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# The Development of Photophosphorylation and Photosynthesis in Greening Bean Leaves

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## ABSTRACT

**Photophosphorylation and oxygen evolution were measured in 8-day-old dark-grown bean leaves (*Phaseolus vulgaris*) after various times of greening in far red light and in white light. The sequence of development was the same for both greening regimes, but the processes were much more rapid in white light. The capacity for photophosphorylation, as assayed by the firefly luciferase assay, appeared after 12 hours in far red light. At this stage and for times up to 24 hours, photophosphorylation was not inhibited by $10^{-5}$ M 3-(3,4-dichlorophenyl)-1,1-dimethylurea. At 24 hours, the capacity for oxygen evolution appeared and photophosphorylation became partially inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea at concentrations which inhibited oxygen evolution. In white light photophosphorylation appeared after 15 minutes, and oxygen evolution at one hour. Photophosphorylation became partially sensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea when oxygen evolution appeared. Carbonylcyanide *m*-chlorophenylhydrazone inhibited photophosphorylation and photosynthesis at low concentrations, $10^{-6}$ M, with immature leaves, but the leaves developed resistance to carbonylcyanide *m*-chlorophenylhydrazone as they greened.**

A previous study (4) of the development of etiolated bean leaves in far red light showed that chlorophyll accumulated and the capacity for photosynthesis developed but much more slowly than with leaves greened in white light. With 8-day-old dark-grown seedlings where photosynthesis could be detected after 1 to 2 hr of white light, oxygen evolution and photophosphorylation were not detected until 24 hr of far red light. The continuous far red light provided conditions where the rate-limiting step is well defined. A low level of phytochrome is maintained in the far red-absorbing form so that all of the synthetic reactions controlled by phytochrome are fully operative. During 5 days (12 hr light-12 hr dark) of greening in far red light the leaves expand 20-fold in area (as much as a leaf in white light), 8-fold in fresh weight, and 4-fold in dry weight. The rate-limiting step controlling the development of the photosynthetic apparatus is the slow transformation of protochlorophyll to chlorophyll by the far red light. Studying the development of etiolated leaves in far red light has the advantage that the development is greatly prolonged and sequential processes may be better separated. The sharp onset of the capacity for oxygen evolution indicated that a high degree of synchrony was maintained in the developing plastids even though the development time was prolonged.

In the previous paper (4) no attempt was made to distinguish between the onset of oxygen evolution and photophosphorylation or to differentiate between cyclic and noncyclic photophosphorylation. In the present paper we show that cyclic and noncyclic photophosphorylation can be distinguished in the intact leaves during development and that cyclic photophosphorylation appears well before noncyclic photophosphorylation. The onset of noncyclic photophosphorylation is coincident with the onset of oxygen evolution. This sequence of appearance occurs with leaves greened in white light as well but over much shorter periods of time.

## MATERIALS AND METHODS

Primary leaves of *Phaseolus vulgaris* cultivar Topcrop (W. Atlee Burpee Co., Riverside, Calif.) were used for all experiments. The conditions of growth and far red light source were the same as described previously (4). The temperature in darkness was 20 C; in light, 25 C. Greening experiments were made with far red light with 12-hr light, 12-hr dark irradiation cycles (4); with continuous far red illumination; and with continuous white light. The source of white light for greening consisted of 4 cool-white lamps (General Electric F40CW) giving $4 \times 10^3$ ergs $cm^{-2}$ $sec^{-1}$ at the plants.

The chlorophyll *a* and *b* contents of the leaves were determined spectrophotometrically according to the method of Ogawa and Shibata (15). Extractions were carried out under green light.

