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# LIGHT DRIVEN REDOX CHANGES OF CYTOCHROME *f* AND THE DEVELOPMENT OF PHOTOSYSTEMS I AND II DURING GREENING OF BEAN LEAVES

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SUMMARY

1. Light-induced oxidation of cytochrome *f* in greening bean leaves was first observed after 90 min of illumination. Intense red light was needed to drive the oxidation. At 2 h cytochrome *f* oxidation was driven by 675 nm monochromatic light, but not by 703 nm light. By 2.5 h the oxidation was driven by 703 nm light and now 675 nm light was less effective. Both the extent and the initial rate of cytochrome *f* oxidation in 675 nm light were increased more than 10-fold by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). These results show that both Photosystems I and II are active at 2.5 h, which correlates with the end of the lag phase in chlorophyll synthesis. Photosystem I is active ahead of Photosystem II.

2. If etiolated leaves are photoconverted and then returned to darkness for at least 2.5 h, Photosystem I is active at the second photoconversion and Photosystem II develops within a period of minutes. In the presence of DCMU, actinic light of 688 nm is more effective than 667 nm or 675 nm light in photooxidizing cytochrome *f*, even though the absorption maximum of the leaf is at 672 nm.

3. Photosystem II activity, as measured by the ability of 675 nm light to hold cytochrome *f* mainly in the reduced state was not dependent on the presence of grana or cytochrome *b*-559.

## INTRODUCTION

The 'Z' scheme of photosynthetic electron transport consists of two light reactions connected in series by a number of redox carriers[1]. Among the components of Photosystem II in the mature leaf is cytochrome *b*-559. In the etiolated bean leaf cytochrome *b*-559 is absent and it it is not formed until the leaves have been illuminated for some hours[2]. On the other hand cytochrome *f* and cytochrome $b_6$ which are components of Photosystem I are present in the etiolated leaf[2-4]. Thus, there is the possibility that Photosystem I could become active before Photosystem II during greening.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulphate; TCIP, 2,3',6-trichlorophenolindophenol.

Hill reaction activity in plastids isolated from greening leaves using ferricyanide or 2,6-dichlorophenolindophenol (DCIP) as electron acceptor (Photosystem II activity) has been detected after 5–10 h of greening[5–7]. On the other hand, $NADP^+$ reduction either with water or ascorbate–DCIP as electron donor was not observed until 16 h of greening[6]. GYLDENHOLM AND WHATLEY[8] found with bean plastids that cyclic phosphorylation catalyzed by phenazine methosulphate (PMS) (Photosystem I activity) appeared after 5 h of illumination. This preceded the appearance (at 10 h) of non-cyclic phosphorylation with ferricyanide as electron acceptor. There was a further delay before the detection of non-cyclic phosphorylation with $NADP^+$ as electron acceptor[8]. With plastids from greening pea seedlings, the appearance of Hill activity with 2,3',6-trichlorophenolindophenol (TCIP) as oxidant correlated with the formation of grana, which occurred between 4 and 8 h of greening[7,9].

However, studies with leaves suggest that both photosystems may be competent much earlier than indicated by the work with isolated plastids. Oxygen evolution has been detected within 2–3 h of illumination of dark-grown maize and barley seedlings[10]. Fluorescence measurements on greening bean leaves[11] also suggest that both photosystems are functional after 2–3 h. Onset of functional photosystems in leaves appears to correlate with the end of the lag phase and the beginning of rapid synthesis of chlorophyll.

We have investigated the development of the photosystems in greening bean leaves by studying the light-driven redox change of cytochrome *f*. In the Z scheme, cytochrome *f* is situated between the light reactions, closer to Photosystem I than Photosystem II; it is oxidized by Photosystem I and reduced by Photosystem II. Since it is possible to detect a change in absorbance equivalent to $10^{-2}$ nmole of cytochrome *f* the method is a sensitive indicator of photosynthetic electron flow.

## MATERIALS AND METHODS

Bean plants (*Phaseolus vulgaris* "Brown Beauty") were grown in darkness in vermiculite at 25 ± 1° for 10–15 days. At this stage the primary leaves are well developed (2.5 cm × 1.2 cm). The etiolated plants were exposed to light from white fluorescent tubes (intensity at leaf surface, 400 ft candles). The plants either remained in continuous light or were returned to darkness after 3 min.

