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THE GREENING OF ETIOLATED BEAN LEAVES

III. MULTIPLE LIGHT/DARK STEP PHOTOCONVERSION PROCESSES

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SUMMARY

1. Multiple short light/long dark step photoconversions of etiolated bean leaves were made to study the synthesis of chlorophylls *a* and *b* and the carotenoids, together with the formation of the photoactive molecular arrays of Photosystems I and II, as indicated by comparative fluorescence analysis *in vivo* at 77°K.

2. In old etiolated leaves (approx. 21 days), chlorophyll *a* per leaf is formed as a linear function of the number of photoconversions, in accord with a possible protein carrier function of the protochlorophyllide holochrome. The chlorophyll arrays of the first units of Photosystems I and II, taking the chlorophyll *a*/*b* ratio of 3 as a criterion, form up concurrently and are completed with the synthesis of no more than 600 total chlorophyll molecules per holochrome.

3. The fluorescing centres of Photosystems I and II form when chlorophyll *b* is formed within the range 2–7 % of the total chlorophyll. Two sites of location of chlorophyll *b* are indicated in each of the forming arrays of the two photosystems, an inner or trap site, and an outer or peripheral site.

4. In younger leaves (approx. 10 days), chlorophyll *a* forms as before, but leaf expansion (about 10-fold) occurs and chlorophyll *b* formation is almost inhibited.

5. The carotenoid content per leaf is proportional to leaf area, over a 10-fold range, indicating that carotenoid formation, and leaf expansion, follow a parallel development. White and blue light experiments support this concept.

6. Where leaf expansion, with a minimum of chlorophyll formation, has taken place, further greening in continuous white light gives a maximum steady rate of chlorophyll synthesis. During this period, it is calculated that the synthesis of a quantity of chlorophyll *a*, equivalent to the capacity of each holochrome in the leaf, takes place once every 4 min at 25°.

INTRODUCTION

In earlier papers of this series[1,2], *in vivo* studies of the photoconversion processes of etiolated bean leaves, have suggested that the protochlorophyllide holochrome may be regarded as a protein carrier. The protochlorophyllide F655 located on the holochrome is photoconverted to chlorophyll *a* and then possibly transposed from the holochrome to forming membranes. It was shown that the quantity of protochloro-

phyllide reformed in the dark in a leaf was the same at Photoconversions 1, 2 and 3. Using the appearance of the intermediate F674 occurring at the initial fractional photoconversion, it was estimated that each holochrome *in vivo* had an average capacity of some 20 molecules of protochlorophyllide. The formation of chlorophyll *a* in the etiolated leaf was then studied in the earliest stages[2], *in vivo*, by a series of short light/long dark steps.

One approach to an understanding of the molecular organization of the photochemical systems has been to use selective fragmentation of the mature chloroplasts and to study the properties of isolated complexes. BOARDMAN AND ANDERSON[3,4] gave details of the isolated complexes of Photosystems I and II. The fluorescence properties of the complexes were also detailed[5]. A recent summary[6] of other analytic methods that have been used to isolate complexes has also been made.

Another approach, in part made possible by the results of the analytical method, is to study the synthesis of the photosystems in etiolated seedlings. This approach was used by THORNE AND BOARDMAN[7] who studied the formation of the photosystems in etiolated peas on greening in continuous white or red light.

AKOYUNOGLOU AND ARGYROUDI-AKOYUNOGLOU[8] investigated the effects of intermittent light on etiolated plants, by measuring chlorophyll *a* and chlorophyll *b* only, but could only speculate due to insufficient evidence, that under these conditions Photosystem I only may be formed.

The synthesis of the elements of Photosystems I and II chlorophyll arrays may also be followed *in vivo* in the bean leaf, by the use of a series of light/dark steps, each dark period allowing a reformation of protochlorophyllide. This method was used in the present work where the formation of chlorophyll *a*, the chlorophyll *a*/*b* ratio, *in vivo* 77°K fluorescence emission (F) and excitation (E) spectra, leaf expansion, total carotenoid formation and 77°K fluorescence kinetics F_∞/F_0, have been studied as a function of the number of photoconversion light/dark steps of etiolated bean leaves.

MATERIALS AND METHODS

Brown Beauty beans (*Phaseolus vulgaris* L) were dark grown at 25° under conditions previously described[1].

Multiple step light/dark regimes could be selected from synchronous interlocked timing mechanisms such that the light period could be selected within the range 5 sec to 5 min and the dark period with the range 10 min to 24 h. The number of light periods was recorded by a photocell and digital recorder.