The phosphorylation capacity of the leaves was determined by the firefly luciferase assay (9, 16). At selected stages of greening, primary leaves were taken and cut into 10 segments (4 to 9 $mm^2$) under green safelight. Five of the discs were incubated in either a solution of DCMU or a solution of CCCP[2] and the other 5 discs were incubated in distilled water. After 15 to 30 min of incubation the leaf discs were placed in a Petri dish on filter paper (moistened with the same solution they had been incubating in) where they were irradiated with white light filtered through 7 cm of a 1% $CuSO_4$ solution ($1.5 \times 10^5$ ergs $cm^{-2}$ $sec^{-1}$) for 0.5, 1, 3, and 5 min (4). Immediately after each exposure time one leaf disc was submerged in 5 ml of boiling tris buffer (16), pH 7.4, for 1.5 min. A 0.35-ml aliquot of this extract was injected into a cuvette containing 0.1 ml of diluted firefly lantern extract and 0.25 ml of tris buffer (16). The light-measuring apparatus was designed after Holm-Hansen and Booth (9). The ATP value for the sample

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[2] Abbreviations: CCCP: carbonylcyanide *m*-chlorophenylhydrazone; PMS: phenazine methosulfate; DCPIP: 2,6-dichlorophenolindophenol; PS1 and PS2: photosystem I and photosystem II.

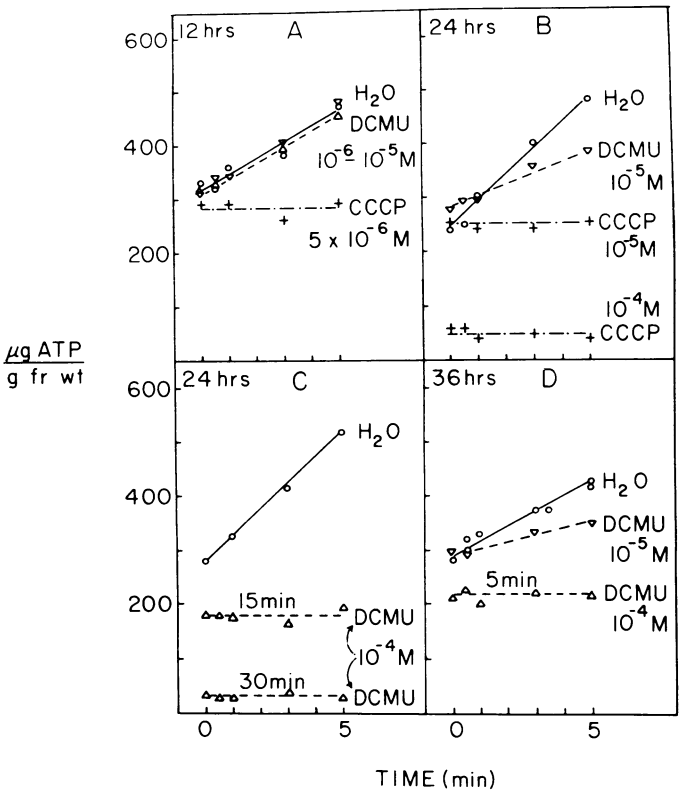


FIG. 1. ATP level in leaf discs during irradiation with white light ($1.5 \times 10^5$ ergs $cm^{-2}sec^{-1}$). A: From a leaf after 12 hr of far red light. Leaf discs were incubated in the solutions indicated for 20 min before the irradiation. B and C: Same with leaves after 24 hr of far red light. D: Same with a leaf after 36 hr of far red light. Irradiation with 12 hr dark period-12 hr light period and measurements were taken at the end of the last light period.

Table I. *Oxygen Exchange, Chlorophyll Content, and Rates of Respiration and Photosynthesis of Leaves after Various Times in Far Red Light*

Photosynthesis was measured as the difference in the rate of oxygen exchange with leaf discs in white light ($1.5 \times 10^5$ ergs $cm^{-2}$ $sec^{-1}$) from the rates obtained in the dark before and after the irradiation.

| Time in Far Red Light | Oxygen Exchange | | Chlorophyll Content | Respiration | Photosynthesis |
|---|---|---|---|---|---|
| | Dark | Light | | | |
| *hr* | *µmole $O_2$ consumed/g dry wt·hr* | | *µg chl/g dry wt* | *µmole $O_2$ consumed/g dry wt·hr* | *µmole $O_2$ evolved/mg chl·hr* |
| 12 | 272 | 272 | 89 | 272 | 0 |
| 24 | 250 | 228 | 153 | 250 | 190 |
| 36 | 208 | 63 | 254 | 208 | 601 |
| 48 | 149 | −177 | 460 | 149 | 715 |

was determined from a calibration curve made with known amounts of ATP.

