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Formation of Chlorophyll *b*, and the Fluorescence Properties and Photochemical Activities of Isolated Plastids from Greening Pea Seedlings

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ABSTRACT

Chlorophyll *b* was first detectable after 10 minutes of illumination of etiolated pea seedlings (*Pisum sativum* L. var Greenfeast) with continuous white light. The chlorophyll *a/b* ratio decreased from 300 at 10 minutes to 15 after 1 hour. There was little change in the chlorophyll *a/b* ratio between 1 and 2 hours, and it declined to 3 between 2 and 5 hours of illumination. In red light, the time courses of total chlorophyll synthesis and chlorophyll *a/b* ratio were similar to those in white light for the first 5 hours of illumination. But with increasing time of illumination with red light, there was an increase in the chlorophyll *a/b* ratio to 7 after 30 hours. Illumination with white light of very low intensity also gave high chlorophyll *a/b* ratios. Seedlings which had been illuminated for varying periods and then returned to darkness always showed an increase in chlorophyll *a/b* ratio during the dark period. It is concluded that the synthesis of chlorophyll *b* is controlled by light.

The onset of the transfer of excitation energy from carotenoids to chlorophyll *a* correlated with the appearance of chlorophyll *b*. The detection of chlorophyll *b* and the onset of energy transfer from carotenoids to chlorophyll *a* preceded by several hours the appearance of Hill activity in the isolated pea plastids. The detection of the large fluorescence emission band at 735 nm at 77 K also preceded the observation of Hill activity, but it correlated with the phase of rapid chlorophyll synthesis. There was a good correlation between Hill activity and the formation of grana. The fluorescence kinetics which are characteristic of mature chloroplasts appeared considerably later than grana or the observation of Hill activity.

It is concluded that the formation of photosynthetic membranes and the assembly of the photosystems is not a single step process, at least in the initial stages of chloroplast development from the etioplast.

In angiosperm seedlings the synthesis of chlorophyll and the formation of photosynthetically active membranes is controlled by light. Following the initial photoconversion of protochlorophyllide to chlorophyllide *a* there is a lag phase of 1 to 3 hr, depending on the age and species of plant, before additional chlorophyll is synthesized. The lag phase in continuous light is controlled to some degree by phytochrome, since it can be eliminated by giving dark-grown seedlings a short exposure to red light followed by 3 to 5 hr of darkness. Continuous irradiation following the dark period causes an immediate rapid formation of chlorophyll. The effect of red light can be partly reversed by far red light (26, 29, 36).

Phytylation of chlorophyllide *a* occurs over a period of 30 to 40 min after the initial photoconversion of protochlorophyllide, and the esterification does not require additional light (5, 37). The appearance of chlorophyll *b* has been correlated with the end of the lag phase and the synthesis of additional chlorophyll *a* (35). Recently, Rudoi *et al.* (30) have reported that chlorophyll *b* is detectable within a few minutes of illumination of etiolated corn seedlings. They further reported that chlorophyll *b* is synthesized in the dark after a brief illumination of dark-grown seedlings sufficient to photoconvert protochlorophyllide (15, 30). Labeling data suggested that chlorophyll *b* is formed from chlorophyll *a* (33).

In the present work, we have used a very sensitive fluorescence method to determine chlorophyll *a/b* ratios in greening pea seedlings. Chlorophyll *b* is detectable after 10 min of illumination of dark-grown seedlings, and light is required for its continued synthesis.

We have also examined a number of parameters in isolated plastids as indicators of the formation of photosynthetic membranes. These include the onset of energy transfer from carotenoids to chlorophyll *a*, the increase in intensity of fluorescence emission at 735 nm at 77 K, the development of light-induced fluorescence changes, and the appearance of Hill-reaction activity.

Plastid development in white light was compared with that in red light. Structural aspects of chloroplast development in white and red light will be reported in a following paper (T. E. Treffry, in preparation).

MATERIALS AND METHODS

Plant Materials. Pea seeds (*Pisum sativum* L. var. Greenfeast) were surface-sterilized with bromine water (3% of saturated), soaked for several hours in running tap water, and planted in vermiculite that had been moistened with a nutrient solution. The plants were grown in darkness at 25 C for 8 to 10 days. Manipulations of the plants before illumination were performed in a dim green safelight.

Plants were illuminated in a room maintained at 25 C with white or red light. White of reasonably high intensity was provided by a bank of 40w fluorescent tubes (Philips, Type White). The intensity at leaf level as measured by a thermopile was 12,600 erg $cm^{-2}sec^{-1}$, or 850 ft-c as measured by a Weston illumination meter. Weak white light was obtained by reflection from a single white fluorescent lamp, the incident intensity at leaf level being 50 erg $cm^{-2}sec^{-1}$ or between 2 and 3 ft-c on the light meter. Moderate white light of intensity 2,200 erg

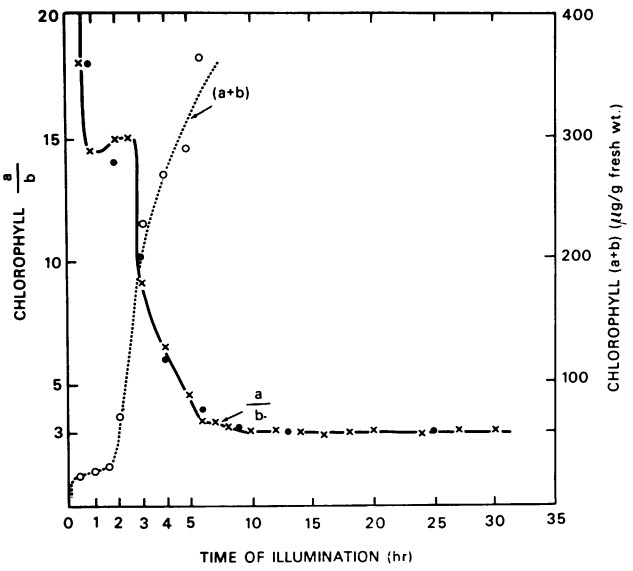

FIG. 1. Formation of total chlorophyll (chl *a* + chl *b*) (----) and chl *a*/chl *b* ratio (——) with time of illumination of etiolated pea seedlings in high intensity white light (850 ft-c). In some experiments (●), the seedlings were grown from seed which had been treated with chloranil. Total chlorophyll was determined on acetone extracts of leaves, and chl *a*/chl *b* ratios on ethanol extracts.

$cm^{-2}sec^{-1}$ (180 ft-c) was also used in some experiments. Moderate red light was obtained from two red fluorescent lamps (Philips TL 20w/15) fitted with red filters to give a band pass between 600 and 700 nm. The incident intensity measured by a thermopile was 1,950 erg $cm^{-2}sec^{-1}$ at leaf level.