All light exposures and growth were carried out in a room maintained at 25°. White light was provided by a bank of 40 W fluorescent tubes (Philips Type White). The intensity at leaf level was 12000 ergs·cm^{-2}·sec^{-1} as measured by a thermopile, or 600 ft candles by a Weston illumination meter. Red light was obtained from a bank of 4 red fluorescent lamps (Philips TL 20W/15) fitted with red filters to give a band pass between 620 and 690 nm, and an intensity at leaf level of 3500 ergs·cm^{-2}·sec^{-1}. Blue light was obtained from a bank of 4 blue fluorescent lamps (Philips Blue 20 W) fitted with blue filters to give a band pass between 420 and 500 nm, and intensity at leaf level of 3200 ergs·cm^{-2}·sec^{-1}. Far-red light was obtained from a 150-W photoflood lamp with water filter and plexiglass FR700 filter and intensity at leaf level of 15000 ergs·cm^{-2}·sec^{-1}.

Fluorescence emission (F) and excitation (E) spectra were recorded in an instrument which automatically corrected the spectra for photomultiplier and monochromator responses, and variation in energy output of the light source, as previously described by BOARDMAN AND THORNE[9]. The excitation monochromator was operated with a bandwidth of $\pm$ 1.5 nm and the fluorescence monochromator with a bandwidth of $\pm$ 1.0 nm.

Absorption spectra were recorded with a Cary Model 14R spectrophotometer fitted with a Cary Model 1462 scattered transmission attachment.

Emission and excitation spectra of leaves could be made at 77°K. Individual leaves were mounted in 1 cm quartz cells, being held gently between two thin clear sheets of perspex (0.5 mm thick) of such a size as to fit diagonally within the cells. Light from the monochromator was then incident on the leaf surface at 45°, and emission viewed from the rear side of the leaf, at 90° to the beam to avoid stray and scattered incident light. The integral of the emission spectrum was also recorded, from which the distribution of the emitted quanta could be expressed as Φ_{680}/Φ_{Tot}. The designation Φ_{680} refers to the emission between 675 and 705 nm, including the 683- and 693-peaks, while Φ_{Tot} refers to the total emission including the 735-nm band. This relative measure Φ_{680}/Φ_{Tot} avoids difficulties due to physiological variables in leaves, and serves to indicate the manner in which the elements of Photosystems I and II may be formed with progressive photoconversions.

Chlorophyll a determinations were made from ethanol extracts of leaves by fluorescence spectroscopy at 77°K. Excitation at 665 nm, with the chlorophyll a peak at 678 nm, avoided the absorbance due to carotenoids and protochlorophyll, and gave the necessary high sensitivity at low photoconversion stages. To minimize physiological variables, extracts were made on 10 leaves at each stage, the chlorophyll a per leaf at photoconversion n, a_n, being expressed relative to the extract at Photoconversion 1, a_1, as a_n/a_1, thus avoiding the need for an absolute calibration. All measurements of a_n/a_1 were made on a per leaf basis, irrespective of the leaf expansion which may have also occurred.

Chlorophyll b was also determined from ethanol extracts of leaves by fluorescence spectroscopy at 77°K. Excitation at 478 nm, enabled the chlorophyll a/b ratio to be measured within the range 3:1 to 100:1. Details of the calibration have been given by BOARDMAN AND THORNE[26]. At each photoconversion stage, where a leaf was used to determine Φ_{680}/Φ_{Tot}, the other leaf of the pair from the same seedling was ground in ethanol to give the extract from which the chlorophyll a/b ratio was measured. This value could then be expressed as the ratio chlorophyll $b/(a+b)$ in percentage.

Total carotenoids were measured on ethanol extracts of leaves by absorption spectroscopy at 472 nm and expressed as c_n/c_1, where c_n and c_1 are the carotenoid content per leaf at the n^{th} and first photoconversion, respectively. Under the conditions used, interferences from chlorophyll a or b were negligible at 472 nm.

Leaf areas were measured from the average dimensions of 10 leaves at each photoconversion stage. The average length l and width w of gently pressed leaves was determined, from which $A_1 = l_1 w_1 f$ and $A_n = l_n w_n f$ where f is a shape factor which could be considered constant, since the ratio l_n/w_n was constant as leaf expansion occurred. The ratio A_n/A_1 was then simply determined from $l_n w_n / l_1 w_1$.