Respiration and photosynthesis were determined by measuring oxygen changes manometrically in Warburg vessels with a Gilson differential respirometer (Gilson Medical Electronics, Inc., Milwaukee, Wisc.). The side arms contained 3 ml of a 3 M carbonate buffer ($KHCO_3/K_2CO_3 = 4$) plus 2 mg of carbonic anhydrase. Thus, atmospheres of approximately 3% $CO_2$ in air were obtained (18). Leaf discs of about 50 to 100 $mm^2$ were placed in the main compartment. Oxygen exchange was measured first in the dark for 30 min, with readings taken every 10 min, then in white light ($1.5 \times 10^5$ ergs $cm^{-2}$ $sec^{-1}$ from Unitron LKR microscope illuminator filtered through 20 cm of water) for 30 min, and finally in the dark for 30 min. The gas exchange during the first 10 min after changing from light to dark or dark to light was not used to compute rates of exchange. The rates of respiration obtained during the two dark periods always agreed and were used as the base from which to calculate the rate of photosynthetic oxygen evolution. For experiments on the effects of CCCP on photosynthesis and respiration, leaf discs were vacuum-infiltrated in the inhibitor solutions for 30 min in darkness prior to the experimental run. With DCMU, incubation (without vacuum infiltration) was used.

## RESULTS

Photophosphorylation was found in the 8-day-old dark-grown bean leaves after 12 hr of far red light (Fig. 1A). The ATP content of the leaves increased linearly during the first 5 min of irradiation with white light but began to decrease at longer irradiation times. Our assay procedure was limited to measuring the increase of ATP in the leaves during the first 5 min of irradiation. At the stage of development reached in 12 hr of far red light, photophosphorylation was not affected if the leaf segments were incubated with $10^{-6}$ or $10^{-5}$ M DCMU for 20 min, but was totally inhibited when the segments were incubated with $5 \times 10^{-6}$ M CCCP (Fig. 1A). At this stage no oxygen evolution was detected during an irradiation with white light (Table I).

After 24 hr of far red light (a 12-hr dark period intervened between two 12-hr irradiation periods) oxygen evolution was detected when the leaves were irradiated with white light (Table I). Photophosphorylation at this stage (Fig. 1B) was partially inhibited by incubation with $10^{-5}$ M DCMU. DCMU at $10^{-6}$ M and $10^{-5}$ M gave the same degree of inhibition of photophosphorylation, and both concentrations inhibited oxygen evolution at this stage and at later stages (Table II). CCCP at $10^{-5}$ M completely inhibited photophosphorylation but did not affect the initial level of ATP. The initial level of ATP, however, was markedly lowered after the leaf segments were incubated with $10^{-4}$ M CCCP, presumably because of uncoupling or inhibition of oxidative phosphorylation at the higher concentration. At the 24-hr stage (Fig. 1C), $10^{-4}$ M DCMU completely inhibited photophosphorylation, and with longer incubation time (30 min) the initial level of ATP was lowered. Complete inhibition of photophosphorylation by $10^{-4}$ M DCMU was also observed after 12 hr of far red light.

After 36 hr of far red light (with 12-hr dark periods following the first two 12-hr irradiation periods) the rate of oxygen evolution had increased almost to the compensation point

Table II. *Relative Rates of Photosynthesis and Respiration of Leaves Greened 36 hr or 60 hr in Far Red Light and Infiltrated 20 min with Solutions of DCMU*

| DCMU | Photosynthesis | | Respiration | |
|---|---|---|---|---|
| | 36 hr | 60 hr | 36 hr | 60 hr |
| *M* | | | | |
| 0 | 100 | 100 | 100 | 100 |
| $10^{-7}$ | — | 84 | — | 99 |
| $3 \times 10^{-7}$ | 68 | 73 | 100 | 101 |
| $10^{-6}$ | 2 | 4 | 102 | 98 |
| $10^{-5}$ | 0 | 0 | 100 | 103 |
| $10^{-4}$ | — | — | 72 | — |

(Table I) while the net rate of photophosphorylation decreased (Fig. 1D). The apparent decrease in photophosphorylation as the leaves develop in far red light was noted previously (4) and was ascribed to the greater utilization of ATP in the later stages. Photophosphorylation in the 36-hr leaves shows approximately the same sensitivity to DCMU and CCCP as was found with the 24-hr leaves.