Chlorophyll Determinations. Total chlorophyll (chl *a* + chl *b*)[1] was determined in acetone extracts by a spectrophotometric method (4). Ratios of chl *a*/chl *b* were determined in ethanol extracts by a sensitive fluorescence method (N. K. Boardman and S. W. Thorne, in preparation). Leaves were ground in ethanol, the extract was clarified by centrifugation and diluted with ethanol to give an absorbance of 0.1 at 436 nm. A sample of the extract was cooled to 77 K in a fluorescence spectrometer, and an emission spectrum was measured with the excitation monochromator set at 474 nm and the absorption maximum of chl *b* in ethanol at 77 K. The chl *a*/chl *b* ratio was obtained from the relative fluorescence intensities at 678 nm (chl *a*) and 658 nm (chl *b*). Full details of the experimental procedure and the method of calculating chl *a*/chl *b* ratios will be given elsewhere (Boardman and Thorne, in preparation). The method enabled chl *a*/chl *b* ratios to be measured over the range of 3 to 300 with an accuracy of ± 3%. Ratios of chl *a*/protochlorophyllide were determined in a similar way, except that the excitation wavelength was 449 nm, and the fluorescence emission was read at 678 nm (chl *a*) and 630 nm (protochlorophyllide).

Plastid Isolation. For plastid isolation, leaves were blended in a Servall Omnimixer for 10 sec at 85% of the line voltage in a medium containing 0.05 M phosphate buffer, pH 7.2, 0.01 M KCl, and 0.3 M sucrose. The plastids were sedimented by centrifugation at 1000*g* for 10 min and washed once with the sucrose-phosphate buffer. Plastids were resuspended in the sucrose-phosphate medium.

Fluorescence Measurements. Fluorescence measurements at 77 K were carried out in a medium containing 37 parts by volume of 0.05 M phosphate buffer, pH 7.2, containing 0.01 M KCl, and 63 parts of glycerol. The absorbance of the plastid suspension was 0.1 at 436 nm. Fluorescence emission and excitation spectra were recorded on a fully corrected fluorescence spectrometer, some details of which were described previously (8). The bandwidths of the excitation and emission monochromators were ±1.5 nm and ±1.0 nm, respectively. Fluorescence quantum efficiencies were measured by an extension of the relative method (8). Fluorescence kinetics were measured as described earlier (8).

Photochemical Activity. Hill-reaction activities were assayed with TCIP as oxidant (3).

RESULTS

Chlorophyll Formation. The time-courses of accumulation of chlorophyll (chl *a* + chl *b*) and the chl *a*/chl *b* ratio on illumination of dark-grown pea seedlings with continuous white light of high intensity are shown in Figure 1. After turning on the light, there was a lag phase of about 0.5 hr before there was any detectable synthesis of chlorophyllide *a*, other than the amount formed by photoconversion of protochlorophyllide existing in the dark-grown seedlings. There was a very slight increase in total chlorophyll between 0.5 and 2 hr, and then rapid chlorophyll synthesis occurred. These results are in general agreement with previous work on chlorophyll formation in other plants (1, 2, 34).

Chlorophyll *b*, however, was first detectable after 10 min of switching on the light. At 10 min, the chl *a*/chl *b* ratio was 300, at 20 min it was 30, and then the chl *a*/chl *b* ratio followed the continuous curve in Figure 1. The ratio remained constant at 15 between 1 and 2 hr, and then it declined reasonably rapidly to 3 after about 5 hr of greening. The decline in chl *a*/chl *b* ratio from 15 to 3 corresponded to the phase of rapid chlorophyll synthesis, but the initial decrease from infinity to 15 occurred over a period where synthesis of total chlorophyll

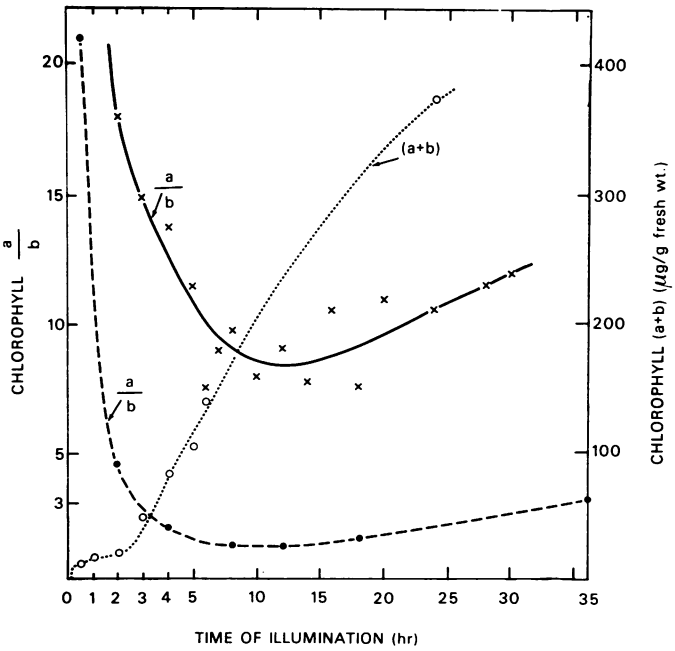

FIG. 2. Formation of total chlorophyll (○) and chl *a*/chl *b* ratio (×) with time of illumination of etiolated pea seedlings in low intensity white light (50 erg cm^{-2} sec^{-1}). Seedlings grown from seed treated with chloranil gave chl *a*/chl *b* ratios (●).

[1] Abbreviations: chl *a*: chlorophyll *a*; chl *b*: chlorophyll *b*; TCIP: 2,3,6-trichlorophenolindophenol.

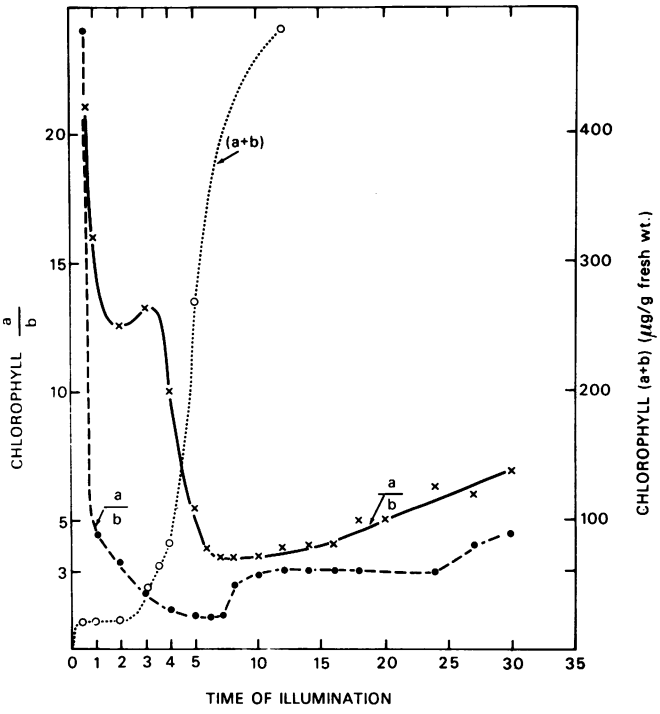

FIG. 3. Formation of total chlorophyll (○) and chl *a*/chl *b* ratio (×) with time of illumination of etiolated pea seedlings in red light of moderate intensity (1,950 erg cm^{-2} sec^{-1}). Seedlings grown from seed treated with chloranil gave chl *a*/chl *b* ratios (●).

