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Greening of Etiolated Bean Leaves in Far Red Light

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ABSTRACT

Eight-day-old dark-grown bean leaves were greened by prolonged irradiation with far red light. Growth, chlorophyll content, oxygen-evolving capacity, photophosphorylation capacity, chloroplast structure (by electron microscopy), and *in vivo* forms of chlorophyll (by low temperature absorption and derivative spectroscopy on intact leaves) were followed during the greening process. Chlorophyll *a* accumulated slowly but continuously during the 7 days of the experiment (each day consisted of 12 hours of far red light and 12 hours of darkness). Chlorophyll *b* was not detected until the 5th day. The capacity for oxygen evolution and photophosphorylation began at about the 2nd day. Electron microscopy showed little formation of grana during the 7 days but rather unfused stacks of primary thylakoids. The thylakoids would fuse to give grana if the leaves were placed subsequently in white light. The low temperature spectroscopy of intact leaves showed that the chlorophyll *a* was differentiated into three forms with absorption maxima near 670, 677, and 683 nanometers at −196 C during the first few hours and that these forms accumulated throughout the greening process. Small amounts of two longer wavelength forms with maxima near 690 and 698 nanometers appeared at about the same time as photosynthetic activity.

The development of etiolated tissue of higher plants in the light involves phototransformations of both phytochrome and protochlorophyll. Phytochrome controls a number of developmental processes from the synthesis of specific proteins (14) to the gross morphological characteristics such as leaf expansion (15). The development of photosynthetically active plastids, however, requires the phototransformation of protochlorophyll and the accumulation of chlorophyll. Even though both pigment systems play decisive roles in directing the development of the plant, there appears to be little interaction between the two systems with the exception that the rate of protochlorophyll synthesis after the initial transformation is influenced by phytochrome (16). Long before the discovery of phytochrome as the photomorphogenic pigment (5), Withrow *et al.* (26) concluded from a detailed study of photomorphogenesis and chlorophyll formation in etiolated bean seedlings under prolonged irradiation with various light sources, including far red sources of different spectral quality and red and blue sources of low intensity, that protochlorophyll and chlorophyll did not participate in the photomorphogenic growth responses.

Phytochrome in dark-grown seedlings is entirely in the Pr[2] form (3). It was shown, however, that a low level of Pfr is established as a photostationary state by far red light (because of the long wavelength absorption tail of Pr), and it was suggested that the photomorphogenic responses obtained with prolonged far red irradiation were due to the maintenance of the low level of Pfr over a long period of time (in darkness Pfr reverts to Pr) (3). Mohr and his co-workers (13) have shown in a number of cases that prolonged irradiation with far red light activates the phytochrome system. Häcker (9) illuminated etiolated mustard seedlings with continuous far red light (740 nm maximum) and followed morphological changes in the etioplasts of the cotyledons. He reported that, under the influence of far red light, storage protein was converted to structural protein of the etioplasts and that the etioplasts expanded to the same size and shape as mature chloroplasts while only traces of chlorophyll were formed.

Development of the photosynthetic apparatus, however, requires the presence of chlorophyll, not only for the photosynthetically active pigment but also for the formation of the lamellar and grana structures of the chloroplasts. In the present work we have studied the development of the photosynthetic apparatus in eight-day-old, dark-grown bean leaves under conditions where the rate of transformation of protochlorophyll to chlorophyll limited the rate of development while the synthetic processes controlled by phytochrome were fully active. This was achieved by using a far red source (incandescent lamps with a 720 nm cut-off filter) which apparently had somewhat more action in transforming protochlorophyll than the source used by Häcker (9). The synthetic processes associated with leaf expansion were fully active, but the accumulation of chlorophyll was very slow and the development of the photosynthetic apparatus was much prolonged. One advantage of extending the period of development was the greater temporal resolution of sequential processes.

METHODS

All experiments were carried out on the primary leaves of *Phaseolus vulgaris* cultivar Topcrop (W. Atlee Burpee Co., Riverside, Calif.). Plants were grown in vermiculite, soaked with tap water, in complete darkness at 25 C for 8 days. On the 8th day, uniform plants were selected and transferred to water cultures with modified Hoagland-Arnon medium. At the beginning of the 9th day plants were taken for the experimental run.

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[2] Abbreviations: Pr and Pfr: red- and far red-absorbing phytochrome, respectively.