Primary leaves were cut from the plants and placed between two thin sheets of perspex for the spectrophotometric observations. Light-induced absorbance changes were measured with an Aminco-Chance dual-wavelength spectrophotometer (American Instrument Corp. Silver Springs Maryland, U.S.A.) fitted with a side-illumination attachment. Actinic light was provided by a 650-W tungsten iodine lamp and passed through a 3-cm layer of water and appropriate glass and interference filters (half-band width, 7–10 nm). Light intensities at the position of the sample were measured with a YSI Kettering Radiometer. The photomultiplier was protected by a Corning glass filter 4-96. Treatment of leaves was carried out by pricking the leaf surface with a needle prior to soaking in $10^{-5}$ M DCMU for 15 min. A difference molar extinction coefficient of $2.5 \cdot 10^4$ at 554 nm was used for cytochrome *f*.

Absorbance spectra of etiolated leaves were measured as described by KAHN *et al.*[12].

For electron microscopy leaf pieces, 1 mm × 2 mm, were fixed in 4% gluta-

raldehyde in 0.025 M phosphate buffer (pH 7.2) overnight at 4°. They were washed in buffer and post-fixed in 2 % osmium tetroxide in 0.1 M phosphate buffer for 3 h at 20°. After washing and dehydration, the leaf pieces were embedded in an araldite–epon mixture and sectioned. Sections were stained with uranyl acetate, followed by lead hydroxide.

## RESULTS

### *Absorbance changes in leaves held continuously in white light*

Immediately after photoconversion of the protochlorophyll(ide), illumination of a bean leaf with high intensity red light (Corning 2-64 filter $\lambda > 660$ nm, intensity $10^6$ erg·cm$^{-2}$·sec$^{-1}$) occasionally caused a small decrease in absorbance ($< 0.0004$) at 554 nm (reference wavelength, 570 nm). However, this could not be attributed to the oxidation of cytochrome *f* and appeared to be a non-specific bleaching throughout the 540–570 nm region. After 4–6 h light when the leaves have a distinct green tinge, exposure to strong red light produced a biphasic absorbance decrease at 554 nm. A monophasic decrease was observed in 703 nm light and a smaller decrease in red light (675 nm). After treatment with DCMU intense red light caused a monophasic decrease in absorbance at 554 nm, and in 675 nm light the absorbance decrease was much larger than in the control (Fig. 1). The light *minus* dark difference spectrum showed a minimum at 554 nm characteristic of the $\alpha$-band of cytochrome *f*. Thus, cytochrome *f* is more oxidised in 703 nm light, which is absorbed more strongly by Photosystem I than in 675 nm light which is absorbed about equally by Photosystems I and II. In 675 nm light, cytochrome *f* is kept mainly in the reduced state by Photo-

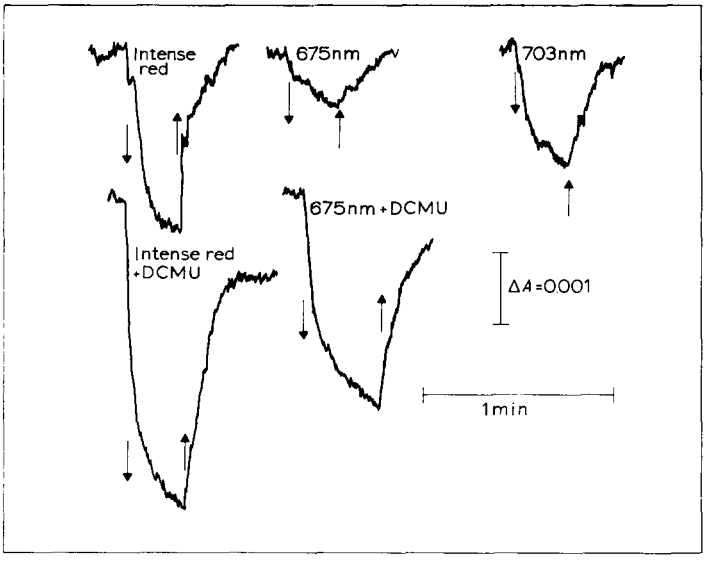


Fig. 1. Light-induced absorbance changes at 554 nm (reference wavelength 570 nm) in a bean leaf from a seedling which was greened in white light for 4 h. The downward arrows indicate actinic light on and the upward arrows light off. The two lower traces were made after treating the leaf with $1 \cdot 10^{-5}$ M DCMU. Light intensities were, Intense red, Corning filter 2-64 $\lambda > 660$ nm, $1 \cdot 10^6$ erg·cm$^{-2}$·sec$^{-1}$; 675 nm, $1.5 \cdot 10^4$ erg·cm$^{-2}$·sec$^{-1}$; 703 nm, $1.4 \cdot 10^4$ erg·cm$^{-2}$·sec$^{-1}$.