RESULTS

Multiple photoconversions of 'old' leaves

14-day-etiolated bean seedlings were subjected to light/dark step photoconversions with 2 min of white light at 600 ft candles followed by 24-h dark periods for 4 conversions then alternating 16-h or 8-h dark periods up to 22 photoconversion

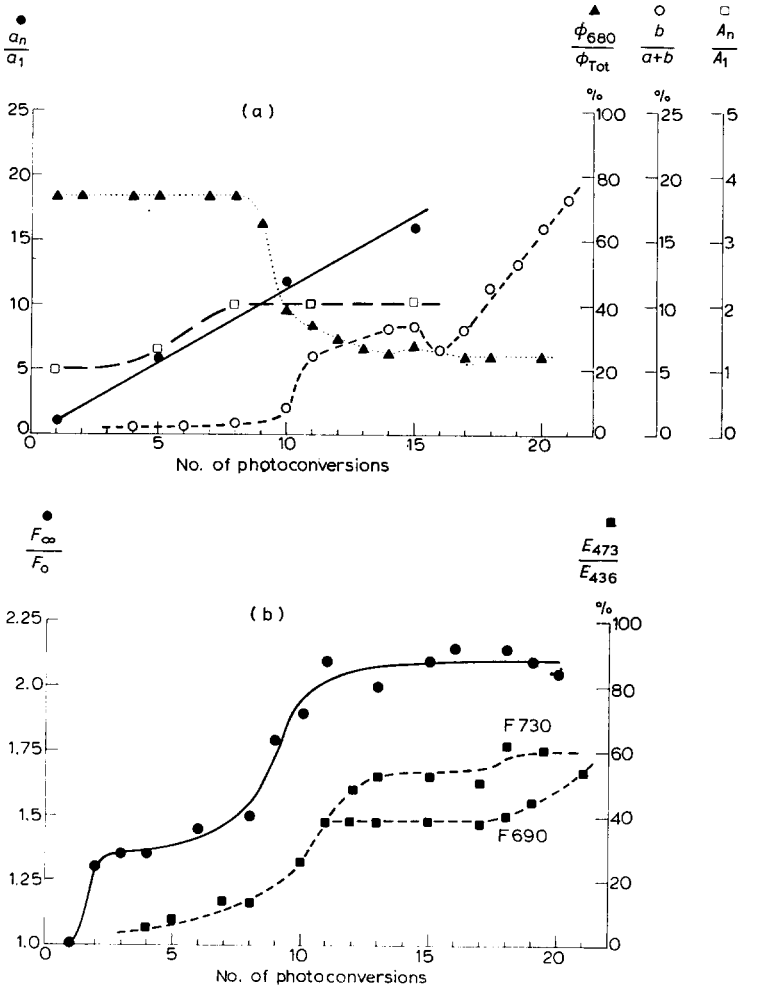


Fig. 1a. 14-day-etiolated bean leaves subjected to light/dark cycles of 2 min white (600 ft candles) followed by 24 h dark at 25° for 4 cycles and then alternately 16-h or 8-h dark periods. ●—●, a_n/a_1 the ratio of chlorophyll *a* content per leaf at photoconversion *n* to that at Photoconversion 1; □--□, A_n/A_1 the ratio of leaf area of the average of 10 leaves; ○---○, $b/(a+b)$ the ratio of chlorophyll *b* to the total chlorophyll $(a+b)$ of the leaf at each photoconversion step; ▲---▲, Φ_{680}/Φ_{Tot} the ratio of the fluorescence quantum efficiency distribution of a leaf at each photoconversion step at 77°K. (b) ■---■, E_{473}/E_{436} the ratio of the peaks in the fluorescence excitation spectra, with the fluorescence monochromator set at 690 nm up to Photoconversion 10, and either at 690 nm or 730 nm beyond that point; ●—●, the fluorescence kinetic ratio F_∞/F_o at 690 nm. The leaf is dark mounted, cooled to 77°K, excitation 436 nm with incident intensity 90 ergs·cm^{-2}·sec^{-1}, F_o initial fluorescence amplitude; F_∞ the value after 2 min in the excitation beam.

steps. Long dark periods were allowed for the complete resynthesis of protochlorophyllide at each step.