Table I shows that respiration of the leaves decreases with time in far red light. A similar decrease of respiration was also found with leaves kept in the dark over the same periods. Although the data in Figure 1 do not demonstrate the effect, the initial level of ATP was also found to decrease with time (days) in far red light (4).

The effects of DCMU on photosynthesis (oxygen evolution) and respiration (oxygen uptake) at two stages of development are shown in Table II. DCMU inhibited oxygen evolution 97% at $10^{-6}$ M and 100% at $10^{-5}$ M. DCMU at $10^{-4}$ M inhibited respiration about 30%.

The time of appearance of the capacities for photophosphorylation and oxygen evolution was examined more closely in the 8-day-old bean leaves which were greened in continuous far red light without the 12-hr dark periods. Figure 2 shows that photophosphorylation began abruptly at 12 hr and that during the early stages, between 12 and 24 hr, the photophosphorylation was insensitive to $10^{-5}$ M DCMU. At 24 hr, the capacity for oxygen evolution appeared, and photophosphorylation became partially sensitive to $10^{-5}$ M DCMU.

A similar pattern for the development of photophosphorylation and oxygen evolution was found with 8-day-old dark-grown bean leaves greened in white light but over much shorter periods of time (Fig. 3). Photophosphorylation was detected after 15 min in white light, and oxygen evolution was detected after 1 hr. Photophosphorylation at times up to 45 min was not sensitive to $10^{-5}$ M DCMU, but a partial sensitivity to DCMU was found at longer times after oxygen evolution appeared.

The effects of CCCP on photosynthesis and respiration are shown in Table III for leaves greened in far red light. It was found with the large leaf segments (50–100 mm$^2$) used for the oxygen measurements that vacuum infiltration was required to get the CCCP solution into the leaf tissue. With the small segments (4–9 mm$^2$) used in the phosphorylation measurements, incubation was sufficient. Vacuum infiltration of $10^{-5}$ M CCCP into leaf segments greened for 36 hr in far red light completely inhibited photosynthesis ($CO_2$ fixation cannot proceed in the absence of photophosphorylation) while incubation of the leaf segments with $10^{-5}$ M CCCP had relatively little effect (11% inhibition of oxygen evolution). However, as the leaves developed, photosynthesis became increasingly resistant to $10^{-5}$ M CCCP even with vacuum infiltration. Table III shows the effects of different concentrations of CCCP on the photosynthesis and respiration of leaves which had been greened for 36 and 60 hr in the 12 hr light-12 hr dark cycles.

Table III. *Relative Rates of Photosynthesis and Respiration of Leaves Greened 36 hr or 60 hr in Far Red Light and Infiltrated 30 min with Solutions of CCCP*

| CCCP | Photosynthesis | | Respiration | |
|---|---|---|---|---|
| | 36 hr | 60 hr | 36 hr | 60 hr |
| M | | | | |
| 0 | 100 | 100 | 100 | 100 |
| $10^{-5}$ | 0 | 48 | 100 | 100 |
| $5 \times 10^{-5}$ | — | 13 | — | 82 |
| $10^{-4}$ | — | 0 | 57 | 57 |