system II, but in the presence of DCMU electron flow from Photosystem II to cytochrome *f* is inhibited and cytochrome *f* is then oxidised in 675 nm light. These results show that after 4–6 h continuous illumination at the time when chlorophyll synthesis is moving out of the lag phase[6], both Photosystems I and II are active and balanced in the same way as in the mature green leaf.

TABLE I

PHOTOOXIDATION OF CYTOCHROME *f* IN BEAN LEAVES DURING EARLY GREENING

Dark-grown seedlings were illuminated in continuous white light for the times indicated. Photooxidation of cytochrome *f* was examined in intense red light (Corning 2-64 filter, $\lambda > 660$ nm) of intensity, $10^6$ erg·cm$^{-2}$·sec$^{-1}$ or in 675 nm light of intensity $1.5 \cdot 10^4$ erg·cm$^{-2}$·sec$^{-1}$. All the decrease in absorbance at 554 nm (reference wavelength, 570 nm) has been attributed to cytochrome *f*, but this may be an overestimate.

| *Illumination time (min)* | *Number of leaves examined* | *Number of leaves showing cytochrome f oxidation* | | *Extent of cytochrome f oxidation (nmole·cm$^{-2}$)* |
|---|---|---|---|---|
| | | *In intense red light* | *In 675 nm light* | |
| 50–70 | 5 | 0 | 0 | — |
| 70–90 | 5 | 1 | 0 | 0.091 |
| 90–110 | 5 | 5 | 0 | 0.01–0.067 |
| 120–130 | 2 | 2 | 1 | 0.04, 0.09 |

Following these preliminary experiments further observations were made on leaves which had been illuminated for less than 4 h. We have been able to positively detect cytochrome *f* oxidation in only 2 leaves from many different experiments in which the plants had been illuminated for less than 1 h. Table I shows some typical results from experiments in which the plants were illuminated for 1–2 h. Between 70–90 min, five leaves were examined and light driven oxidation of cytochrome *f* was observed in one leaf. After illumination for 90–110 min all leaves examined showed

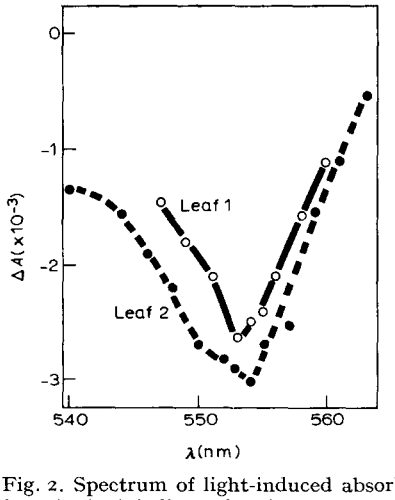


Fig. 2. Spectrum of light-induced absorbance changes in bean leaves from seedlings illuminated for 2 h. Actinic light; Corning 2-64 filter, $\lambda > 660$ nm, $1 \cdot 10^6$ erg·cm$^{-2}$·sec$^{-1}$.

photooxidation of cytochrome *f* in intense red light, but not in 675 or 703 nm actinic light. After 120–130 min, cytochrome *f* oxidation in one of the two leaves examined was driven by 675 nm light, but not by 703 nm light. The spectrum of the light-driven absorbance changes in leaves greened for 2 h is shown in Fig. 2. There is a minimum at 553–554 nm which coincides with the α-peak of cytochrome *f*.