The far red light source used for the greening experiments consisted of a bank of 12 100-w incandescent lamps suspended above a water tank (with 15 cm of water) and a far red plastic filter (Westlake Plastics, FRF-700). The absorption spectrum of the plastic cut-off filter rises sharply from an absorbance of about 0.3 to 720 nm to an absorbance above 7.0 at 670 nm. The intensity of far red light at the level of the plants was 2×10^4 ergs $cm^{-2}sec^{-1}$. The plants were maintained on 12-hr light, 12-hr dark irradiation cycles at 25 C. A source of white light for greening consisted of 4 cool-white (General Electric F 40 CW) and 4 Gro-Lux (Sylvania F40 Gro) fluorescent lamps giving 1.1×10^4 ergs $cm^{-2}sec^{-1}$ at the plants. Greening by repetitive brief irradiation periods was achieved by placing the plants under red fluorescent lamps (General Electric Red F20 T12-R) for 1 min every hour during the 12-hr day. The intensity of red light, 5×10^3 ergs $cm^{-2}sec^{-1}$, was sufficient to transform all of the protochlorophyll in 1 min.

The chlorophyll *a* and *b* contents of the leaves were determined spectrophotometrically with an 80% (v/v) acetone extract by Arnon's method (1). Extractions were carried out in dim green light.

Photosynthetic activity was assayed with a Clark polarographic electrode (Yellow Springs Instrument Co., model 5331), combined with a Hewlett-Packard strip chart recorder, model 7127 A. A 3-ml sample chamber was filled with equal parts of CO_2-buffer (7 parts 0.1 M $NaHCO_3$ + 3 parts 0.1 M Na_2CO_3) and Avron's medium (15 mM tris buffer, 20 mM NaCl, 4 mM $MgCl_2$, and 4 mM potassium phosphate buffer, pH 7.8). Two leaf discs of 9-mm diameter sliced into strips 1 mm wide were used as the sample. A constant rate of oxygen uptake in the dark due to respiration was established before irradiation and served as the basal rate from which O_2 evolution was calculated. The sample chamber was then irradiated with a Unitron LKR microscope illuminator, filtered through 7 cm of 1% $CuSO_4$ solution, giving 1.75×10^5 ergs $cm^{-2}sec^{-1}$. In each measurement the first response to white light was taken as representative for the photosynthetic activity at a given developmental state.

The photophosphorylation capacity of the far red-irradiated leaves was determined by the firefly luciferase assay (10). At zero time and at the end of each 12-hr period of far red light 5-mm discs were excised from the primary leaves under green safelight. The leaf discs were placed on moistened filter paper in a Petri dish and exposed to white light filtered through 7 cm of a 1% solution of $CuSO_4$ (7.0×10^4 ergs $cm^{-2}sec^{-1}$) for specific periods up to 5 min. Immediately after each exposure time one leaf disc was submerged in 5 ml of boiling tris-HCl buffer, pH 7.75, for 1 min. This extract was used for the ATP assay. The amount of ATP in 0.2 ml of the extract was determined from a calibration curve made with known amounts of ATP.

Low temperature absorption spectra and derivative spectra were measured with a single thickness of an intact leaf by methods described previously (4).

For electron microscopy the leaf tissue was cut into strips 0.5 mm wide in dim green light and placed in a 1% glutaraldehyde solution in 0.1 M phosphate buffer (320 milliosmoles, pH 7.3) at 0 C for 3 hr. The tissue was then rinsed three times with cold phosphate buffer and further fixed in 2% osmium tetroxide in 0.1 M phosphate, pH 7.5, for 2 hr. After rinsing with cold water the tissue was dehydrated with a graded series of acetone concentrations in six steps from 50 to 100% at 0 C followed by four changes of pure acetone at room temperature, each step involving a 15-min soaking period. The tissue was then embedded in Epon as described by Ledbetter and Porter (12) and sectioned.

RESULTS

Prolonged illumination of 8-day-old dark-grown bean leaves with far red light resulted in the accumulation of chlorophyll and the development of etioplasts into photosynthetically active chloroplasts. The development processes, however, were much slower in far red light than in white (or red) light. Photosynthetic activity (oxygen evolution) which normally appears in 8-day-old etiolated bean leaves between 1 and 2 hr after the onset of illumination with white light did not appear until after 2 days of illumination (each day had 12 hr light and 12 hr dark) with far red light.

Figure 1 shows the development of photosynthetic activity as well as chlorophyll accumulation and leaf expansion as a function of time in far red light. During the first 5 days leaf expansion as measured by leaf area or fresh weight increased as rapidly in far red light as in white light. In white light, however (data not shown), leaf expansion continued beyond 5 days, whereas in far red light expansion ceased after 5 days when the cotyledonary food reserves were exhausted. The percentage dry weight of the far red-irradiated leaves decreased linearly from 21% initially to 12% after 5 days, where it remained constant. During the first 5 days in far red light the dry weight increased from 3.8 to 16.0 mg per leaf.