Following each photoconversion, values of a_n/a_1, $b/(a+b)$, and A_n/A_1 were measured, whilst from fluorescence spectroscopy on whole leaves at 77°K values of Φ_{680}/Φ_{Tot}, F_∞/F_0 and E_{473}/E_{436} were determined. The values are plotted against photoconversion step n in Figs. 1a and 1b. The ratio a_n/a_1 determined on a per leaf basis is a linear function of n for $1<n<20$, thus justifying the extension of the concept that the pool of protochlorophyllide is resynthesized to the same level in the leaf after each photoconversion, despite the fact that some leaf expansion A_n/A_1 occurs. The relationship of the fluorescence emission at 77°K, $\Phi/_{680}/\Phi_{Tot}$ and that of the formation chlorophyll b is interesting. The major change in the ratio $\Phi/_{680}\Phi_{Tot}$ occurs due to the rise of emission at 735 nm when chlorophyll b is formed in the leaf in the range 2–7 % of the total chlorophyll. Fig. 2a compares the fluorescence emission and red excitation spectra of a leaf at Photoconversion 8, prior to the onset of chlorophyll b formation, with Photoconversion 13 where the chlorophyll b is about 7 % of the total chlorophyll with a chlorophyll a/b ratio of about 14:1. The excitation spectra with the fluorescence set at 735 nm show that an additional band at 678 nm together with a band at 705 nm have developed. BUTLER[10] has previously associated the 705-nm band in mature chloroplasts with the 735-nm emission at 77°K. In this step greening system *in vivo*, it then becomes apparent that the 705 nm excitation and the 735-nm emission arise when chlorophyll b is formed in the range 2–7 % of the total chlorophyll. Prior to the changeover of Φ_{680}/Φ_{Tot}, the excitation spectrum for F_{735} shows that the E_{705} band is absent. Also prior to the changeover the main emission peak shifts slowly from 683 to 686 nm as n increases, but after the changeover appears as 693 nm together with the new 735-nm emission band. Emission at 683 and 693 nm has been associated with Photosystem II whilst emission at 735 nm has been associated with Photosystem I in mature chloroplast fragmentation studies[5].

Excitation spectra over the range 400–520 nm were also taken at 77°K at each Photoconversion stage, with the fluorescence monochromator set at 690 or 735 nm. Fig. 2b shows two such spectra, normalised at 436 nm. The excitation peak at 473 nm is associated with chlorophyll b formation, whilst that at 493 nm is associated with energy transfer from the carotenoids. Fig. 1b also shows the way in which the ratio E_{473}/E_{436} increases as n increases. Although not plotted, E_{493}/E_{436} also follows a similar pattern of development. The excitation spectra were recorded with the fluorescence set either at F_{690} or F_{735}, the ratio E_{473}/E_{436} being shown for both cases in Fig. 1b. This clearly indicates that chlorophyll b is being formed in both of the forming photosystems, with energy transfer from the carotenoids and chlorophyll b to chlorophyll a and thence to the fluorescing centres of both of the forming photosystems.

At these early development stages, it is also possible to observe a fluorescence kinetic effect shown as F_∞/F_0 in Fig. 1b. A leaf is dark mounted at each photoconversion step, and cooled to 77°K in the dark, then with the conditions given in Fig. 1b, the emission at 690 nm following continuous excitation at 436 nm, rises from an initial value F_0 to a value F_∞ in a period of 2 min, indicating that some work of reduction is being performed by the incident light[11], the ratio F_∞/F_0 being a measure of this. At the changeover of the $\Phi/_{680}/\Phi_{Tot}$ curve, the value of F_∞/F_0 also changes from a value of about 1.3–2.1. It should perhaps be emphasized that this kinetic effect is observable once only at 77°K following excitation The ratio of F_∞/F_0 against photocon-

version stage is shown in Fig. 1b to be 1.0 following Photoconversion 1, and 1.3 following Photoconversion 2. This point was investigated as a function of dark time following Photoconversion 1, values of F_∞/F_0 and the resynthesis of protochlorophyllide being shown in Table I. It is clear that F_∞/F_0 and the resynthesis of protochlorophyllide are both subject to a similar lag phase. This supports the view[2] that the chlorophyll *a* may be transposed to a new environment, thus allowing a resynthesis of protochlorophyllide on the holochrome.

