. The similar effect of added mitochondria or ATP on an etioplast fraction suggests that mitochondria were supplying ATP as the active ingredient.

The combined results of these two sets of experiments suggest an organelle interaction between mitochondria and developing etioplasts. Furthermore, the implication is that ATP is supplied by the mitochondria and utilized by the etioplasts during the light dependent step of PChle photoconversion. While the light induced oxygen assay alone does not conclusively prove the occurrence of a bonafide
interaction, these results both add credence to the proposals of Horton and Leech as well as offer indirect proof for organelle interaction.

The results of the inhibitor experiments with atractyloside also favor the proposal of an adenylate interaction. This is suggested by a greater inhibition observed during the light cycle (29%) compared to the dark cycle (22%). This seven percent difference is presumed to be a reflection of a lowered ATP pool concentration resulting in a less stable PChle-650 holochrome as well as a decrease in the rate of regeneration of phototransformable PChle.

Finally, it is possible to formulate a general scheme to illustrate photoconversion of PChle-650 to Chle-678 together with protochlorophyllide regeneration of PChle-650 from PChle-630. The scheme in Figure 25 is supported by evidence from the present study as well as that from other pertinent works. The conversion of PChle-630 to PChle-650 requires ATP (Horton and Leech 1975) but is regulated by the rate of photoconversion of PChle-650 to Chle-678. This has been suggested by Sundquist (1973) to be the result of availability of a limited number of sites for PChle-650 formation. The ATP required in photoconvertible PChle-650 regeneration is very possibly supplied by the mitochondria. Such a role on the part of the mitochondria has been indicated by the results of the mixing experiments.
Figure 25. Diagram illustrating proposed interaction where the mitochondrion supplies ATP to the etioplast reaction of PChle-650 regeneration.

In dark grown leaves, this interaction is stimulated by red light due to PChle-650 photoreduction. The letters "A" and "B" are oxidized substrates in the etioplast and mitochondrion respectively.
Figure 25 also illustrates the need for two distinct dark oxidation reactions. This was suggested by the studies of mitochondrial respiratory inhibition in conjunction with those of light induced oxygen uptake. Results of these experiments showed that even though mitochondrial oxidation had been inhibited, a separate oxidation reaction could be stimulated by light conditions. Evidence presented earlier inferred that this unknown dark reaction is located in the etioplasts where it supplies the hydrogen atoms needed in photoconversion of PChle-650 to Chle-678 (Boardman 1962). The source of these hydrogen atoms is as yet unknown; however, kinetic studies of the photoconversion process favor the hypothesis that the hydrogen donor is in close association with the holochrome protein (Thorne and Boardman 1972). With this in mind, a likely source for the hydrogen needed for PChle photoreduction might logically include an oxidation step occurring earlier in the chlorophyll pathway. The oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX both requires molecular oxygen and occurs prior to the photoreduction step. This oxidation step theoretically could supply the hydrogen atoms needed for the subsequent photoreduction step. If such a conjecture were correct, it would also offer a control mechanism built into the chlorophyll biosynthetic pathway. This could be envisaged as a feedback system where the light dependent reduction step located near the end of the pathway would regulate an earlier
dark oxidative step. The actual regulation would be based on the supply and demand for hydrogen protons via an electron carrier molecule such as nicotinamide adenine dinucleotide phosphate (NADP).
SUMMARY

Although the dependence of plants on light for growth and development has long been obvious, the intermediary events between photoabsorption and altered development patterns is only now becoming known. For example, early botanists knew that dark grown seedlings when exposed to a few hours of light lost their characteristic pale yellow appearance and turned green. However, the biochemical nature of the reaction was unknown. Later, it was understood that the absence of green color in darkness indicated a regulatory dependence on light for chlorophyll biosynthesis. To discover the nature of the light regulation mechanism, investigators studied chlorophyll biosynthesis by pigment extraction analyses as well as in vivo leaf spectrophotometric measurements. By the 1960's, results of these studies revealed the existence of a light dependent reaction in chlorophyll biosynthesis in the PChle to Chle step. The present study has further investigated chlorophyll biosynthesis by a new approach which measures metabolic activity associated with early light induced changes. This research forms a two-part study aimed at investigating (1) the photoinduced oxygen uptake in isolated etioplasts, and (2) the etioplast-mitochondrial interaction which enhances oxygen uptake under light conditions.
The first of these objectives concerns the fact that isolated etioplasts, when illuminated, demonstrate increased oxygen uptake. Since etioplasts could not be completely separated from mitochondria, it was necessary to show by other means that this phenomenon was indeed peculiar to etioplasts. This was inferred by noting that only fractions containing etioplasts, i.e., those from primary leaves or cotyledons, possessed the photoinduced oxygen response. Furthermore, an inhibitor of mitochondrial respiration, potassium cyanide (KCN), inhibited mitochondrial uptake but had little or no effect on the light induced oxygen uptake. These two experiments suggested that etioplasts were responsible for the light phenomenon. In effect, this information provided evidence that the light receptor pigment was located in the etioplast. Further confirmation was obtained when protochlorophyllide-650 holochrome, an etioplast pigment, was found to be the light receptor. This discovery was the result of a series of experiments designed to delineate the physical parameters necessary for maximum oxygen uptake in light conditions. One such experiment which proved to be an important key was the determination of the action spectrum. The receptor pigment for light induced oxygen uptake was found to have maximum absorption between 640 and 680 nm. When the action spectrum plus four other experimental results were compared with those obtained by spectrophotometric and pigment analyses of PChle-650, the indication was that the same pigment,
i.e., PChle-650, was involved. The above examples in conjunction with a lack of evidence for phytochrome involvement, led to the conclusion that the pigment, PChle-650, was the light receptor molecule and that it was located in the etioplast.

The chemical nature of the light phenomenon can be explained by hypothesizing a dark oxidation reaction coupled to a light reduction reaction. The light reaction, the photoreduction of PChle-650, drives a yet unknown dark oxidative reaction to supply protons necessary for photoreduction. Evidence supporting this possibility comes from the observation of a lag period following light exposure before oxygen consumption increases. This lag period was viewed as the time interval required for the light reduction step to assert its influence either by mass action effect or control of the dark oxidative step.

The second area investigated in this study involved the role of the mitochondria in the light phenomenon. Mixing experiments showed that mitochondria indirectly participate in this light effect. A scutellar mitochondria fraction that by itself did not increase oxygen uptake in the light, when mixed with an etioplast-containing fraction, caused increased etioplast oxygen uptake. It was shown that pure ATP could substitute for the mitochondrial fraction with the same result. That ATP was the effective molecule in the organelle
interaction was confirmed by inhibitor studies with atractylloside.

It was possible to propose mechanisms for the ATP effect from a comparison of the results of the present study with previous work of Horton and Leech (1975). The proposed interaction suggested that mitochondrial oxidative phosphorylation supplied ATP to the etioplast where the reaction of PChle-650 regeneration occurred. In dark grown leaves this reaction is driven by red light stimulation of PChle-650 photoreduction. As the pool of PChle-650 is reduced, its regeneration from PChle-630 requires ATP.

The implications of the above findings are important in that they provide increased insight into organelle interactions at the biochemical level. In addition, new avenues of investigation have been opened up in the areas of metabolic measurements of photoinduced reactions as well as of light regulation mechanisms in plant development.
LITERATURE CITED