The rate of chlorophyll accumulation in the far red-irradiated leaves was much slower than normal. In white light the concentration of chlorophyll increased to a plateau of about 2000 μg of chlorophyll *a* per g fresh weight in 2 days while in far red light the concentration reached about 200 μg of chlorophyll *a* per g fresh weight after 7 days. The total chlorophyll per leaf in the leaves greened in far red light increased 50- to 100-fold during the 7 days, but the leaves remained yellow to yellowish green in appearance. At each point for the chlorophyll determination one batch of leaves was extracted in dim green light while another batch was illuminated for 15 min with white fluorescent light before extraction. The difference between the two measurements, which indicates the amount of protochlorophyll per leaf, is shown by the length of the vertical arrows in Figure 1.

Photosynthetic activity on a chlorophyll basis (μmole O_2/mg chlorophyll·hr) began after 24 hr in far red light, went through a maximum, and declined with further greening. The early maximum and later decline of photosynthetic activity on a chlorophyll basis were even more pronounced in leaves greened in white light. The same phenomenon was observed much earlier by Willstätter and Stoll (noted by Smith [24]) and more recently by Wieckowski (25). The decline of activity with greening may be related to an increase in the size of the photosynthetic units. Chlorophyll *b*, which normally appears at about the same time as photosynthetic activity in leaves greened in white light, did not appear until after 5 days in the far red light, well after the onset of photosynthetic activity.

Photophosphorylation began at the same time as photosynthetic oxygen evolution. Leaf discs were taken after various periods of illumination with far red light and irradiated for 5 min with white light to test for photophosphorylation. The total ATP pool was measured by the firefly luciferase assay at different times during the 5-min irradiation period. With dark-grown leaves or with leaves irradiated 12 hr with far red light, the pool of ATP was relatively high and was not altered by irradiation (Fig. 2). At 24 hr near the onset of photosynthetic activity the pool of ATP was somewhat lower initially but increased substantially during the irradiation period owing to

photophosphorylation. At later stages of greening in far red light the ATP pool was lower and did not increase so much, probably because of the greater drain on the ATP pool by synthetic activity.

The development of the photosynthetic apparatus in far red light was also followed by electron microscopy. The electron micrographs of the 8-day-old dark-grown bean leaves (Fig. 3a) were typical of such tissue, showing large crystalline prolamellar bodies with a few surrounding lamellae. After 12 hr of far red light (Fig. 3b) the picture was essentially the same except that the etioplasts contained appreciably more lamellae. Short regions of fusion between adjacent lamellae occur at all stages including the completely dark-grown leaf. Up to this time the cross sections of most of the lamellae or thylakoids were discontinuous and appeared to consist of numerous small segments as if the process by which small vesicles coalesce to form large thylakoids were not completed. At 24 hr (Fig. 3e), the time of onset of photosynthetic activity, large uniform thylakoids began to appear, and some of these lined up in close parallel juxtaposition. This pattern of stacks of parallel thylakoids, separated by about the thickness of a thylakoid, remained throughout the greening process in far red light. We will use the term "primary thylakoid" introduced by Sironval *et al.* (23) to indicate these large uniform thylakoids that have not fused into grana. On the average the prolamellar bodies at 24 hr were somewhat smaller in size, but they remained numerous and well formed. By 42 hr (Fig. 3d) the number of parallel thylakoids had increased, and the size of the prolamellar bodies, still highly ordered, was much smaller. Irradiation with white light for 30 min at any of these stages caused the complete disappearance of the prolamellar bodies. By 60 hr in far red light (Fig. 3e) the prolamellar bodies were scarcely visible. Figure 3f (84 hr in far red light) shows the large unfused stacks of parallel thylakoids which develop under prolonged irradiation with far red light.

Low temperature absorption spectra and derivative spectra were measured with intact leaves after various periods of illumination in far red light (Fig. 4) to determine if the appearance of any chlorophyll pigments correlated with the development of activity or structure. The transformation of protochlorophyll by far red light was very inefficient so that chlorophyll accumulated very slowly. The rate of synthesis of protochlorophyll was almost as rapid as its rate of conversion. The absorption spectrum of the leaf after 4 hr of far red light showed almost as much protochlorophyll as was present initially and a very broad band for the chlorophyll *a* which had accumulated. The derivative spectrum of the 4-hr leaf showed that the chlorophyll was differentiated into three forms even at this early, nonactive stage. These were not the early transient forms of chlorophyll which appear shortly after transformation (20) but were stable forms which accumulated throughout the greening process. The spectra of the leaves at 12 hr and at 24 hr showed the same components with progressively less protochlorophyll and more of the three forms of chlorophyll. At 24 hr a shoulder in the 660 nm region of the derivative spectrum was somewhat more prominent, but this appeared to be a gradual transition from earlier stages. In the spectrum of the leaf after 48 hr, two additional long wavelength absorbing forms with maxima near 690 and 698 nm were apparent in the derivative spectrum. These bands were also detected as small shoulders in the derivative spectrum of some leaves after 24 hr in far red light when the measurements were made at higher sensitivity. The 690 and 698 nm bands were seen previously in low temperature derivative spectra of spinach chloroplasts (6). The absorption band at 650 nm in the 78-hr spectrum was due mainly to chlorophyll *b*. This band