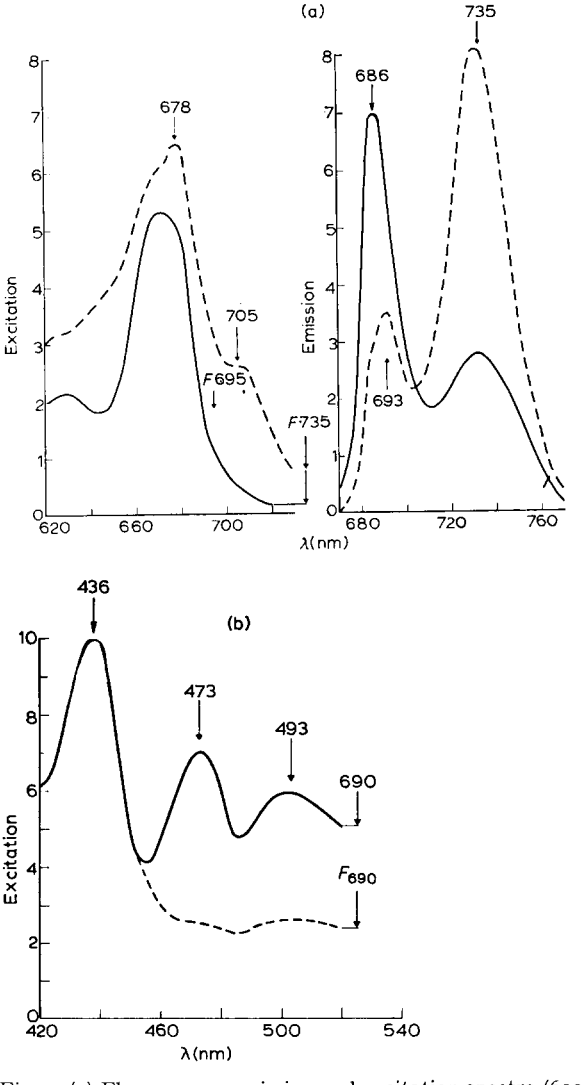


Fig. 2. (a) Fluorescence emission and excitation spectra (600 nm onwards) at 77 °K with conditions as in Fig. 1. ———, following Photoconversion 8 *plus* 1 h dark; – – – –, following Photoconversion 13 *plus* 1 h dark. $F\lambda$ indicates setting of the fluorescence monochromator. (b) Fluorescence excitation spectra (400–520 nm) at 77 °K with conditions as in Fig. 1. – – – –, following Photoconversion 11 *plus* 1 h dark; ———, following photoconversion 18 *plus* 1 h dark. Fluorescence monochromator set at 690 nm, the two curves being normalised at E_{438}.

Following the changeover of Φ_{680}/Φ_{Tot} as shown in the emission spectra of Fig. 2a, as the number of photoconversion steps increased, the ratio chlorophyll $b/(a+b)$ reached about 18 % at Photoconversion 21. Measurements were stopped at this stage due to increasing leaf age (about 27 days). Extrapolation of the chlorophyll $b/(a+b)$ curve of Fig. 1a, showed that some 25 photoconversions would be necessary to give a chlorophyll a/chlorophyll b ratio of 3.

Multiple photoconversions of 'young' leaves

In order to avoid the age affect of leaves, 10-day-etiolated bean seedlings were subjected to multiple step light/dark photoconversions. Comparisons were made with cycles of 2 min white light/8 h dark, 2 min white/3 h dark, 2 min red light/3 h dark and 2 min blue light/3 h dark, with up to 30 photoconversions at 25° in each case. Results for the case of 2 min white/8 h dark are shown in Fig. 3a, values of a_n/a_1, A_n/A_1, $b/(a+b)$ and Φ_{680}/Φ_{Tot} being plotted at each photoconversion step as before. Chlorophyll a, measured as a_n/a_1 on a per leaf basis shows a linear increase, however this regime is characterized by a large leaf area increase, A_n/A_1 following a sigmoid growth curve to give a 10.5-fold increase in leaf area after some 25 photoconversions. Under these conditions very little chlorophyll b is formed. At Photoconversion 20 for example, less than 1 % chlorophyll b is formed, the emission from the leaf at 77°K being mostly at 687 nm with no significant increase in the 735-nm band. Fig. 3b compares the leaf emission and red band excitation spectra at Photoconversions 19 and 22. At Photoconversion 22, about 3 % chlorophyll b has formed, and at this point there is again a correlation with the increase in emission at 735 nm with a corresponding excitation at 705 nm. Leaf expansion then in some way, delays the formation of chlorophyll b on the membranes.

The multiple step photoconversions of 10-day-etiolated bean seedlings with regimes of 2 min white light/3 h dark or 2 min red light/3h dark were almost identical to each other and closely followed the results of 2 min white/8 h dark. At Photoconversion 30, the value of A_{30}/A_1 was 8.5, with a linear increase in a_n/a_1 on a per leaf basis with chlorophyll b about 1% of total chlorophyll only, with no changeover of Φ_{680}/Φ_{Tot}. Once again leaf expansion seems to inhibit the formation of chlorophyll b.