Table I. *Chl a/Protochlorophyll(ide) Ratios of Pea Seedlings in Red Light*

Etiolated pea seedlings were illuminated with moderate red light of intensity 1,950 erg cm^{-2} sec^{-1}. Leaves (1-g samples) were extracted with ethanol, and chl *a*/protochlorophyllide ratios were determined spectrofluorimetrically at 77 K.

Time of Illumination	Chl *a*/Protochlorophyll(ide)
hr	
0.5	9.8
2	18
4	33
8	96
12	100
19	>100
24	>100
36	>100

was not measurable. At 5 hr the total chlorophyll content of the leaves had increased 15-fold as compared with the protochlorophyllide content of etiolated leaves. Plants greened in moderate white light of 180 ft-c (2,200 erg $cm^{-2}sec^{-1}$) gave a time-course for chl *a*/chl *b* ratio which was similar to that shown in Figure 1. In very weak white light, total chlorophyll formation was slower, and the synthesis of chl *b* was inhibited compared with greening in high or moderate intensity light (Fig. 2). The chl *a*/chl *b* ratio declined more slowly, reaching 9 after about 10 hr of illumination. On further illumination, however, the chl *a*/ chl *b* ratio increased slowly and after 30 hr it was 14.

In red light of moderate intensity, the time courses of chlorophyll synthesis and chl *a*/chl *b* ratio were similar to those in white light (moderate or high intensity) for the first 5 hr of greening (Fig. 3). A surprising feature of the red light treatment was the gradual increase in the chl *a*/chl *b* ratio after 7 hr of illumination. After 30 hr the chl *a*/chl *b* ratio was 7. In the red light, photoactive protochlorophyllide of the dark-grown seedling was converted to chlorophyllide *a* in less than 5 min. Protochlorophyll(ide) did not accumulate in the subsequent greening in continuous red light (Table I).

To inhibit mold growth, some batches of seed were shaken with chloranil powder (2,3,5,6-tetrachloro-1,4-benzoquinone)

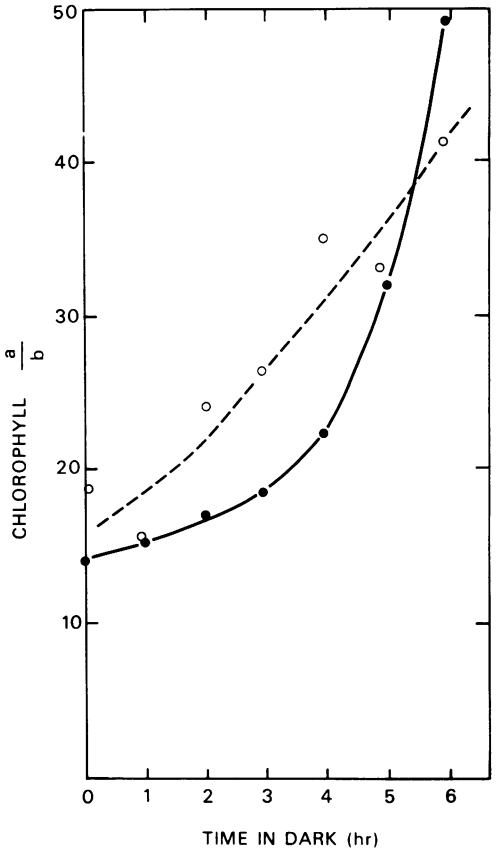

FIG. 4. The effect of dark periods on the chl *a*/chl *b* ratio of greening pea seedlings. Etiolated seedlings were illuminated for 1 hr with moderate white light (300 ft-c) (●) or moderate red light (1,950 erg cm^{-2} sec^{-1}) (○) and returned to darkness.

Table II. *Effect of Dark Period on Chl a/Chl b Ratio*

Etiolated pea seedlings were illuminated at 25 C in white light of 300 ft-c before returning to darkness. Chl *a*/chl *b* ratios were determined spectrofluorimetrically at 77 K in ethanol extracts of the leaves.

Time in White Light	Time in Dark	Chl *a*/Chl *b*
hr	*hr*	
6.5	0	3.8
6.5	1	3.6
6.5	5	7.8
6.5	7	8.1
6.5	24	15.7
16	0	3.0
16	2	3.1
16	4	3.2
16	7	3.6
16	14	9.6

prior to soaking in running water. Chloranil treatment of the dry seed was found to decrease the chl *a*/chl *b* ratio of the greening seedling in the weak white light and red light, but not in high or moderate white light. After 5 hr of greening, the chl *a*/chl *b* ratio in weak white light or red light was 1.5 (Figs. 2 and 3), which is below the chl *a*/chl *b* ratio of green pea leaves. The conclusion is reached that chloranil, a strong oxidant, is taken up by pea seedlings and under certain conditions of illumination influences the chl *a*/chl *b* ratio either by increasing the rate of oxidation of chl *a* to chl *b* or by preferentially destroying chl *a*.

Since the formation of chl *b* relative to chl *a* is influenced by light intensity, it is apparent that the synthesis of chl *b* is controlled by light. To decide whether light is required, either directly or indirectly, for the synthesis of chl *b*, we measured chl *a*/chl *b* ratios on greening pea seedlings which had been placed in various regimes of dark and light. Figure 4 shows chl *a*/chl *b* ratios for seedlings which had been illuminated for 1 hr in white light of moderate intensity and then returned to darkness. At the end of the light period, the chl *a*/chl *b* ratio was 14; after 1 hr in darkness it increased to 15, and with increasing periods of darkness the chl *a*/chl *b* ratio gradually increased to 50 after 6 hr of darkness. A similar trend in chl *a*/chl *b* ratio was observed when etiolated seedlings were illuminated for 1 hr with red light and returned to darkness (Fig. 4). It is apparent that either chl *b* is degraded more rapidly than chl *a*, or that chl *b* formed in the light is reverting to chl *a* in the dark.

On the basis of analysis for chl *a* and chl *b* in greening bean seedlings which had been returned to darkness, Kupke and Dorrier (23) suggested that chl *b* reverts to chl *a* in the dark. Analysis for chl *a* and chl *b* on the greening pea were not sufficiently reproducible to decide whether chl *b* is degraded in the dark or changed to chl *a*. In any batch of pea seedlings, there is an inherent variability in the rate of greening between seedlings, and it is not possible to attach significance to small variations in absolute contents of chl *a* and chl *b*.