After illumination for 2.5 h almost all leaves examined show absorbance changes characteristic of cytochrome *f* oxidation, which can now be driven by 703 nm light. Red light (675 nm) is now less effective than 703 nm light. Intense red light causes the biphasic oxidation as shown in Fig. 1. These results indicate that both photosystems were operative and this conclusion was confirmed by the effect of DCMU (Table II). Both the extent of cytochrome *f* oxidation and the initial rate of oxidation in 675 nm light were increased more than 10-fold in the presence of DCMU. In 703 nm actinic light, the stimulation of cytochrome *f* oxidation by DCMU was much smaller.

TABLE II

EFFECT OF DCMU ON CYTOCHROME *f* OXIDATION

Seedlings were greened for 2.5 h in continuous white light. Leaves were treated with DCMU as indicated in MATERIALS AND METHODS. Intensities of actinic light (in erg·cm$^{-2}$·sec$^{-1}$) were: $1.5 \cdot 10^4$ at 675 nm; $1 \cdot 10^4$ at 688 nm, $1.4 \cdot 10^4$ at 703 nm and $1 \cdot 10^6$ for intense red light (Corning 2-64 filter, $\lambda > 660$ nm).

| *Actinic light* | *Extent of cytochrome f oxidation (nmole·cm$^{-2}$)* | | *Initial rate of cytochrome f oxidation (nmole·cm$^{-2}$·min$^{-1}$)* | |
|---|---|---|---|---|
| | *Control* | *+ DCMU* | *Control* | *+ DCMU* |
| 675 | 0.009 | 0.091 | 0.12 | 1.73 |
| 688 | 0.016 | 0.091 | 0.17 | 1.27 |
| 703 | 0.016 | 0.029 | 0.08 | 0.23 |
| Intense red | 0.037 | 0.117 | — | — |

The conclusion is reached that the ability to photooxidize cytochrome *f* (by Photosystem I) and photoreduce cytochrome *f* (by Photosystem II) develops rapidly after 2–3 h of greening. This period coincides with the end of the lag phase of chlorophyll synthesis[6]. Photoreduction of cytochrome *f* by Photosystem II is inhibited by DCMU. From these greening experiments in continuous light, we conclude that Photosystem I activity develops ahead of Photosystem II, but only by about 30 min.

*Absorbance changes in leaves exposed to white light and returned to darkness*

Dark-grown seedlings were illuminated with white light for 3 min and returned to darkness for varying periods. In the experiment reported in Fig. 3a, a leaf was harvested after a 6-h dark period and transferred under a green safe light to the Aminco-Chance spectrophotometer where it was illuminated intermittently with high intensity red light. The large absorbance decrease at 554 nm, observed at the initial illumination with the red light is due partly to the photoconversion of protochlorophyllide which is synthesized during the 6-h dark period. The reversible component of the absorbance change, which is observed on turning off the light is due to cytochrome *f*. A second illumination with the intense red light produces an absorbance

change due only to cytochrome *f*, as protochlorophyllide is synthesized slowly in the dark. With successive pulses of red light, the extent of the absorbance decrease at 554 nm is progressively smaller, and the biphasic character more pronounced. For example, at the sixth illumination, the absorbance decrease at 554 nm is less than one-half of the absorbance decrease at the second illumination. Treatment of the leaf with