Leaf expansion is known to be associated with phytochrome, summaries being given by HILLMAN[12] and more recently by SMITH[13]. White or red light establishes a high photostationary state of P_{fr}/P_r, the P_{fr} being responsible for the mediation of leaf expansion. 10-day-etiolated bean seedlings were then subjected to photoconver-

TABLE I

14-day-etiolated bean leaves after Photoconversion 1. Comparison of the fluorescence kinetic ratio F_∞/F_o, conditions as Fig. 1, and the resynthesis of protochlorophyllide with subsequent dark time at 25°. Relative protochlorophyllide was determined by ethanol extraction of leaves by fluorescence at 77°K, excitation 440 nm, from the fluorescence emission peak at 630 nm. The 15-h dark period being taken as a unit reference (see ref. 1).

	Dark time(h):					
	1	*2*	*3*	*4*	*6*	*15*
F_∞/F_o	1.0	1.04	1.08	1.20	1.35	1.36
F_{630}	0	0.02	0.25	0.79	1.0	1.0

sion cycles of 2 min blue light/3 h dark. Values of a_n/a_1, A_n/A_1, $b/(a+b)$ and Φ_{680}/Φ_{Tot} for these conditions at 25° are shown in Fig. 4. Blue light served to retard leaf expansion yet still giving photoconversion, the value of A_{30}/A_1 being about 2.5. Chlorophyll *b* formed in the manner shown, up to about 5 % at Photoconversion 20, then increasing leaf expansion caused this value to fall at higher photoconversions. A comparison of the emission and red band excitation spectra at 77°K of individual leaves at various stages during the blue light cycles, showed a correlation between

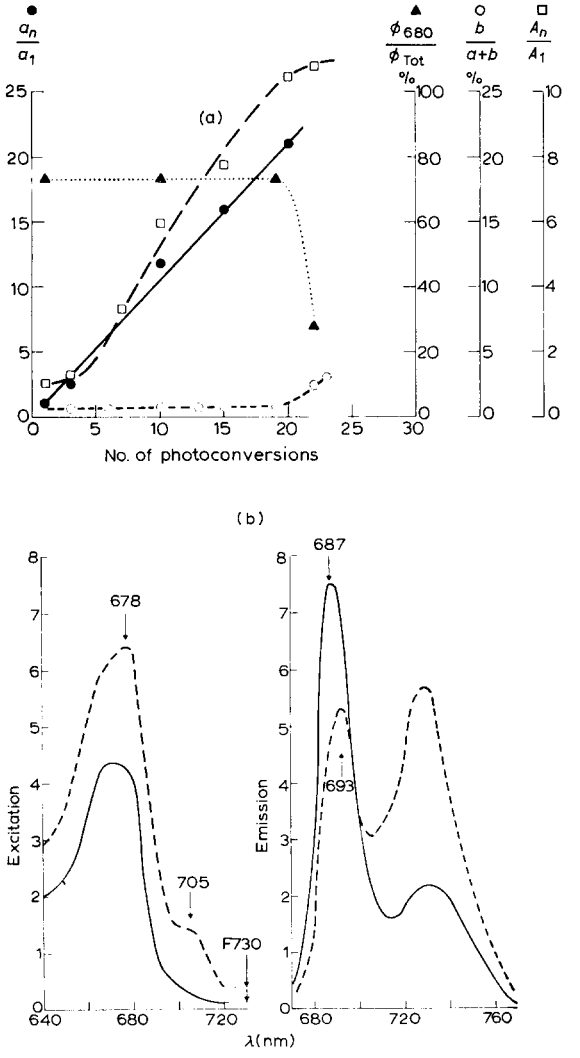


Fig. 3. (a) 10-day-etiolated bean leaves subjected to light/dark cycles of 2 min white (600 ft candles) followed by 8 h dark at 25°. ●—●, a_n/a_1 chlorophyll *a*; □—□, A_n/A_1 ratio of leaf area; ○---○, $b/(a+b)$ ratio of chlorophyll *b* to total chlorophyll $(a+b)$ ▲---▲, Φ_{680}/Φ_{Tot} fluorescence distribution at 77°K, at each photoconversion step. (b) Fluorescence emission and excitation spectra (600 nm, onwards) at 77°K of individual leaves ———, following Photoconversion 19 *plus* 1 h dark; – – – –, following Photoconversion 22 *plus* 1 h dark. *Fλ* indicates setting of fluorescence monochromator.

chlorophyll *b* formation with the formation of the emission at 735 nm with a corresponding excitation band at 705 nm.

Multiple step photoconversions were also made on 10-day-etiolated bean leaves with cycles of 2 min white followed by 2 min blue light with 3-h dark periods and also with 0.5 min white followed by 2 min blue light and 3-h dark periods, in each case the results were identical to the case of 2 min white/3 h dark, the sequential blue light treatment being completely ineffective, for leaf expansions of 8.5 were recorded for some 30 photoconversions. Thus limited blue light did not partially reverse the initial effect of the white light treatment.