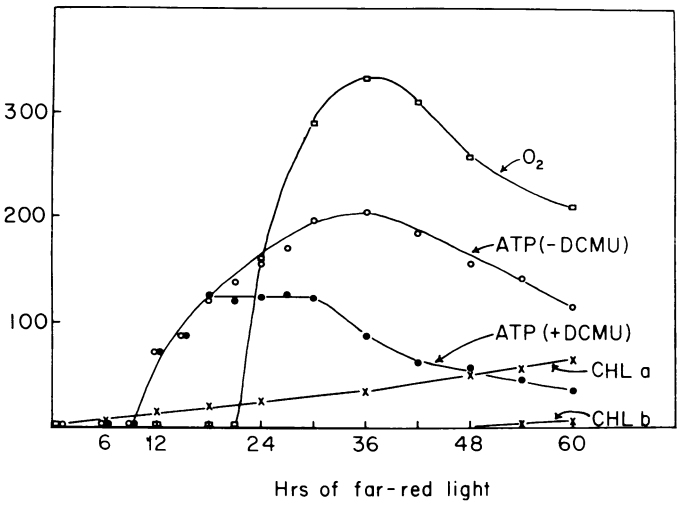


FIG. 2. The accumulation of chlorophylls *a* and *b* (μg chlorophyll/g fresh wt), the rate of oxygen evolution in white light (μmole $O_2$/mg chl·hr), and the rate of phosphorylation in white light (μg ATP/g fresh wt ·5 min). ○: In the absence of DCMU; ●: after incubation with $10^{-5}$ M DCMU as a function of time in continuous far red light.

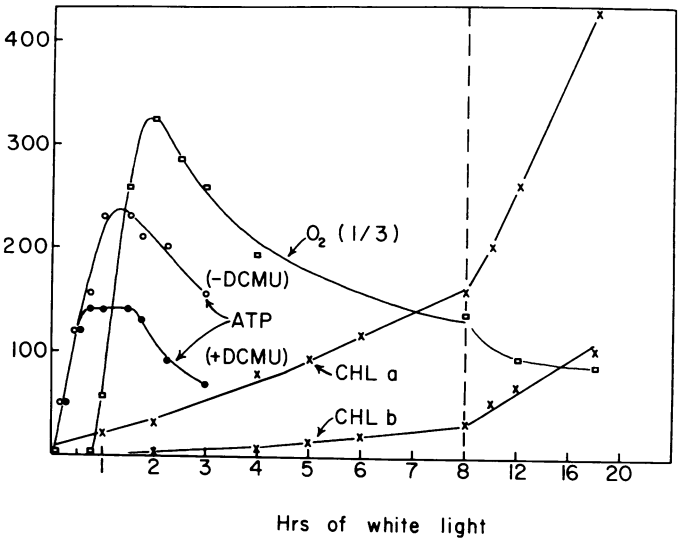


FIG. 3. Same as Figure 2 but with leaves greening in continuous white light. Values of rates of oxygen evolution reduced by one-third to stay on scale. Peak value is 1000 μmole $O_2$/mg chl·hr.

Oxygen evolution was inhibited 52% by $10^{-5}$ M CCCP in the 60 hr leaf whereas inhibition was complete in the 36 hr leaf. The same effect was found with 8-day-old dark-grown leaves greened in white light. Photosynthesis at very early stages of development was completely inhibited by $10^{-5}$ M CCCP but after 18 hr of greening, infiltrating the leaves with $10^{-5}$ M CCCP inhibited photosynthesis by only 15 to 20%.

## DISCUSSION

The measurement of photophosphorylation with intact leaves during development was feasible because of the low drain on ATP at early stages of development. At later stages of development ATP was utilized almost as rapidly at it was produced so that pool size measurements of ATP provided a less sensitive assay of photophosphorylation. Santarius and Heber (17) showed that there were transient increases in the

ATP pool in mature leaves at the beginning of an irradiation: the ATP increased rapidly during the first 30 sec and then decreased in the next 30 sec to a steady state level that was still higher than the dark level. The immature leaves may be showing a similar kind of transient but more prolonged increase with the maximum appearing at about 5 min.

In the studies during development it was possible to distinguish both cyclic and noncyclic photophosphorylation. (Forti and Parisi [6] previously demonstrated cyclic photophosphorylation in mature leaves during irradiation in the presence of CMU by pool size measurements.) Photophosphorylation appeared well before oxygen evolution in the developing leaves, and, at these early stages, photophosphorylation was characteristic of the cyclic process, both by its independence from oxygen evolution and by its insensitivity to $10^{-5}$ M DCMU. At later stages when the oxygen evolution was present, photophosphorylation was partially sensitive to DMCU concentrations which inhibited oxygen evolution. The simultaneous appearance of noncyclic photophosphorylation and oxygen evolution showed that phosphorylation sites associated with the noncyclic pathway were competent at the time that the electron transport commenced.