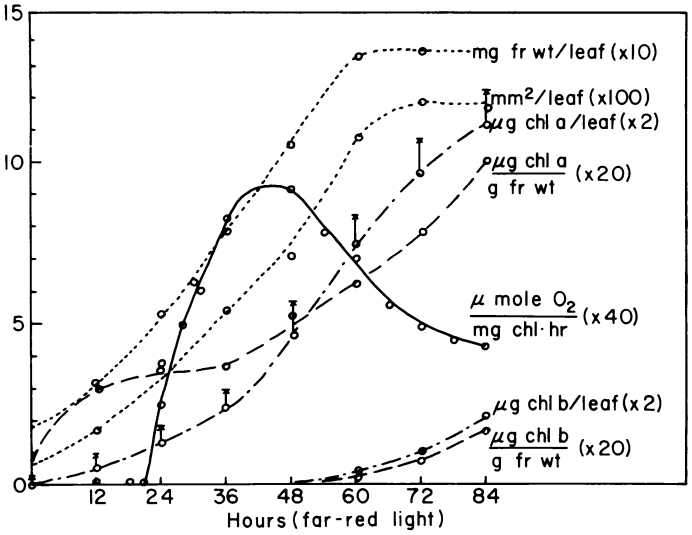

FIG. 1. Growth (fresh weight and area) of the primary leaves, chlorophyll *a* and *b* content (per leaf and per gram fresh weight), and rate of oxygen evolution (in white light) as a function of time of irradiation in far red light. The 12 hr of darkness which followed each 12 hr of far red light are not plotted in the time base. The abscissa values should be multiplied by the factors given in parentheses for the various parameters measured. The values along the curve of chlorophyll *a*/leaf were obtained with leaves extracted in dim green light. The heights of vertical arrows indicate the additional amount of chlorophyll *a* obtained when the leaf was irradiated with white light just prior to extraction.

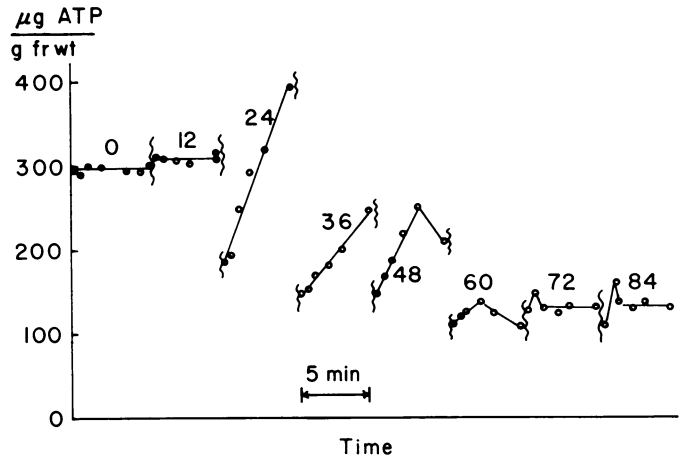

FIG. 2. Amount of ATP per gram fresh weight of the etiolated bean leaves after various periods of far red light (hours of far red light indicated by the numbers above the curves). The leaves were irradiated with white light for 5 min to stimulate photophosphorylation, and small leaf discs were taken at various periods during the 5-min irradiation to assay for ATP by the firefly luciferase method.

at 48 hr and earlier was due to photochlorophyll. The two 650 nm-absorbing pigments could be distinguished by the phototransformability of protochlorophyll.

Low temperature absorption and derivative spectra of leaves greened in white light were also measured (Fig. 5). Appreciable photosynthetic activity was measured after 2 hr of white light but not after 1 hr of light. The leaves greened in white light showed the same pigments as those greened in far red light but with a somewhat different distribution. The far red greened leaves had relatively more of the 684 nm-absorbing form than the normally greened leaves.