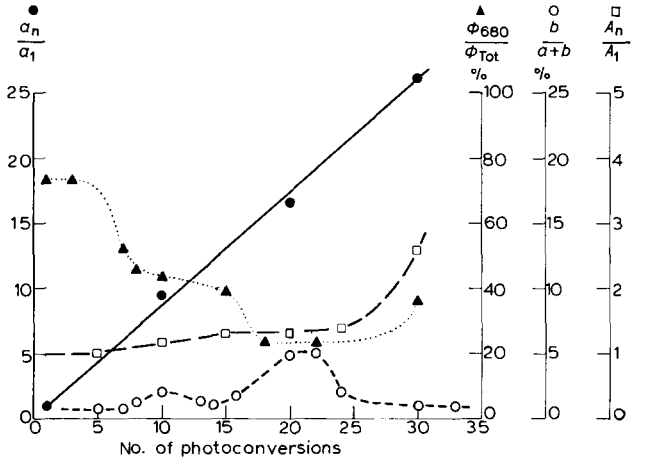


Fig. 4. 10-day-etiolated leaves subjected to light/dark cycles of 2 min blue (3200 ergs·cm^{-2}·sec^{-1}) with 3 h dark at 25°. ●—●, a_n/a_1 chlorophyll *a*; □ — — □, A_n/A_1 ratio of leaf area; ○----○, $b/(a+b)$ the ratio of chlorophyll *b* to total chlorophyll; ▲---▲, Φ_{680}/Φ_{Tot} fluorescence distribution at 77°K at each photoconversion step.

Multiple step exposures were also made on 10-day-etiolated bean leaves with cycles of 2 min far-red radiation with 3-h dark periods. At exposure 30, the value of A_{30}/A_1 was 1.6, with only about 2 % of the initial pool of leaf protochlorophyllide being converted to chlorophyll *a*. For comparison multiple step photoconversions were made with cycles of 2 min white light immediately followed by 10 min of far-red radiation with 3-h dark periods. The results for this treatment closely followed those for the blue light photoconversions as in Fig. 4, but with a value of $A_{30}/A_1 = 1.9$. Thus sequential far-red exposure effectively retarded leaf expansion, for up to 30 photoconversion cycles, implicating a leaf phytochrome system with multiple reversibility.

Multiple photoconversion of 21-day-etiolated leaves

The best conditions for the study of the formation of chlorophyll *b* arise when leaf expansion is minimized, for this reason 21-day-etiolated bean seedlings were subjected to multiple photoconversion cycles of 2 min white light/3 h dark. Values of a_n/a_1, $b/(a+b)$, A_n/A_1 and Φ_{680}/Φ_{Tot} were determined. The depletion of reserves due to the initial age of the leaves led to a leaf expansion of A_{30}/A_1 of only about 1.5. Although the age and conditions were different, the results closely followed a pattern somewhat similar to Fig. 1a. The changeover of Φ_{680}/Φ_{Tot} occurred with <5 % chloro-