The pattern of emerging activities was very similar between leaves greened in far red light and those greened in white light even though the time base was markedly different (see Figs. 2 and 3). Since the rates of development are so different in the two greening regimes, we tend to interpret common features to be inherent characteristics of the development process rather than fortuitous correlations. For instance, the rate of oxygen evolution expressed on a chlorophyll basis goes through a maximum early in development and decreases as the leaf matures. We interpret this to indicate the early appearance of small photosynthetic units with the accumulation of bulk chlorophyll in those units as the leaf matures. Cyclic photophosphorylation precedes noncyclic, showing that PS1 activity appears before PS2 activity in the course of development. Cyclic photophosphorylation reaches a maximal rate (which is the same in both white light and far red light) at about the time that oxygen evolution commences. Cyclic photophosphorylation then remains constant while noncyclic photophosphorylation increases. The curves for photophosphorylation in the absence of DCMU look as if they would reach a plateau at about twice the maximal rate that was found in the presence of DMCU, but the increasing rate of ATP utilization starts to limit the net rate of phosphorylation. The latter correlations are much more tenuous but could indicate the formation of two sites of phosphorylation in a linear electron transport chain.

DCMU entered the intact leaves with no difficulty. The sensitivity of oxygen evolution in intact leaves to low concentrations of DCMU was essentially the same as the Hill reaction of chloroplasts. At higher concentrations, $10^{-4}$ M, DMCU inhibited cyclic photophosphorylation as well. Good (7) reported that DCMU at concentrations above $10^{-4}$ M inhibited the PMS-mediated cyclic photophosphorylation in chloroplasts, and Izawa (11) presented evidence that the inhibition was due to uncoupling of phosphorylation rather than inhibition of cyclic electron transport. DCMU at $10^{-4}$ M also partially inhibited respiration and lowered the level of ATP in the cells. An inhibition of respiration in yeast by $10^{-3}$ M CMU has been reported previously (14). Low concentrations of CCCP ($10^{-5}$ M) were effective in uncoupling the photophosphorylation in intact leaf tissue at early stages of development when the photophosphorylation was most prominent. At early stages of oxygen evolution, photosynthesis was completely inhibited by $10^{-5}$ M CCCP because of the uncoupling of photophosphorylation, but at later stages, when the photosynthetic apparatus was better developed, CCCP was less effective. At higher concentrations, $10^{-4}$ M, CCCP partially inhibited respiration, similar to the effect of $10^{-4}$ M DCMU.

The appearance of cyclic photophosphorylation in the 8-day-old etiolated leaves after only 15 min of white light was earlier than we had expected. The appearance of oxygen evolution after 1 hr of white light is fairly typical of young etiolated tissue. Unfortunately, we do not have electron micrographs of the tissue greened in white light, but, from the work of others, little structural development other than the dispersal of the prolamellar body into vesicles would be expected during the first 15 min. Electron micrographs of the 8-day-old etiolated bean leaves greened in far red light have been compared with the activities at different stages (4). The correlations between structure and function in the far red greened leaves indicate that cyclic photophosphorylation commences before the formation of primary thylakoids, perhaps while vesicles are fusing into primary thylakoids, and that oxygen evolution and noncyclic photophosphorylation require the primary thylakoids but not grana.

Several laboratories have examined photosynthetic activities of plastid preparations isolated from etiolated leaves after various times of greening. Such studies, however, have the inherent uncertainty of possible artifacts introduced by the isolation procedures. Gyldenholm and Whatley (8) and Bradbeer *et al.* (3) measured photophosphorylation with plastids from 14- to 16-day-old dark-grown bean leaves after different times of development in white light. They detected cyclic photophosphorylation with PMS as cofactor after 10 hr of greening, noncyclic photophosphorylation with ferricyanide as the Hill acceptor at 15 hr, and noncyclic photophosphorylation with NADP as the acceptor and either water or DCPIP plus ascorbate as the electron donor at 20 hr. They concluded that PS1 became functional in the developing leaves before PS2 and that the last part of the photosynthetic electron transport system to be completed was that between PS1 and NADP. Anderson and Boardman (1) also found the NADP photoreduction appeared later (after 16 hr of greening) than ferricyanide photoreduction (6 hr) in plastid preparations from developing bean leaves. However, Hung *et al.* (10), using plastid preparations from dark-grown barley in which they claimed a high percentage of intact plastids, found that NADP photoreduction appeared first, after 2 hr of greening, while cyclic photophosphorylation with PMS appeared after 4 hr and noncyclic photophosphorylation with NADP as acceptor after 10 hr.