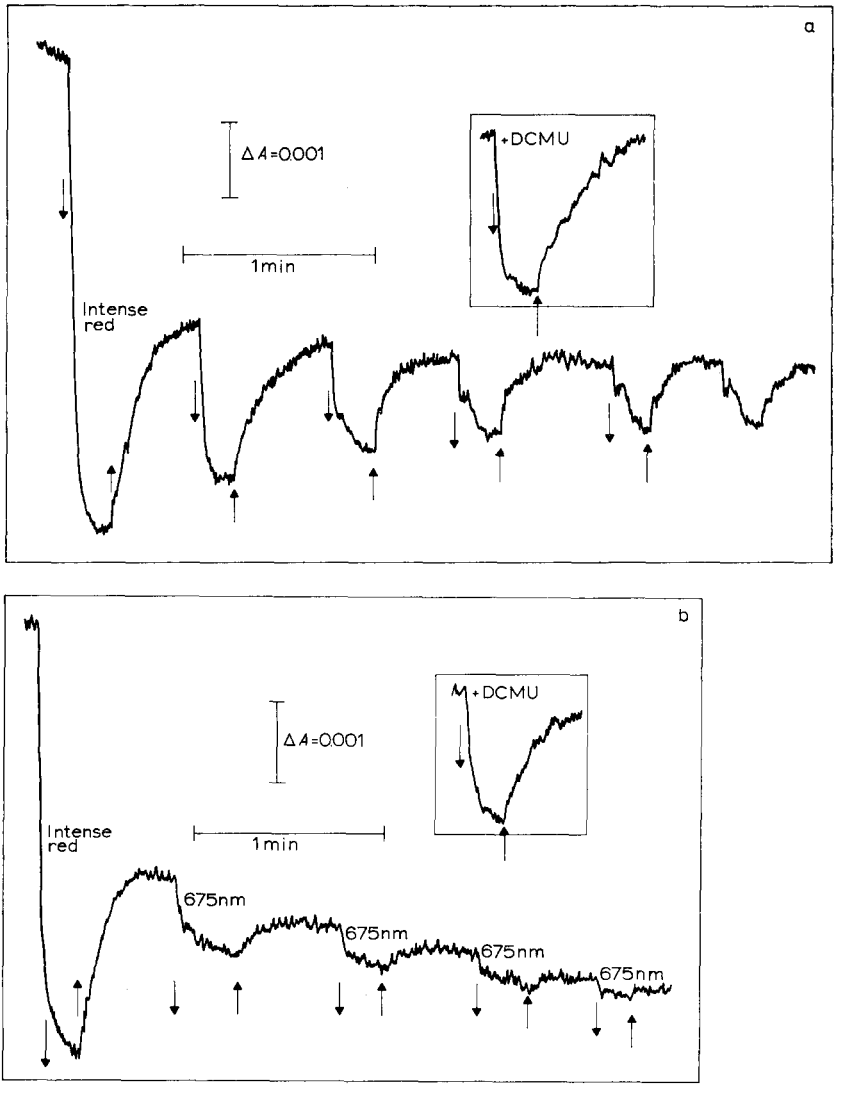


Fig. 3. Light-induced absorbance changes at 554 nm (reference wavelength, 570 nm) in bean leaves from seedlings which were illuminated for 3 min in white light and returned to darkness. (a) The leaf was harvested after a dark period of 6 h and given pulses of intense red light ($\lambda > 660$ nm; intensity $1 \cdot 10^6$ erg·cm$^{-2}$·sec$^{-1}$ as indicated by the arrows. (b) The leaf was harvested after a dark period of 16 h and given an initial pulse of intense red light and then pulses of 675 nm light of intensity $1.5 \cdot 10^4$ erg·cm$^{-2}$·sec$^{-1}$, as shown by the arrows. In both (a) and (b), the large absorbance decrease at the first pulse of light is due to the photoconversion of protochlorophyllide. The inset trace shows the absorbance changes in the same leaf after a subsequent treatment with $1 \cdot 10^{-5}$ M DCMU.

DCMU after the sixth illumination increased the extent of the absorbance change at 554 nm to the level observed at the second illumination. As described for leaves greened in continuous white light (Fig. 1), DCMU abolished the biphasic kinetics of the absorbance decrease at 554 nm.

From the experiment in Fig. 3a we conclude that Photosystem I develops in the dark period, subsequent to the initial photoconversion of protochlorophyllide. Photosystem I is active at the first illumination following the dark period, and Photosystem II develops rapidly within a few minutes. The experiment shown in Fig. 3b, in which the intermittent illuminations were carried out in 675 nm monochromatic light after a 16-h dark period, supports this conclusion. At the fifth illumination, the absorbance decrease at 554 nm was just detectable, and DCMU had a marked effect on increasing the extent of the absorbance change.

The minimum period in the dark in order to observe absorbance changes similar to those shown in Fig. 3 was about 3 h. This period corresponds to the lag phase in chlorophyll synthesis, and to the appearance of Photosystem II activity in leaves illuminated continuously in white light (*cf.* Table II).

During some of our experiments in which leaves were returned to darkness for at least 3 h it was noted that the rate of oxidation of cytochrome *f* in the presence of DCMU was as rapid in 688 nm light as in 675 or 667 nm light. This was somewhat surprising as at this time the *in vivo* absorption maximum of the leaf is at 672 nm. However, it was difficult to be certain of the result since the rate of photooxidation of cytochrome *f* may not be a linear function of light intensity.

