Title

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J. Biggins and R. B. Park

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CO_2 assimilation by etiolated Hordeum vulgare seedlings during the onset of photosynthesis.[1,2]

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Seedlings, germinated and grown in darkness, develop at the expense of energy liberated by the breakdown of seed reserves. Exposure of such etiolated plants to light results in the conversion of the existing protochlorophyll to chlorophyll *a* followed by net synthesis of chlorophyll in the proplastid. After the initial pigment conversion, the proplastid undergoes a marked structural reorganization concurrent with the formation of the photosynthetic apparatus and development of an autotrophic metabolism.

Several biochemical and ultrastructural parameters have received attention during such an onset of autotrophy in etiolated plants and algae. The proplastid in the etiolated organism is bounded by a double membrane and contains small vesicles (3,11,14,21). These appear to aggregate to form prolamellar bodies (11). At this stage the proplastids contain protochlorophyll (16), cytochromes f and b_6 (8,10) and carotenoids (17). Exposure of the etiolated plants to light results in a rapid formation of lamellae, differentiation into grana and stroma lamellae, and development of the proplastid into a mature chloroplast.

Oxygen evolution does not occur until the plants have received 30-minute irradiation (17) and net chlorophyll synthesis has commenced (4,18). In Euglena the uptake of carbon dioxide appears to occur concurrently with the synthesis of chlorophyll and evolution of oxygen and with the formation of lamellae within the proplastid (18).

Light-induced fluorescent yield changes were first detected in etiolated bean leaves after 2-hour illumination (6). At the same time, chlorophyll *a* differentiated into *a*-670 and *a*-680, and C_{705} and chlorophyll *b* were observed. It was concluded that the two photosystems of the higher plant photosynthetic apparatus develop at the same time and

that electron transport commences as soon as the reaction centers are organized.

Consistent with these data are the observations that photo-induced absorption changes attributable to cytochrome f (5), and the photo-induced electron paramagnetic resonance signal in a greening Chlamydomonas mutant (1) do not appear until net chlorophyll synthesis starts. This takes place after a lag phase subsequent to the initial illumination.

Tolbert and Gailey (19) observed that significant CO_2 fixation in etiolated wheat does not occur until some two hours after continuous, rapid chlorophyll synthesis commences. The initial fixation was into malic, aspartic and glutamic acids. Also, a further two-hour illumination was found to be necessary before hexose phosphates and sucrose appeared labeled. It was concluded that the availability of ribulose diphosphate is a limiting factor during the greening process. In the present investigation we find significant increases in CO_2 fixation after only one to two hour illumination. The operation of the Calvin cycle appears to be concurrent with the photo-induced increases in CO_2 uptake and with chlorophyll synthesis. We attribute these variations to differences in plant material and experimental design.

Materials and Methods

Preparation of etiolated barley seedlings: Seeds of Hordeum vulgare (1956 crop, var. Tennessee Winter) were imbibed in aerated water for 24 hours and grown hydroponically in complete darkness for six days using 1/200 strength Hoagland's solution. For the greening experiments the seedlings were illuminated by a bank of unfiltered fluorescent tubes giving an incident light intensity of 500 foot candles. Samples of seedlings were taken at intervals of time during this illumination period

and their capacity for CO_2 assimilation was measured in the light and dark.

In vivo $C^{14}O_2$ assimilation: $C^{14}O_2$ assimilation was conducted in an apparatus shown in Figure 1 which contained three whole seedlings. The apparatus and seedlings were equilibrated for 10 minutes in the dark or 5000 foot candle white light (photoflood and infrared filter) and gassed with 1% CO_2 in air. At the start of the reaction, the apparatus was closed and 200 µl 0.06 M $NaHC^{14}O_3$ (1.98 mC/ml) was injected through the serum cap into the phosphoric acid in the side arm liberating 12 µmoles $C^{14}O_2$ (396 µc in 20 ml volume). The seedlings were exposed to $C^{14}O_2$ for 4 minutes and the reaction was stopped by rapidly disassembling the apparatus in a safety hood, cutting off the leaves and plunging them into liquid nitrogen. The leaves were then homogenized in warm 80% methanol using a mechanically driven glass pestle and homogenizer. The resulting macerate was transferred quantitatively to a test tube and boiled for one minute.