The relatively late appearance of NADP photoreduction in the plastid preparations from bean leaves (1, 3, 8) could be due to a soluble component needed for the reduction of NADP which was lost more readily from immature plastids. Bradbeer *et al.* (3) presented evidence that NADP photoreduction occurred in the intact leaves at earlier stages of development than could be detected with the isolated plastid preparations, and they suggested that the same was true for photophosphorylation. If inactivations do occur during the isolation procedures at early stages of development, then conclusions as to the relative appearance of PS1 and PS2 may be questioned because PS2 is known to be much more fragile than PS1. The most susceptible site in the electron transport system appears to be the site between water and PS2 which can be inactivated by tris buffer (20), chaotropic agents (13), heat (12, 21), and aging. The electron transport reactions of plastids from developing leaves could be examined with artificial electron donors specific for PS2, but such experiments have not been reported. However, the conclusion by Gyldenholm and Whatley (8) that PS1 appears before PS2 in develop-

ment is confirmed by the work on the intact leaves reported here.

The measurements on the intact leaves indicate that noncyclic photophosphorylation appears as soon as oxygen evolution while the work on the barley plastids (10) places the onset of noncyclic photophosphorylation well after the beginning of NADP photoreduction. The late appearance of noncyclic photophosphorylation in the barley plastid preparation and the relatively low ATP to NADPH ratios after it does appear (10) suggest that coupling factors or other phosphorylation components may have been lost or inactivated in the young barley plastids even though they appear intact.

Attempts have also been made to correlate structural changes of the plastids developing in the leaves with the appearance of photosynthetic activities of plastid preparations isolated from the leaves (1, 8). It has generally been concluded that a good correlation exists between the development of Hill activity of the plastids from higher plants and the development of grana in the plastids. This correlation, however, may merely reflect the greater stability of grana against inactivation of PS2. The activity may have been present in the leaves before the appearance of grana but was inactivated by the isolation procedures. In such experiments the time of onset of oxygen evolution by the intact leaves should also be determined and compared with the onset of Hill activity by the plastids.

Chloroplasts from bundle sheath and mesophyll cells of $C_4$ plants have been used to demonstrate a correlation between grana and PS2 activity (5, 19). Later PS2 activity was found in the agranal bundle sheath chloroplasts, but these chloroplasts appeared to lack a component needed to connect electron transport between PS2 and PS1 (2). However, the possibility exists that the bundle sheath chloroplasts were inactivated by the isolation procedures (19) or by the procedures needed to get electron acceptor and donor compounds into the cells (5). At one stage of the isolation procedures, intact bundle sheath cells attached to vascular tissue are obtained free of contamination by mesophyll cells. The above considerations suggest that these cells should be examined for photosynthetic oxygen evolution prior to their fractionation for chloroplasts.

Electron transport cannot be separated from photophosphorylation *in vivo* because the reduction of the electron acceptor, $CO_2$, requires ATP. Thus, the onset of oxygen evolution might reflect the appearance of either an electron transport step or a reaction needed for phosphorylation. If activities could be demonstrated in plastids from immature leaves before they appeared *in vivo*, *e.g.*, if the Hill activity in the plastids appeared before oxygen evolution by the whole leaf, the plastid preparations might provide valuable information about the development processes. However, if plastid activities lag behind the whole cell activities, inactivations which occurred during the plastid isolation and sequence of developmental changes may be confounded.

Studies of the development of photosynthetic activities in intact leaves have the advantage of avoiding artifacts due to the isolation procedure but have the disadvantage that only changes in the pool size of ATP can be measured rather than the rate of synthesis of ATP. However, the sensitivity of photophosphorylation in the immature leaves to low concentrations of CCCP, the insensitivity of the reaction to $10^{-5}$ M DCMU before the appearance of oxygen evolution and the partial sensitivity after oxygen evolution, and the apparent distinguishability of photophosphorylation from oxidative phosphorylation are strong evidence that photosynthetic reactions are involved directly in the phosphorylation rather than indirectly through some unknown mechanisms by which light might alter the pool sizes of the nucleotides.

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