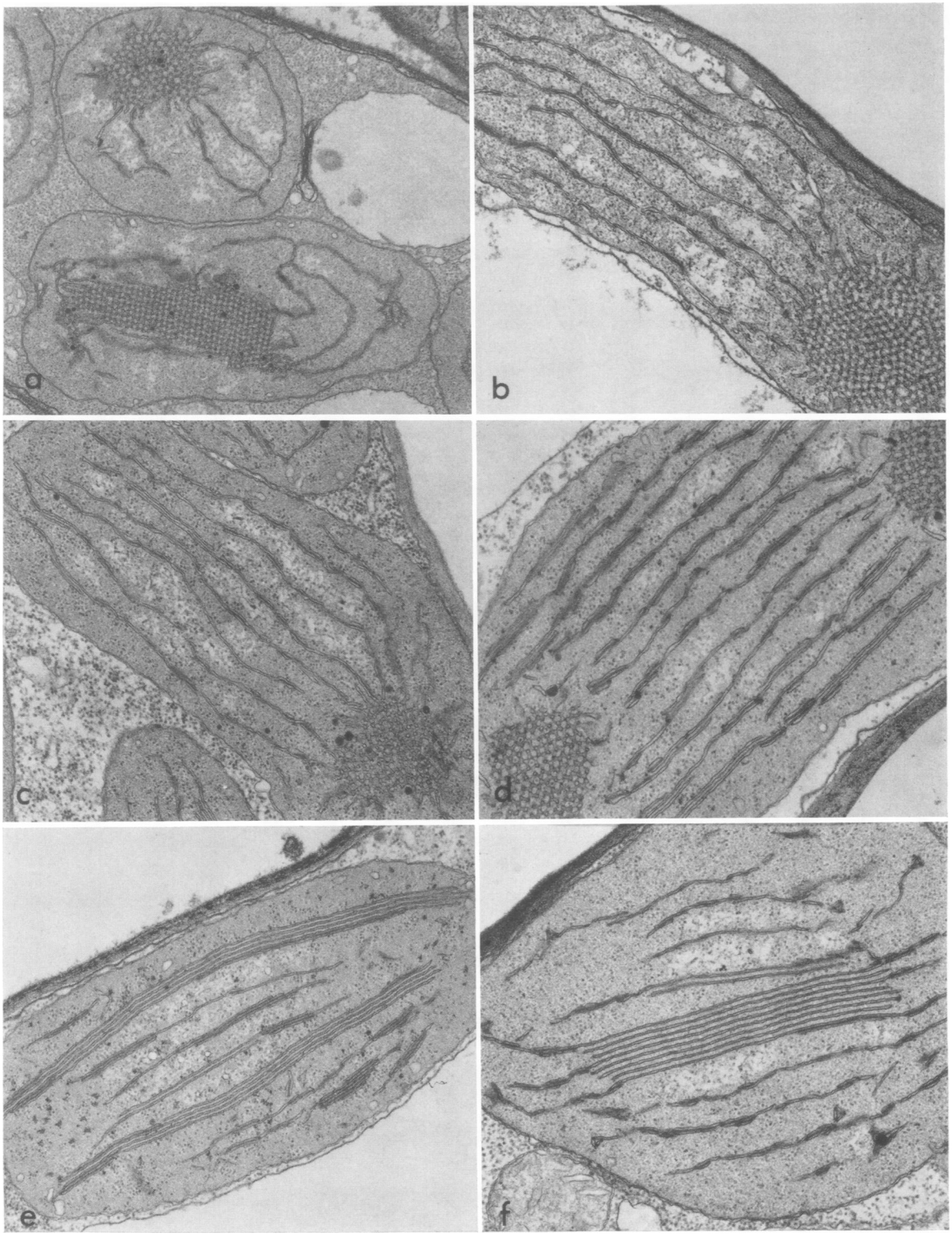

FIG. 3. Electron micrographs of 8-day-old etiolated bean leaves after various periods in far red light. a: 0 hr; b: 12 hr; c: 24 hr; d: 42 hr; e: 60 hr; f: 84 hr.

The parallel stacks of primary thylakoids seen after prolonged illumination in far red light could be induced to fuse into grana-like assemblages by placing the leaves in white light. Figure 6 is an electron micrograph of a leaf which had received 3 hr of white light following 60 hr of far red light. Such leaves should provide an excellent experimental system to explore the light requirements for the fusion process. Low temperature absorption spectra taken before and after the white light treatment (Fig. 7) did not reveal any particular pigment changes that could be causally related to the fusion proc-

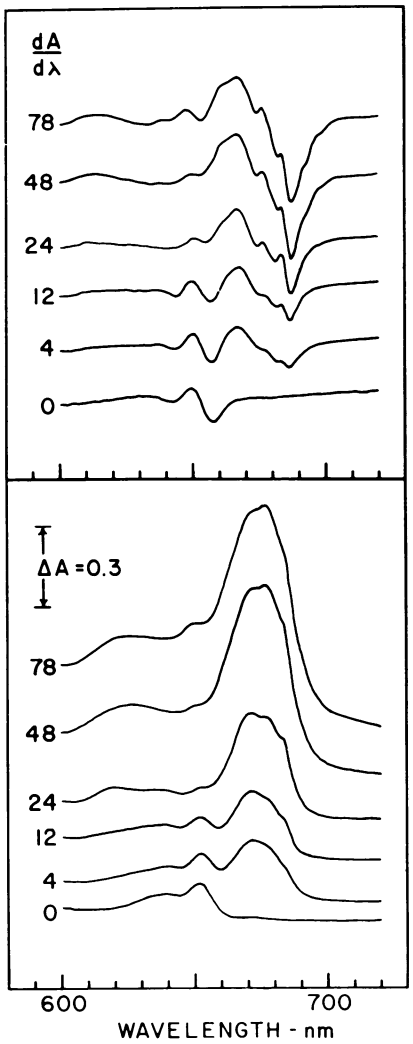

FIG. 4. Absorption and first-derivative absorption spectra of single leaves at −196 C. The numbers next to the spectra indicate the number of hours the leaf was in far red light before the measurement.