Extraction of plant residues: The residues were extracted successively with 50% and 20% methanol followed by warm water. The residue dry weights were then measured. The combined supernatants were reduced in volume by distillation in vacuo following procedures developed in this laboratory (2). The radio-carbon contents of 100 µl aliquot samples were determined by means of a Packard Tri-carb automatic liquid scintillation spectrometer using 10 ml scintillation solution* and one drop of commercial bleach (NaOCl) to decolorize the pigments. After the initial counting, an internal standard (250 µl C^{14}-toluene, 123 dpm/µl) was added to each vial and the vial was recounted.

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*(2000 ml toluene, 2000 ml n-dioxane, 1200 ml ethanol, 200 g naphthalene, 26 g 2,5-diphenyloxazole and 0.5 g 1,4-bis-2-(5-phenyloxazol benzene)

The distribution of radiocarbon in the products of CO_2 assimilation was determined in early samples by chromatographic analysis. Aliquot samples of the extracts were placed on Whatman No. 4 paper and chromatographed in two dimensions using a new solvent containing ammonia, isobutyric acid, water and several aliphatic alcohols in the long dimension (7) and n-butanol-propionic acid-water (2) in the other dimension. Radioautograms were prepared and then the radioactive spots were located, cut out and counted automatically (13).

Results and Discussion

Figure 2 shows the total $C^{14}O_2$ assimilated in light and dark during the greening period. Differences between light and dark exposures are apparent after one hour illumination and very significant increases in assimilation occur after 2 hours. At this time a net increase in chlorophyll was first observed. Plants after 24 hours' greening are capable of photosynthesizing at 78% the rate of control seedlings grown from germination in continuous light.

These data are consistent with those on O_2 evolution in barley leaves (17), photo-induced fluorescent yield changes in bean leaves (6) CO_2 uptake and O_2 evolution in Euglena (18) and oat seedlings (4), and photo-induced absorption changes in mung beans (5).

Table I shows the distribution of assimilated carbon in the individual components of the soluble fraction as analyzed by paper chromatography and radioautography (2). In the etiolated plant, the products of CO_2 assimilation are malic, citric/iso-citric, aspartic and glutamic acids. This distribution of C^{14} suggests that either phosphoenolpyruvate carboxylase or carboxykinase coupled to malic dehydrogenase (12), or malic enzyme (20) are the operative carboxylation enzyme systems in the

etiolate. All three enzymes are widely distributed in plant tissues (20) including barley (9).

This pattern of carboxylation compares closely with the fixation of CO_2 by roots and coleoptiles of Avena (15), etiolated wheat leaves (19) and a Chlamydomonas mutant lacking chlorophyll (1). However, in this investigation there appears to be a large quantity of assimilated C^{14} in citrate/iso-citrate. Similar quantities were observed in Avena (15), but not in wheat (19).

Throughout the greening period it is apparent that of the CO_2 assimilated in the dark, the distribution of C^{14} in the soluble fraction remains essentially the same as that of the etiolate. However, the pattern of assimilation in the light changes quite markedly. Phosphoglyceric acid and hexose mono- and di-phosphates appear labeled after one hour illumination and became more preponderant with time. The increase in labeling of sucrose occurs with a decrease in hexose phosphates. Concurrent with the appearance of these intermediates is the decrease of C^{14} entering the organic and amino acids. This suggests that the respiratory carboxylations, which are characteristic of the etiolated and dark reactions, are replaced by the Calvin cycle during development of the plastid.

These results are in agreement with those of Hall et al. (9) who investigated certain enzyme activities in greening barley leaves. They showed that the activity of phosphoenolpyruvate carboxylase and carboxykinase decreased during the greening period, whereas the activities of carboxydismutase, phosphoribulokinase and phosphoriboisomerase increased.