This point was reinvestigated by measuring the rates of cytochrome *f* oxidation in the presence of DCMU over a 10-fold range of light intensities at each actinic wavelength (except for 714 nm). In this range of light intensity ($0.15 \cdot 10^4$–$1.5 \cdot 10^4$ erg·cm$^{-2}$·sec$^{-1}$), the rate of cytochrome *f* oxidation was proportional to light intensity. The rates at all intensities were normalised to an incident intensity of $10^4$ erg·cm$^{-2}$·sec$^{-1}$ to permit statistical confirmation of the relative effectiveness of 688

TABLE III

EFFECTIVENESS OF ACTINIC LIGHT FOR THE PHOTOOXIDATION OF CYTOCHROME *f*

Dark-grown seedlings were illuminated for 3 min in white light and returned to darkness for 6 h. Leaves were illuminated a second time and then treated with DCMU for 15 min as indicated in MATERIALS AND METHODS. Light-induced absorbance changes were measured over a 10-fold range of light intensities ($0.15 \cdot 10^4$–$1.5 \cdot 10^4$ erg·cm$^{-2}$·sec$^{-1}$). Results are normalised at each incident light intensity to $1 \cdot 10^4$ erg·cm$^{-2}$·sec$^{-1}$, and are given as the mean, standard deviation and number of experimental observations. The values for the absorbance ratios at 703 and 714 nm relative to 672 nm must be considered as rough approximations due to light scattering and the low absorbance. The absorbance at 672 nm of the intact leaves ranged from 0.05–0.10.

| *Actinic light (nm)*: | *667* | *675* | *688* | *703* | *714* |
|---|---|---|---|---|---|
| Initial rate of cytochrome *f* oxidation (nmoles·cm$^{-2}$·min$^{-1}$) | 1.80 ± 0.19 (6) | 1.86 ± 0.14 (5) | 2.01 ± 0.3 (7) | 0.62 ± 0.09 (5) | 0.17 ± 0.01 (3) |
| Ratio of leaf absorbance at actinic wavelength/leaf absorbance at 672 nm | 0.92 | 0.92 | 0.33 | 0.04 | 0.01 |

and 667 nm light. The results are shown in Table III. Actinic light at 688 nm is slightly more effective than 675 or 667 nm light in photooxidizing cytochrome *f* in the presence of DCMU, even though the leaf absorbance at 688 nm is only about one-third that at 675 and 667 nm. There was also a marked effect of DCMU on the rate of oxidation in 703 nm actinic light. The rate was increased 2–6-fold by DCMU as compared with that in control leaves.

*Ultrastructure of plastids*

As previous workers have described in detail the structural changes which occur in plastids during the greening of etiolated bean plants[10], a brief description only will be given of our observations. The etioplast usually contained a single crystalline centre surrounded by a few single lamellae outside the prolamellar body. After 3 min illumination and a further 60 min in light or dark, the crystallinity of the prolamellar body was lost and replaced by a ball of knotted tubules. This situation remained unchanged for a further 120 min. After 6 h continuous illumination the tubules in the prolamellar body were partly dispersed and the beginnings of grana formation were visible in the outer part of the plastid. In contrast, leaves returned to darkness for 6 h showed a close similarity to the etiolated leaf with the crystalline prolamellar bodies completely reformed. A second illumination with white light for 3 min after a dark period of 6 h did not cause a loss of crystallinity of the prolamellar bodies. We were unable to detect any ultrastructural features whose appearance correlated with the onset of the light-driven redox changes of cytochrome *f*.

## DISCUSSION

BONNER AND HILL[13] observed a light-induced oxidation of cytochrome *f* during the greening of etiolated mung bean leaves. Photooxidation of cytochrome *f* was detectable after 1 h, but the maximal response was after 3–4 h when the leaves were visibly green. They also reported that the oxidation of cytochrome *f* in partially greened leaves was driven by 700 nm light, but 640, 660 and 680 nm light were ineffective. The time of greening was not given in this experiment, but it seems likely that in light of the shorter wavelength Photosystem II was active in holding cytochrome *f* in the reduced state. Our results are in general agreement with those of BONNER AND HILL[13] but further suggest that Photosystem I becomes active ahead of Photosystem II. The development of Photosystem II activity, as measured by the relative ineffectiveness of 675 nm actinic light (compared to 703 nm light) in photooxidising cytochrome *f* in the absence of DCMU correlates with the end of the lag phase in chlorophyll synthesis. Oxidation of cytochrome *f* by Photosystem I, however, is observed before the end of the lag phase.