ess. Some chlorophyll *b* accumulated during the 3-hr fusion period, but more work is needed to establish the significance of such correlations with the fusion process.

The 8-day-old dark-grown bean leaves were also greened with a series of 1-min red light exposures given once each hour. Figure 8 shows the development of photosynthetic activity and the accumulation of chlorophyll. Chlorophyll was measured immediately after the 1-min light period, and activity measurements were made at the end of the 1-hr dark period. A 12-hr dark period followed the 13th irradiation. Photosynthetic activity increased rapidly during the early flashes. No chlorophyll *b* appeared until near the end of the 2nd day. An electron micrograph of a leaf after 17 1-min irradiations (Fig. 9) shows a number of primary thylakoids with little fusion. The low temperature absorption spectrum of such a leaf (Fig. 10) is more similar to the spectrum of a leaf greened in far red light than that of one greened to a similar chlorophyll content in continuous white or red light. Sironval *et al.* (23) have shown electron micrographs and low temperature spectra of dark-grown bean leaves greened with up to 300 flashes separated by 15-min dark periods. Such leaves show large stacks of the primary thylakoids with very little fusion.

DISCUSSION

A previous study of the greening of 8-day-old dark-grown bean leaves in white light showed that a number of pigment changes were coincident or nearly coincident with the onset of photosynthetic activity (2). These included the differentiation of the bulk of the chlorophyll *a* into two forms, chlorophyll *a*-670 and chlorophyll *a*-680, the appearance of chlorophyll *b* (although this may have been complicated to some extent by the resynthesis of protochlorophyll) and the appearance of C-705 in the fluorescence excitation spectrum. In the present

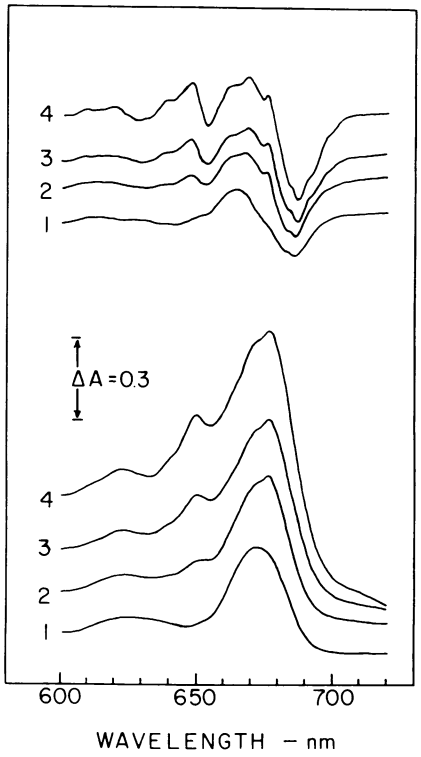

FIG. 5. Low temperature absorption and derivative spectra of leaves greened for the indicated number of hours in white light.

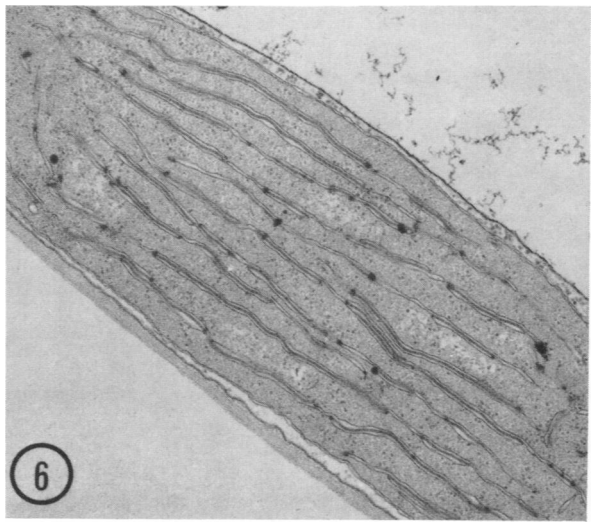

FIG. 6. Electron micrograph of an etiolated leaf irradiated for 60 hr with far red light followed by 3 hr of white light.