Seedlings greened for longer periods (6.5 hr and 16 hr) before returning them to darkness showed a greater stability in their chl *a*/chl *b* ratios (Table II). For example, 4 hr of darkness following 16 hr in white light changed the chl *a*/chl *b* ratio from 3.0 at the end of the light period to 3.2. However, irrespective of the time of illumination, plants always showed an increase, never a decrease, in the chl *a*/chl *b* ratio in a subsequent dark period.

Table III shows the chl *a*/chl *b* ratios of batches of seedlings which had been illuminated with red light for various times, followed by periods of darkness. Ethanol extracts were made at the end of 1 hr in all experiments and chl *a*/chl *b* ratios determined. It is apparent that the longer the time in the light, the lower the chl *a*/chl *b* ratio.

Following illumination in red light for 10 min, some seedlings were exposed to far red light (>730 nm) for 10 min before returning to darkness for 40 min. The far red illumination had no effect on the chl *a*/chl *b* ratio; control plants which had 10 min of red light and 50 min of darkness showed the same chl *a*/chl *b* ratio as the plants treated with far red light.

From our experiments, we conclude that in pea seedlings chl *b* is not formed to any significant extent in the dark following the photoconversion of protochlorophyllide to chlorophyllide *a*. Light, therefore, appears to be essential for the synthesis of chl *b*. Phytylation of chlorophyllide *a* is known to occur in the dark, and in the case of bean seedlings it is complete in about 45 min at 25 C (5, 37). To test whether chl *b* is formed by a rapid photo-oxidation of some of the molecules of chl *a* formed from chlorophyllide *a* in the dark, seedlings were illuminated for 2 min with high white light followed by 60 min in the dark and a further 2 min in the light. Chlorophyll *b* was not detectable either at the end of the dark period or after the subsequent period in the light.

Table III. *Effect of Red Light and Dark Periods on Chl a/Chl b Ratio*

Etiolated pea seedlings were illuminated in moderate red light (intensity: 1,950 erg cm^{-2} sec^{-1}) and returned to darkness. Leaves were harvested during the dark period and samples (1 g) were extracted with ethanol at the end of the dark period.

Time in Red Light	Time in Dark	Chl *a*/Chl *b*
min	*min*	
5	55	...[1]
10	50	90
15	45	39
30	30	24
45	15	15
60	0	13

[1] Chl *b* not detectable.

The experiments of Rudoi *et al.* (30), in which chl *b* formation was observed to occur in the dark following a brief illumination of dark-grown plants, were performed with corn seedlings. We have also studied the formation of chl *b* in corn seedlings, using our sensitive spectrofluorimetric method to determine chl *a*/chl *b* ratios. Dark-grown corn seedlings (*Zea mays*) were illuminated for 1 min with high white light and returned to darkness. Negligible formation of chl *b* occurred in the dark, and high chl *a*/chl *b* ratios were observed (>100). Detection of very small amounts of chl *b* in extracts of corn seedlings are complicated by the synthesis of additional protochlorophyllide during the first 2 hr of darkness, but we are sure that the amount of chl *b* formed in the dark in our experiments is extremely small if, in fact, any is synthesized.

Fluorescence Properties of Developing Plastids at 77 K. Spinach chlorplasts show a three-banded fluorescence emission spectrum at 77 K with maxima at 683, 695, and 735 nm (9, 16). Similar results are obtained with pea chloroplasts (Thorne and Boardman, unpublished observations). A prominent feature of the emission spectrum of chloroplasts is the band at 735 nm, which accounts for 75% of the total fluorescence emission. An examination of the fluorescence properties of subchloroplast fragments enriched in photosystems I and II, respectively, indicated that the emission band at 735 nm originates mainly from photosystem I. The bands at 683 and 695 nm at 77 K come from photosystem II (9). The fluorescence at 735 nm emanates from a form of chlorophyll absorbing at 705 nm (11). Butler (12) examined the appearance of a band at 705 nm in the excitation spectra of the long wavelength fluorescence (>730 nm) of greening bean leaves. An increase in excitation at 705 nm was found after 2 hr of illumination.

In the present work with developing pea plastids, we have investigated the appearance of the large fluorescence emission band at 735 nm in relation both to the synthesis of chl *a* and chl *b* and to the development of photochemical activity. Excitation spectra for the fluorescence emission at 77 K were also measured to determine the extent of energy transfer from carotenoids to chl *a*.

Fluorescence emission spectra at 77 K of plastids isolated from greening pea seedlings are shown in Figure 5. After illumination of the seedlings for 20 min in high intensity white light, the plastids showed a major fluorescence peak at 680 nm and a satellite band at about 735 nm, the latter accounting for about 25% of the total fluorescence emission. Chl *a* in organic solvents emits a similar proportion of its fluorescence at 77 K

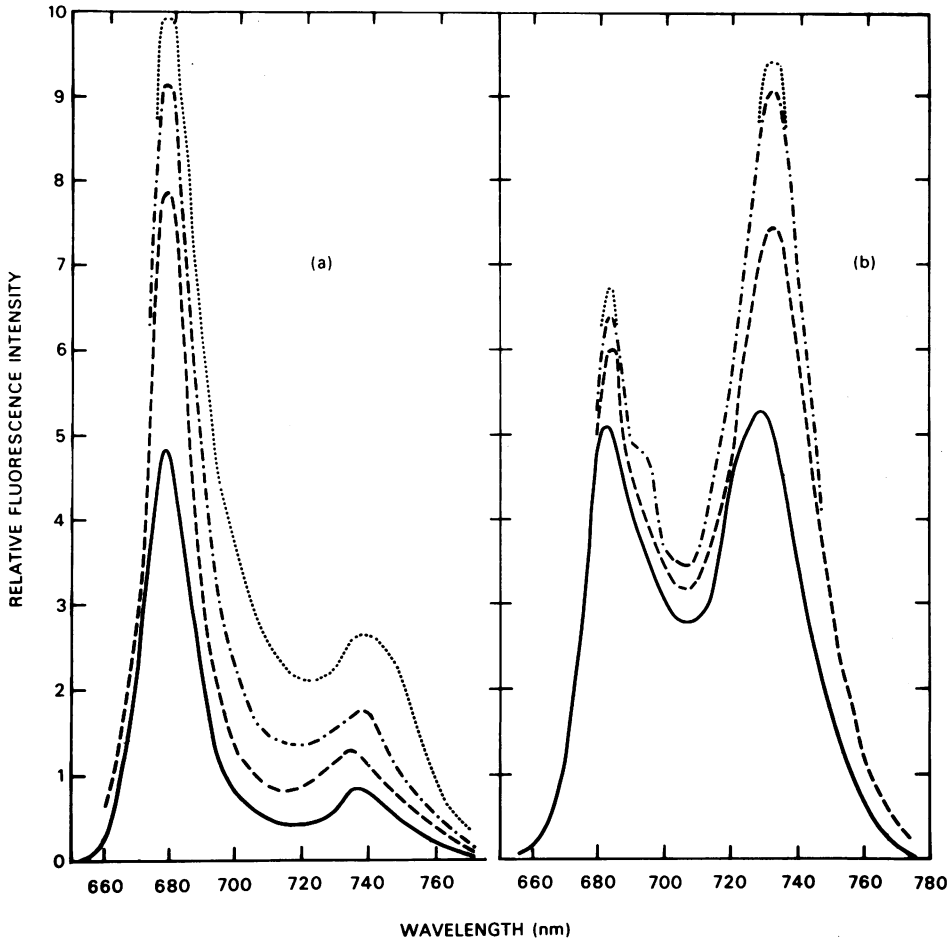

FIG. 5. Fluorescence emission spectra at 77 K of plastids from pea seedlings greened in high white light (850 ft-c). Excitation wavelength was 436 nm. Absorbancy of plastids at 436 nm; 0.1. Times of greening: (a). ——: 20 min; – – –: 45 min; –·–: 90 min; -----: 2 hr. (b). ——: 3 hr; – – –: 4 hr; –·–: 6 hr; -----: 48 hr.