The results here show that photosynthetic phosphorylation and reducing mechanisms are operative at a very early stage in the greeting process. These data are in accord with those of Butler, who showed that electron transport commences as soon as the reaction centers for quantum conversion are assembled (6). A direct result of this onset of quantum conversion is a reorganization of the plastid leading to higher photosynthetic rates and an autotrophic metabolism.

Summary

The assimilation of CO_2 by etiolated Hordeum vulgare seedlings during an illumination period indicates a conversion of the organisms to autotrophy.

After one hour illumination, increases in the photo-assimilation of CO_2 are observed and the distribution of C^{14} in the soluble fraction of the plants is predominantly in intermediates of the Calvin cycle. It is concluded that photosynthetic phosphorylation and reducing mechanisms are operative at very early stages of plastid development and that subsequent increases in photosynthetic rates are concurrent with maturation of the plastid.

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Legends

Figure 1. Apparatus for *in vivo* $C^{14}O_2$ assimilation by *Hordeum vulgare* seedlings.

Figure 2. Total assimilation of $C^{14}O_2$ by *Hordeum vulgare* seedlings during the illumination period. Results expressed on a dry weight basis. Reactions were for 4 minutes in the light or dark.

Table I. Distribution of C^{14} in soluble fraction of *Hordeum vulgare* seedlings after exposure to $C^{14}O_2$.

Running title: Biggins and Park -- CO_2 fixation by greening seedlings.

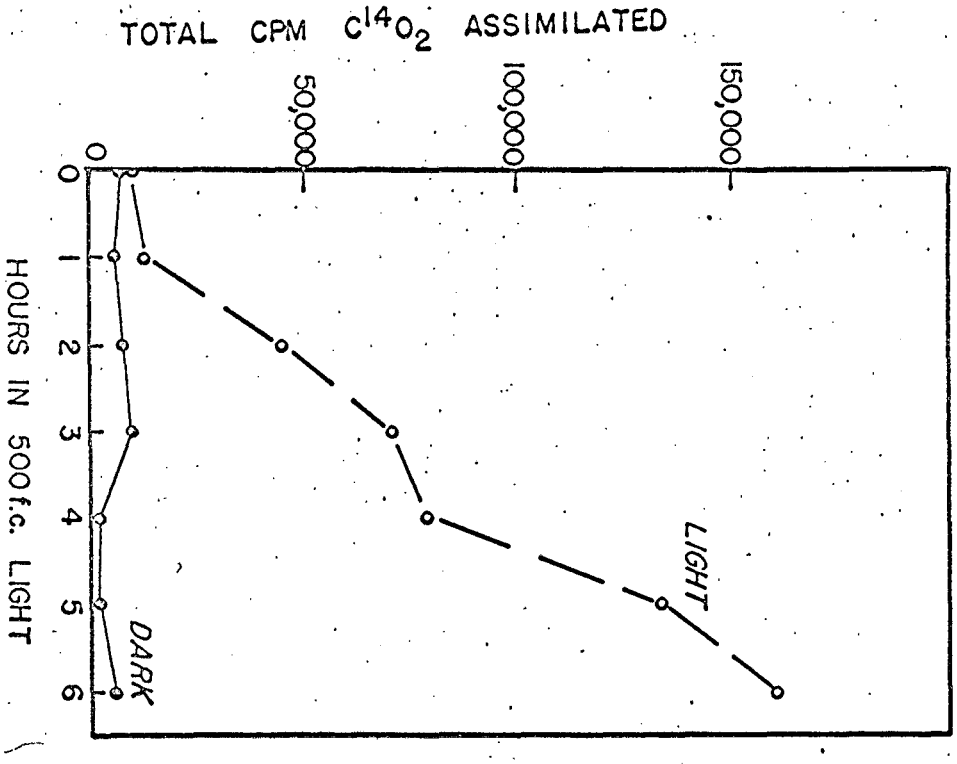
Table I.