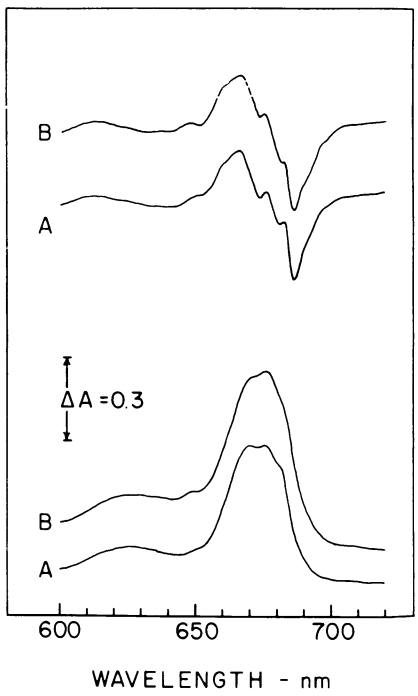

FIG. 7. Low temperature absorption and derivative spectra of etiolated leaves irradiated 60 hr with far red light before (A) and after (B) an additional 3 hr of white light.

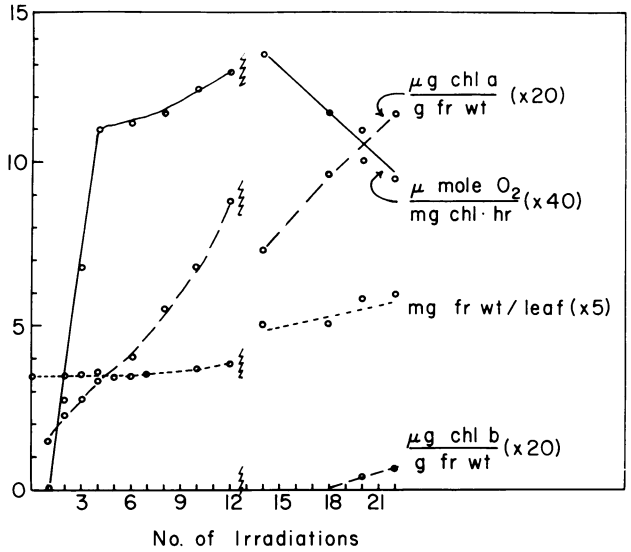

FIG. 8. Growth (fresh weight), chlorophyll *a* and *b* content (per gram fresh weight), and rate of oxygen evolution (in white light) of etiolated leaves after a series of brief irradiations (1 min) with red light given once every hour. A 12-hr dark period is indicated by the break in the curves.

work where the period of development was markedly prolonged by greening the leaves under far red light, the various pigment changes were well separated in time. The bulk of the chlorophyll *a* was differentiated into three forms with low temperature absorption maxima near 670, 677, and 683 nm well before the capacity to evolve oxygen apppeared. (The leaves greened in far red light were much enriched in the 683 nm-absorbing form compared to leaves greened in white light.) Chlorophyll *b* did not appear until long after the leaves were photosynthetically competent. It should be noted that our results on the late appearance of chlorophyll *b* both in far red-irradiated leaves and in flash-irradiated leaves are at variance with the reports of Rudoi *et al.* (17) and Shlyk *et al.* (21, 22) that chlorophyll *b* appeared within a few minutes after the initial transformation of protochlorophyll in etiolated maize leaves. However, they also suggest that there are species differences and that the synthesis of chlorophyll *b* is more rapid in maize than in barley.

The most meaningful correlation between the onset of photosynthetic activity and the appearance of pigments was

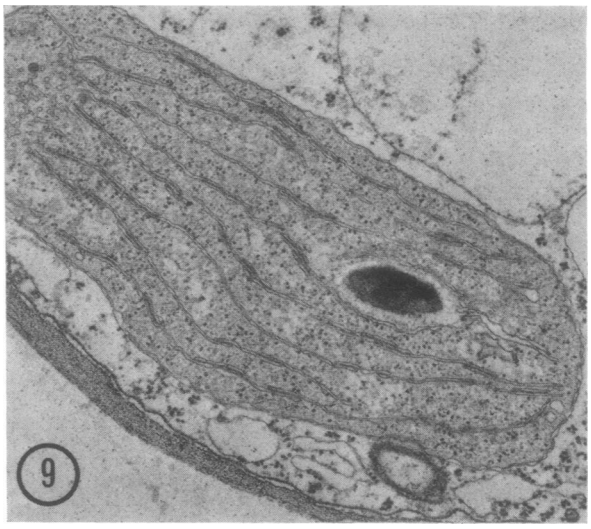

FIG. 9. Electron micrograph of an etiolated leaf after 17 of the brief irradiations used for Figure 8.