at the long wavelength band (7). In the case of chl *a* in solvents, the long wavelength band is ascribed to electronic transitions from the first singlet excited state to upper vibrational levels of the ground state (17).

There is little change in the proportion of fluorescence emitted at the 735 nm band during the first 2 hr of greening (Fig. 5). During the first 20 min of greening, the maximum of the fluorescence emission changed from 690 nm to 680 nm, a shift which corresponds to the absorption spectral change discovered by Shibata (32). The increase in fluorescence yield, which is observable between the 20 min and 45 min plastids (Fig. 5), appears to lag slightly behind the Shibata shift. The lag in the increase in quantum yield of fluorescence has been studied more fully with bean seedlings, and it is discussed elsewhere (Thorne, submitted for publication). After 3 hr of greening, there is a substantial increase in the fluorescence emission at 735 nm and a decrease in the region of 680 nm. The peak of the fluorescence emission shifts from 680 to 683 nm; the latter coincides with the wavelength of maximal fluorescence of mature pea chloroplasts. A distinct shoulder is observed at 695 nm after 6 hr of greening, but it should be mentioned that the intensity of the fluorescence band at 695 nm is somewhat variable even for mature chloroplasts.

The fraction of fluorescence emitted at the 683 nm band plus the 695 nm band is plotted as a function of time of greening in Figure 6. The fluorescence ratio ϕ (683 + 695)/ϕ total (where ϕ (683 + 695) is the quantum yield of fluorescence at the 683 and 695 bands, and ϕ total is the total quantum yield of fluorescence) declined sharply between 2 and 4 hr of greening, and this corresponds closely to the time of rapid chlorophyll synthesis and the decline in the chl *a*/chl *b* from 15 to 3 and 4. When the plastids were isolated from seedlings greened in moderate red light, the decline in ϕ (683 + 695)/ϕ total took place more gradually over a longer time (Fig. 6), in spite of the similarity in the time courses of chlorophyll accumulation and chl *a*/chl *b* ratios in white and red light.

Excitation spectra for the fluorescence emitted at 680 and 683 nm are shown in Figure 7. After 20 min of greening, there is some indication of energy transfer from carotenoids, but the efficiency of transfer is low, since the carotenoids comprise a large proportion of the pigments at the early stage of greening. The carotenoid/chl *a* ratio is about 20 during the lag phase. Chl *b* absorbs at 470 nm *in vivo*, but the proportion of chl *b* after 20 min of greening (chl *a*/chl *b* = 30) seems to be too low for it to give a significant excitation band at 470 nm, even though energy transfer from chl *b* to chl *a* may be highly efficient at this stage. We conclude that the excitation bands at 470 and 495 nm are due to carotenoids. However, as indicated in the following experiment, the presence of chl *b* facilitates the transfer of energy from carotenoids to chl *a*. This point is considered again in the Discussion section. The spectra shown in Figure 7a indicate that energy transfer from carotenoids to chl *a* increases strongly during the first 2 hr of greening.

Butler (10) observed with greening bean seedlings that the onset of energy transfer from carotenoids to chl *a* correlated with the phytylation of chlorophyllide *a*, and he suggested

that the phytol group was necessary for efficient energy transfer. The methods used previously to detect chl *b* were less sensitive than the spectrofluorimetric method used in the present study. In greening pea seedlings, the transfer of excitation energy from carotenoids to chl *a* coincides not only with phytylation of chlorophyllide *a*, but also with the appearance of chl *b*.

As mentioned earlier, chl *b* was not detectable in pea seedlings which had been illuminated in high intensity white light for 3 min and returned to darkness for 1 hr. Phytylation of chlorophyllide *a*, however, occurs in the dark (5, 37). We measured, therefore, the fluorescence excitation spectrum of plastids isolated from pea seedlings illuminated for 3 min and placed in darkness for 1 hr to determine whether chl *b* plays a role in the transfer of energy from carotenoids to chl *a*. The spectrum (Fig. 8) shows small bands at 470 and 495 nm, indicating that transfer of energy from carotenoids to chl *a* can occur in the absence of chl *b*, but the efficiency of transfer is greater when chl *b* is present. (Compare Fig. 8 and the 45 min curve in Fig. 7a).

We also investigated the kinetics of the fluorescence emission at 680 nm at 77 K. Plastids from greening seedlings were dark-adapted for a few minutes at 20 C before cooling to 77 K. For the first 3 hr of greening, the rise in fluorescence at 683 nm on illumination of the plastids at 77 K was virtually instantaneous. A delay was observed in the fluorescence rise after 6 hr of greening, but it was only a fraction of the delay observed with mature chloroplasts.

Fluorescence Properties of Developing Plastids at 20 C. On excitation of mature pea chloroplasts at 20 C, the fluorescence intensity at 683 nm rises from an initial value (F_0) to a steady state value (F_∞). The kinetics of the rise in fluorescence are biphasic and similar to those reported previously for spinach chloroplasts (24). The steady state fluorescence is quenched by an oxidizing agent such as ferricyanide and increased by a strong reducing agent such as dithionite. The fluorescence yield of mature chloroplasts is thought to be related to the redox state of a quencher (Q) in photosystem II. When Q is oxidized, fluorescence is quenched, and when Q is fully reduced, fluorescence is maximal (13).

The kinetics of the rise in fluorescence were determined for developing pea plastids obtained from seedlings greened in moderate white light of 300 ft-c. Results obtained with an excitation wavelength of 470 nm at two exciting intensities are presented in Table IV.