An interesting finding of the present studies is the rapid development of Photosystem II activity on the second photoconversion in leaves which were returned to darkness for 6 h after the initial photoconversion of protochlorophyllide. The nature of this process is as yet unknown. The ability to photooxidize cytochrome *f* by Photosystem I develops in the dark period following the first photoconversion. At the second photoconversion cytochrome *f* photooxidation and protochlorophyllide conversion take place simultaneously. We conclude, therefore, that the photooxidation of cytochrome *f* requires no chlorophyll apart from that formed at the first photo-

conversion. When bean leaves are illuminated initially, they contain approximately 25 $\mu$g chlorophyllide *a* per g fresh weight (or approximately 1 $\mu$g chlorophyllide *a* per $cm^2$ leaf area), and a molar ratio of chlorophyllide *a* per cytochrome *f* of about 3 (ref. 2). If returned to the dark for 6 h and then given a second photoconversion the chlorophyll content increases to approximately 40 $\mu$g/g fresh weight. The chlorophyll(ide) *a*/cytochrome *f* ratio is now about 5. Since about 30–50 % of the cytochrome *f* can be photooxidized by Photosystem I, and since Photosystem II develops rapidly at this time, the size of the light-harvesting assemblies of Photosystems I and II must be small compared with those in the green leaf. The chlorophyll synthesized during the first few hours following the lag phase may serve to increase the size of the lightharvesting assemblies rather than increasing the number of reaction centres.

Both photosystems are developed well ahead of the earliest time at which isolated plastids will carry out the Hill reaction with ferricyanide or DCIP as oxidants[6–8]. In the earlier studies from this laboratory[6], the plastids were isolated from leaf material which was comparable to that used in the present work. The time discrepancy between Hill reaction activity of isolated plastids from bean leaves and the observation of redox changes of cytochrome *f* indicative of Photosystem II activity might be explained if the electron donor to Photosystem II in the early stages of greening were not water but some endogenous donor. The same explanation may be given for the development of Photosystem II after 2 h greening as deduced from the kinetics of chlorophyll fluorescence[11]. An alternative explanation is that at this early stage, plastids lose their ability to evolve oxygen during isolation. In some recent experiments with greening bean leaves, oxygen evolution was first detected after 5–6 h of illumination (J. T. O. KIRK, unpublished observations).

Previously, it was shown that the onset of Hill activity in isolated pea plastids correlated with the formation of grana[7,9]. The present studies indicate that the photoreduction of cytochrome *f* is not dependent on the presence of grana. Leaves returned to darkness following the initial photoconversion reform crystalline prolamellar bodies. In continuous light, photoreduction of cytochrome *f* precedes formation of grana, which occurs between 6 and 10 h of illumination (N. A. PYLIOTIS, unpublished observations). It may also be noted that Photosystem II activity is not dependent on cytochrome *b*-559, as this cytochrome is not present in bean etioplasts and it is not formed during the first 6 h of illumination[2].

In *Chlamydomonas reinhardi*, photooxidation of cytochrome *f* was not observed during the first two hours of greening[14]. But, in contrast to our studies with beans Photosystem II activity as measured by the Hill reaction in cell fragments was detected before the photooxidation of cytochrome *f*. Cyclic photophosphorylation with phenazine methasulphate as cofactor was also observed before the photooxidation of cytochrome *f*.

Two of our observations on the effect of DCMU following the second photoconversion require further explanation. First, in many experiments with 703 nm actinic light, DCMU caused a 2–6-fold increase in the rate of photooxidation of cytochrome *f*. (In the fully greened leaf the increase would be only 10–20 %). This may have been due in part to further development of Photosystem I following the second photoconversion, but more probably indicates that 703 nm light is absorbed equally well by Photosystem I and II at this stage of development. Secondly, at equal

incident intensities cytochrome *f* can be photooxidized at a higher rate in 688 nm light than in 667 nm light suggesting that a high proportion of the chlorophyll absorbing at 667 nm must be ineffective. This problem is under further investigation.

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