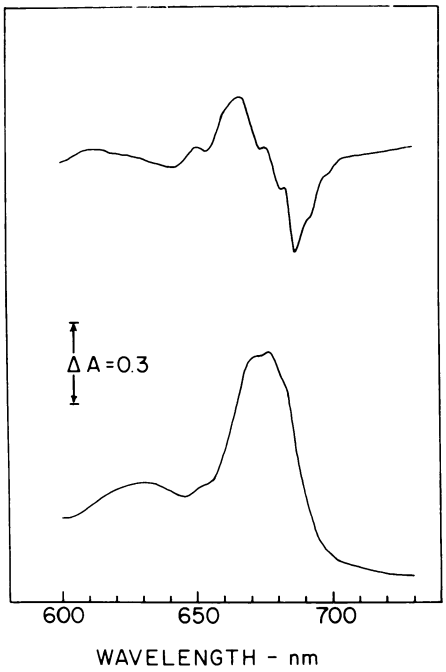

FIG. 10. Low temperature absorption and derivative spectra of an etiolated leaf after 17 brief irradiations (same treatment as that used for Fig. 9).

probably with the 690 and 698 nm-absorbing forms of chlorophyll, but this correlation could not be established sharply in time, probably because the spectrophotometric assay was not as sensitive to small amounts of the pigments as the activity assay. It is of interest that the time course curve for the oxygen evolution rises abruptly at 24 hr, indicating a sharp onset of activity with some degree of synchrony among the chloroplasts, rather than increasing gradually, which would indicate a gradual accumulation of active chloroplasts starting at early times.

There were no sharp correlations between the development of structure and the beginnings of photosynthetic competence. Grana formation, or fusion between adjacent thylakoids, was not necessary for the development of activity or the accumulation of chlorophyll. The most reasonable correlation was probably with the appearance of well developed primary thylakoids. The close parallel alignment of these thylakoids began at the same time as oxygen evolution and may be significant, but the appearance of the thylakoids is probably more important than their parallel alignment. There was relatively little parallel alignment of the thylakoids in the leaf which had received 17 brief irradiations with red light, yet the activity was present and had been present since the third flash.

A great deal of synthetic activity occurred in the far red light under control of phytochrome. The leaves expanded markedly and increased over 4-fold in dry weight. The synthesis of chlorophyll (and yellow pigments) per leaf was also pronounced since the leaves increased 20-fold in area, and the pigment content per unit area, as indicated by the absorption spectra on intact leaves, increased several fold. It is unlikely that the synthetic activity in far red light was sustained by photosynthesis because very little of the far red light was absorbed by the leaves. Thus, in far red light, the photosynthetic capacity developed in the absence of photosynthetic activity. It had been noted previously in etiolated bean leaves (11) and in dark-bleached *Euglena* cells (20) that the photosynthetic apparatus developed in the presence of dichlorophenyl dimethylurea, which would block normal photosynthesis.

The absence of fusion between thylakoids in the far red greened leaves appears to be due to the low amount of energy absorbed during the greening process. Unfused stacks of thylakoids were observed by Sironval *et al.* (22) with leaves greened by a series of brief flashes of red light and by Eilam and Klein (7) with leaves greened with continuous low intensity (2 ft-c) white light. Eriksson *et al.* (8) had previously noted that grana formation in etiolated leaves began at the end of the lag phase in chlorophyll synthesis when chlorophyll began to increase steeply. They concluded that a high energy light reaction was involved with the grana formation process, but it was not clear whether such a light reaction was directly involved in grana formation or whether grana formation occurred as a consequence of the accumulation of chlorophyll. The results of the present work, showing that the unfused stacks of thylakoids formed during 60 hr in far red light are induced to fuse by a relatively short period of white light, suggest that the fusion is not solely a function of chlorophyll accumulation. However, it could require ATP from photophosphorylation. Whether chlorophyll *b* plays any special role is still to be determined. The far red greened leaves should provide useful experimental material to explore the fusion process further.

If the primary thylakoids in the unfused stacks are comparable to the stroma lamellae of normal chloroplasts, the work of Sane *et al.* (18) suggests that the leaves greened in far red light might be enriched in photosystem 1 activity. Previous low temperature absorption and derivative spectra of chloroplasts and of photosystems 1 and 2 subchloroplast particles obtained by digitonin fractionation showed that the long wavelength forms of chlorophyll, especially the 684, 690, and 698 nm-absorbing forms are associated with photosystem 1 particles (6). A comparison of the absorption spectra of leaves greened to similar extents in far red and in white light indicates that the far red greened leaves have relatively more of the long wavelength forms of chlorophyll, at least the 684 nm form. Such correlations suggest that the leaves greened in far red light or chloroplast fragments from such leaves should be examined for relative photosystem 1 and photosystem 2 activities. Although we would not be surprised to find a relative enrichment of the photosystem 1 activity in such chloroplasts, our results do not support the contention of Woo *et al.* (27) that grana are required for photosystem 2 activity.

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