At the lower excitation intensity, the fluorescence emission of developing plastids at 683 nm was influenced to only a small extent by time of excitation. Even after 24 hr of greening, the F_∞/F_0 ratio of the plastids was 1.11, compared with 1.82 for mature pea chloroplasts. Increasing the excitation intensity 10-fold enhanced the time-dependent rise in fluorescence. The ratio F_∞/F_0 increased gradually from 1.25 for 4-hr plastids to 2.16 for 24-hr plastids, but the latter ratio was still well below the value (3.44) observed for mature pea chloroplasts. Plastids isolated from plants which had been illuminated for four periods of 12 hr or five periods of 16 hr gave fluorescence kinetics which more closely resembled those of mature chloroplasts. The initial fluorescence (F_0) declined during plastid development, whereas F_∞ either remained reasonably constant (at the lower excitation intensity) or increased 2-fold (at the higher intensity). Similar trends in F_∞/F_0 to those shown in Table IV were obtained if fluorescence was excited at 650 nm or 670 nm, instead of 470 nm.

One possible explanation for the low F_∞/F_0 ratios of developing plastids is that the steady state redox level of Q is more oxidized than in mature chloroplasts, due either to a preponderance of chlorophyll in photosystem I units or to a low

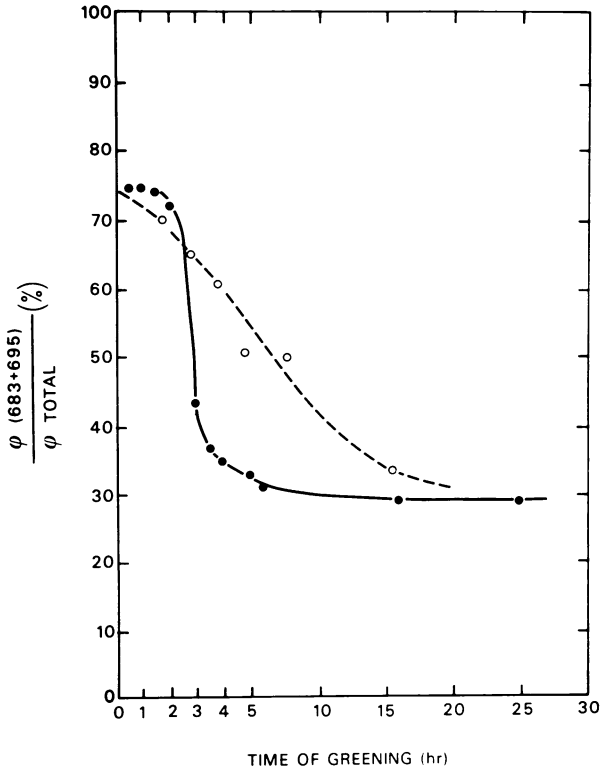

FIG. 6. The distribution of fluorescence emission at 77 K of plastids from greening pea seedlings, $\phi(683 + 695)$ is the integrated emission at the 683 and 695 nm bands, and ϕ_{total} is the total integrated emission. Seedlings were greened in high intensity white light of 850 ft-c (●) or moderate red light (○). Conditions were as for Figure 5.

quantum efficiency for the reduction of Q by photosystem II units. In the experiments reported in Table V, the fluorescence yield of plastids in the presence of sodium dithionite (Q reduced) was compared with the yield in the presence of ferricyanide (Q oxidized). For mature pea chloroplasts, the ratio of the fluorescence yield under reducing and oxidizing conditions (F_{red}/F_{ox}) was 4.85, but developing plastids gave considerably smaller ratios. After 4 hr of greening, the F_{red}/F_{ox} ratio was 1.77, and it gradually increased to 3.40 for plastids from seedlings greened for 24 hr. Illumination of plants for 80 hr (5- × 16-hr light periods with intervening dark periods) gave plastids which resembled plastids from plants grown in a greenhouse. We conclude that the low ratios of F_∞/F_0 for developing plastids are due to factors other than the redox state of Q in the illuminated steady state. Further discussion of this point is reserved until later.

Photochemical Activity of Developing Plastids. Plastids from greening pea seedlings showed no detectable Hill activity until the plants had been illuminated for 5 hr (Fig. 9). There was a rapid increase in activity between 5 and 8 hr to a level which was 80 to 90% of the rate of dye reduction obtained with chloroplasts from plants grown in a greenhouse. Dye reduction was completely inhibited by 1-*p*-chlorophenyl 3'3'-dimethyl urea at a concentration of 3×10^{-5} M. The development of photochemical activity was similar for plants illuminated in high white light and in moderate red light (Fig. 9).

DISCUSSION

The studies of Shlyk and his coworkers (30, 33) suggest that chl *b* is formed from newly synthesized molecules of chl *a*, rather than from the general pool of chl *a* molecules which

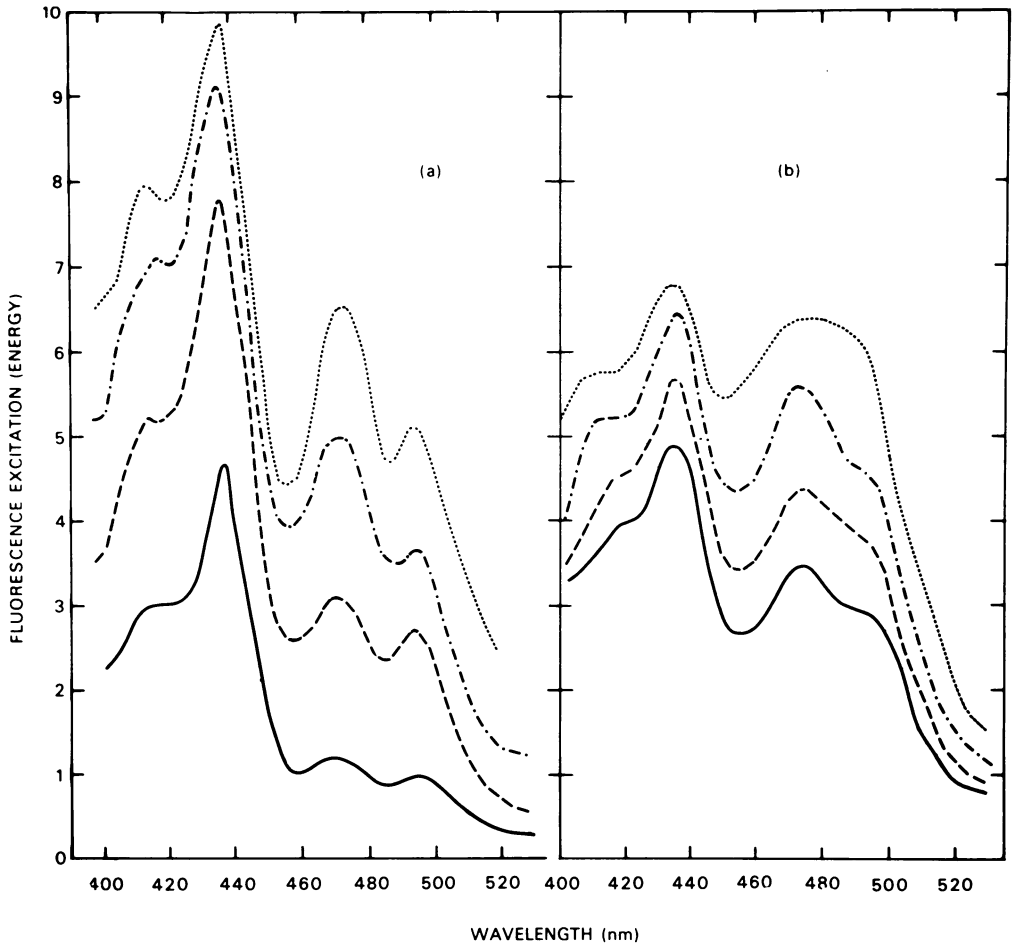

FIG. 7. Fluorescence excitation spectra at 77 K of plastids from pea seedlings greened in high intensity white light (850 ft-c). Absorbancy of plastids at 436 nm was 0.1. Times of greening: (a). ——: 20 min; – – –: 45 min; –·–: 90 min; -----: 2 hr. (b). ——: 3 hr; – – –: 4 hr; –·–: 6 hr; -----: 48 hr. Emission wavelength: (a): 680 nm; (b): 683 nm.