Time in light(hr)	Reaction system	C^{14} found (%)								
		Malate	iso-citrate/ citrate	Aspartate	Glutamate	Alanine	PCA*	HMP**	HDP***	Sucrose
0	Light	33	32	26	8	0	0	0	0	0
	Dark	43	23	23	8	0	0	0	0	0
1	Light	12	18	11	2	1	4	34	16	1
	Dark	43	23	20	9	0	0	0	0	0
2	Light	8	15	11	1	1	7	33	15	8
	Dark	37	28	27	8	0	0	0	0	0
3	Light	6	5	18	1	2	10	24	13	18
	Dark	33	32	26	8	0	0	0	0	0

*PGA = 3-phosphoglyceric acid

**HMP = hexose monophosphates

***HDP = hexose diphosphates


TOTAL CPM $C^{14}O_2$ ASSIMILATED
0
50,000
100,000
150,000
HOURS IN 500 f.c. LIGHT
0
1
2
3
4
5
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DARK
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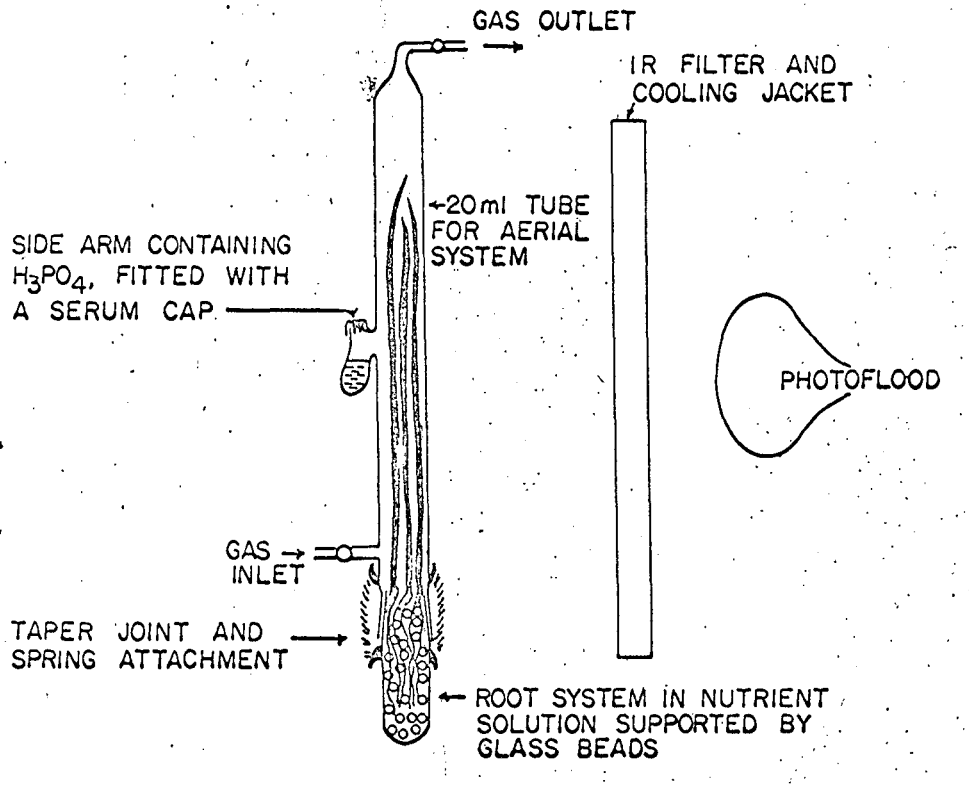
GAS OUTLET
IR FILTER AND COOLING JACKET
←20ml TUBE FOR AERIAL SYSTEM
SIDE ARM CONTAINING H_3PO_4, FITTED WITH A SERUM CAP
PHOTOFLOOD
GAS → INLET
TAPER JOINT AND SPRING ATTACHMENT
← ROOT SYSTEM IN NUTRIENT SOLUTION SUPPORTED BY GLASS BEADS
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