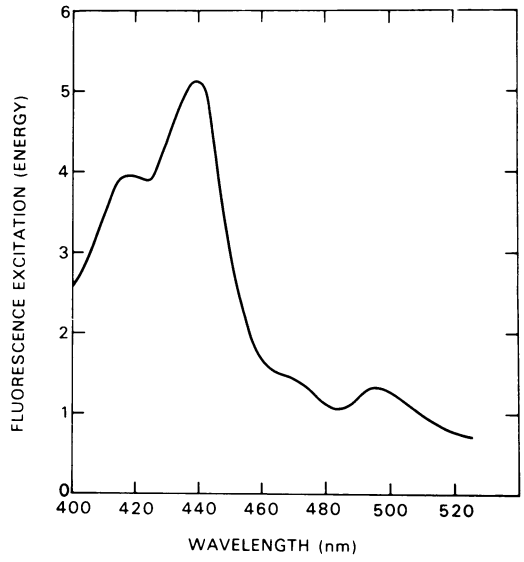

FIG. 8. Fluorescence excitation spectrum at 77 K of plastids from pea seedlings which were illuminated for 3 min with high intensity white light, followed by 60 min of darkness. Emission wavelength was 680 nm.

are formed by photoconversion and phytylation of protochlorophyllide already existing in the etiolated plants. In the present work, chl *b* is observable before the detectable synthesis of additional chl *a* molecules, but our method for detecting chl *b* in the presence of chl *a* is far more sensitive than the analytical procedures for observing additional molecules of chl *a*. We cannot exclude, therefore, the possibility that chl *b* is formed only from newly synthesized molecules of chl *a*.

Phytylation of chlorophyllide *a* is a reaction which occurs in the dark, but chl *b*, which is thought to be formed from chl *a*, does not accumulate in the dark, at least in pea seedlings. Thus, the synthesis of chl *b* is controlled by light. Light is directly required for the conversion of protochlorophyllide *a* during the entire greening process, but in addition there is considerable evidence (26, 29, 36) that phytochrome controls plastid development. Phytochrome may control the rate of synthesis of the developing photosynthetic membranes by controlling the synthesis of a particular component or components of the membranes. It seems possible that chl *b* is formed at particular sites in the developing pigment assemblies, which are an integral part of the photosynthetic membranes. Inhibition of membrane assembly may then lead to an inhibition in the rates of chl *b* formation and phytylation of chlorophyllide *a*, and in turn to the rate of synthesis of protochlorophyllide.

Marmé (25) has provided evidence for the destruction of phytochrome in continuous red light. Membrane formation,

therefore, may be slower in red light than in white light, and this may provide an explanation for the increases in chl *a*/chl *b* ratio and in the chlorophyllide *a*/chl *a* ratio (Treffry, in preparation). In white light of low intensity, the rate of photoconversion of protochlorophyllide to chlorophyllide *a* appears to be the rate limiting step in the synthesis of chl *a* and chl *b*, and in membrane formation.

We conclude that there is an indirect effect of light on the formation of chl *b*, resulting from a requirement for light for plastid development. Our experiments do not exclude the possibility that light also is directly required for the conversion of chl *a*, or other possible chlorin precursor, to chl *b*.

The transfer of excitation energy from carotenoids to chl *a* is facilitated by the presence of chl *b*. Förster (14) has shown that the probability of energy transfer from a donor molecule in an excited state to a neighboring acceptor molecule is dependent on the degree of overlap between the fluorescence spectrum of the donor and the absorption spectrum of the acceptor. A dilute solution of β-carotene in ethanol (10^{-5} M) is weakly fluorescent, the emission spectrum showing a small peak at 490 nm with a long tail extending to 600 nm (Thorne, unpublished observations). The overlap between the Soret absorption band of chl *b* (466 nm in ethanol and 474 nm *in vivo*) and the emission band of β-carotene is considerably greater than the overlap between the Soret absorption band of chl *a* (429 nm in ethanol and 436 nm *in vivo*) and the emission band

Table IV. *Fluorescence Kinetics of Developing Pea Plastids*

Etiolated pea seedlings were illuminated with moderate white light (300 ft-c) for the periods indicated. Seedlings illuminated for 4 × 12 hr were given 12-hr dark periods between illuminations, and those illuminated for 5 × 16 hr were given 8-hr dark periods. Plastids were isolated as described and diluted to an absorbancy of 0.2 at 470 nm with sucrose-phosphate medium. Mature chloroplasts were isolated from pea plants grown in a controlled glasshouse. Fluorescence was excited at 470 nm with an intensity of 100 erg cm^{-2} sec^{-1} (×1) or 1000 erg cm^{-2} sec^{-1} (×10). The time course of fluorescence emission was measured at 683 nm. F_0 is the initial fluorescence intensity and F_∞ the steady-state intensity. The steady-state level was reached within 2 min at the lower exciting intensity and in a few seconds at the higher intensity.

Time of Greening	Fluorescence Excitation Intensity	Fluorescence Emission at 683 nm		
		F_0	F_∞	F_∞/F_0
hr		*relative units*		
4	× 1	39	41	1.05
6	× 1	47	50	1.06
8	× 1	39	44	1.12
12	× 1	34	38	1.12
24	× 1	27	30	1.11
4 × 12	× 1	26	40	1.54
5 × 16	× 1	28	39	1.44
Mature pea chloroplasts	× 1	22	40	1.82
4	× 10	520	650	1.25
6	× 10	570	880	1.54
8	× 10	530	840	1.58
12	× 10	450	840	1.87
24	× 10	460	1000	2.16
4 × 12	× 10	360	1030	2.87
5 × 16	× 10	440	1300	2.96
Mature pea chloroplasts	× 10	340	1170	3.44

Table V. *Fluorescence Properties of Developing Pea Plastids*

Conditions as for Table IV. Fluorescence was excited by 470 nm light of intensity 100 erg cm^{-2} sec^{-1}. The fluorescence emission at 683 nm was measured in the presence of 5 × 10^{-5} M ferricyanide (F_{ox}) or dithionite (F_{red}).

Time of Greening	Fluorescence Emission at 683 nm		
	F_{ox}	F_{red}	F_{red}/F_{ox}
hr	*relative units*		
4	39	69	1.77
6	50	87	1.74
8	42	85	2.02
12	36	97	2.52
24	28	95	3.40
4 × 12	28	124	4.42
5 × 16	29	138	4.75
Mature pea chloroplasts	23	111	4.85

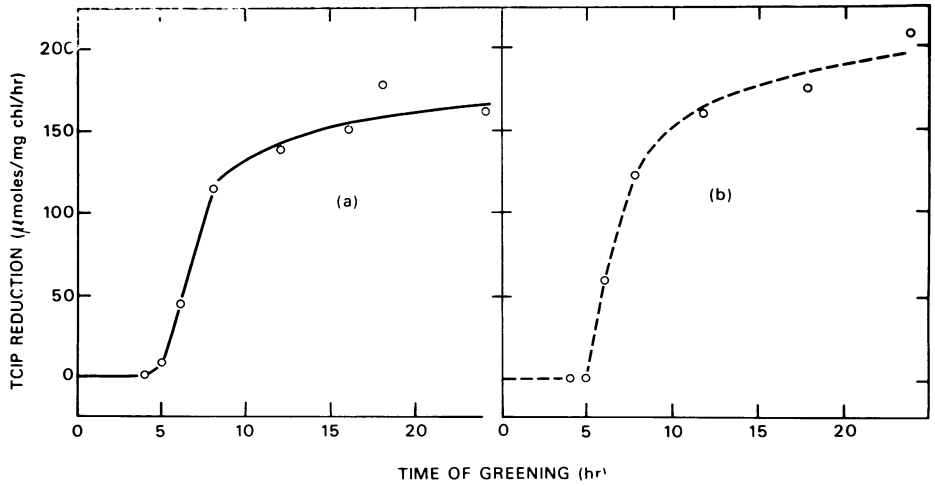

FIG. 9. Hill-reaction activity of plastids isolated from greening pea seedlings, illuminated with (a) high intensity white light (850 ft-c) and (b) moderate red light (1,950 erg cm^{-2} sec^{-1}). Reaction mixture contained in 3 ml, plastids containing 5 to 10 μg of chlorophyll and (in μmoles) tris-HCl buffer, pH 7.8, 40; NaCl, 70; TCIP, 0.06. The reaction mixture was illuminated with white light (4000 ft-c) from a 250-w photoflood lamp for 45 sec. Hill reaction rates were calculated from the decrease in absorbance at 620 nm.

of β-carotene. We would expect, therefore, from a consideration of the Soret band region of the spectra that the efficiency of energy transfer between carotenoid and chl *b* would be much greater than the transfer efficiency between carotenoid and chl *a*. Energy is readily transferred from chl *b* to neighboring chl *a* molecules because of the overlap of the bands in the red region of the spectrum.

These studies with the greening pea indicate that the assembly of the photosynthetic membranes in the developing plastid of a higher plant is not a single step process. The appearance of chl *b* and the observation of energy transfer from carotenoids to chl *a* precedes by several hours the appearance of Hill activity in the isolated plastid. The appearance of the large fluorescence band at 735 nm at 77 K correlates with the phase of rapid chlorophyll synthesis, but it is earlier in the greening process than the onset of Hill activity and the appearance of grana. Photo-oxidation of cytochrome *f*, a measure of activity in photosystem I, is first observed in the greening pea after 30 to 40 min of illumination and the extent of the photo-oxidation increases during the first 2 to 3 hr of greening (6; and Boardman, unpublished observations).

There is excellent correlation between the increase in Hill activity and the formation of grana (6), thus lending some support to the suggestion of Homann and Schmid (21) that appressed or stacked membranes are necessary for photosystem II activity. Homann and Schmid (21) observed that the yellow sections of a varigated mutant of *Nicotiana tabacum* showed essentially no photosystem II activity, but plastids from these sections photoreduced NADP with ascorbate-dichlorophenol-indophenol as electron donor (a photosystem I activity). There was no evidence for appressed lamellae in the plastids of the yellow sections.

A further correlation between grana and the presence of photosystem II has come from studies (38) with plants with the C_4-dicarboxylic acid pathway of photosynthesis (C_4-plants). The bundle sheath chloroplasts of certain species of C_4-plants lack grana (agranal), whereas in other species grana are present both in the bundle sheath and mesophyll chloroplasts. The agranal bundle sheath chloroplasts were deficient in photosystem II, but they contained an active photosystem I (38). Grana-containing chloroplasts, whether mesophyll or bundle sheath, had a functional photosystem II.

However, studies with a barley mutant lacking chl *b* (7, 18, 20) apparently do not support the view that appressed lamellae are essential for photosystem II activity, since the barley mutant exhibited high photosystem II activity, but a greatly reduced amount of appressed lamellae. A mutant of *Chlamydomonas reinhardi* (ac-31) has also been described (19) which has normal photosystem II activity, but essentially no stacking of lamellae.

The development of fluorescence kinetics characteristic of mature chloroplasts occurs considerably later than the appearance of grana and the observation of Hill activity. It appears that during chloroplast development, there is present in the plastid some chlorophyll which has not yet been incorporated into the pigment assemblies either of photosystem I or photosystem II. This chlorophyll has a higher quantum yield of fluorescence than the chlorophyll of the completed pigment assemblies, and its fluorescence yield does not respond either to changes in redox potential or to the photoreduction of the electron carriers, Q and P, associated with photosystem II. In this way we may explain why the initial fluorescence yield (F_0) of a developing plastid is higher than the F_0 for mature chloroplasts, and why the F_∞/F_0 ratio is lower.

Boardman *et al.* (6) have presented a model for the assembly of the photosystems based on observations of energy transfer between protochlorophyllide and the various spectroscopic forms of chlorophyll(ide) *a*. In the model it is proposed that newly synthesized chlorophyllide *a* and chl *a* are associated for a time with the protein of the protochlorophyllide holochrome before the chl *a* is incorporated into the photosynthetic units of photosystem I or photosystem II. Chlorophyll associated with the holochrome may be expected to have a relatively high quantum yield of fluorescence, which is independent of redox conditions. On transfer to the pigment assemblies of photosystem I or photosystem II, the fluorescence of the chlorophyll is quenched, and for molecules incorporated into photosystem II, the fluorescence yield is influenced by the redox state of a quencher.

Extensive studies (22, 27, 28, 31) have been carried out on the assembly of photosynthetic membranes and the development of photochemical activities during the greening of mutants of *Chlamydomonas reinhardi*. From their earlier work, Ohad *et al.* (27) concluded that the synthesis of photosynthetic membranes in *Chlamydomonas* occurred in a single step operation, but more recent studies (22, 28, 31) have shown that the assembly of the membranes can occur stepwise.

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