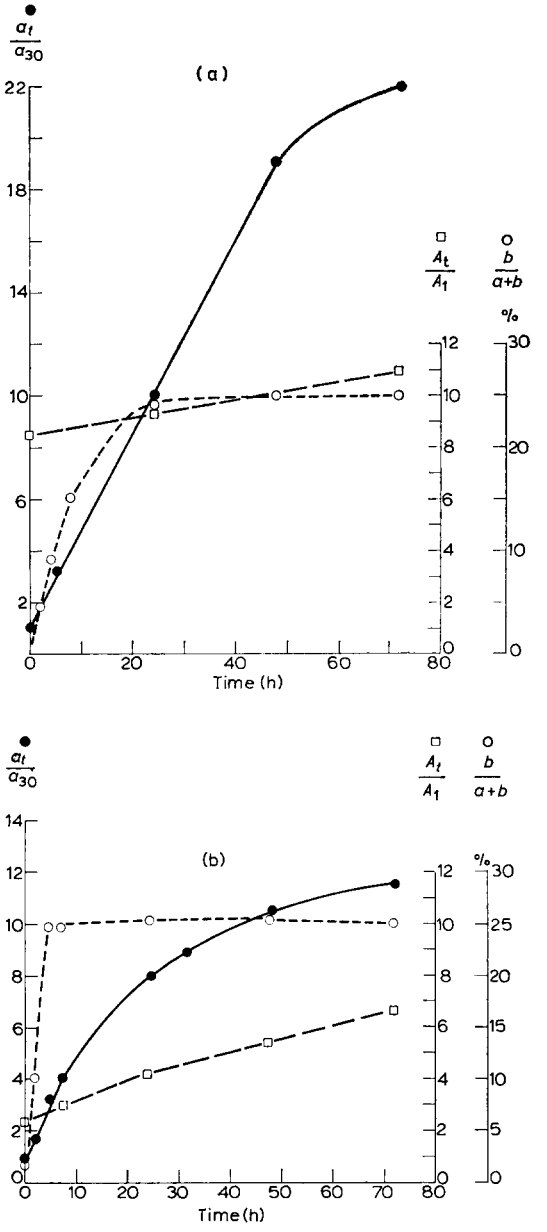
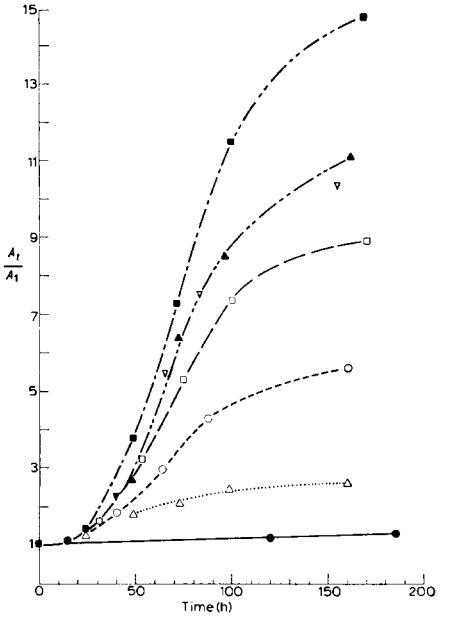
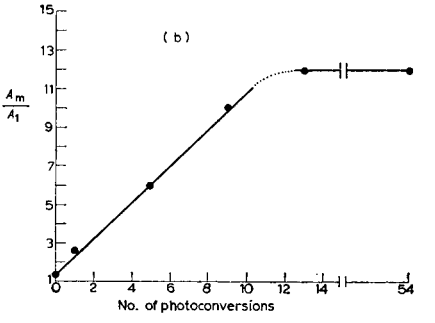


Fig. 5. (a) 10-day-etiolated bean leaves, subjected to 30 photoconversions of 2 min white light (600 ft candles) with 3-h dark periods, then followed by continuous illumination in white light at 600 ft candles for up to 80 h. ●—●, the ratio a_t/a_{30}, the chlorophyll *a* content per leaf, where a_{30} is the chlorophyll *a* per leaf at the 30th photoconversion and a_t is the chlorophyll *a* per leaf at following time *t* of continuous illumination; □--□, A_t/A_1 the ratio of leaf area at time *t* of continuous illumination to that at the first photoconversion; ○---○, $b/(a+b)$ the ratio of chlorophyll *b* to total chlorophyll at time *t* of continuous illumination. (b) 10-day-etiolated bean leaves subjected to 30 photoconversions of 2 min blue light (3200 ergs·cm^{-2}·sec^{-1}) with 3-h dark periods, then followed by continuous illumination in white light at 600-ft candles for up to 80 h. Plotted details as for (a).





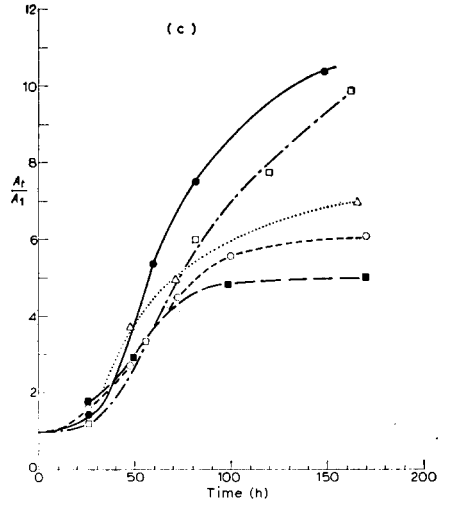


Fig. 6. (a) A comparison of leaf expansion of 10-day-etiolated bean leaves at 25° under various conditions. ■——·——■, under continuous white light at 600 ft candles; ▲——·——▲, under repeated photoconversion cycles of 2 min white light at 600 ft candles with 3-h dark periods; ▽——·——▽, leaves given 13 photoconversions of 2 min white/3 h dark followed by continuous dark; □ – – □, leaves given 9 photoconversions of 2 min white/3 h dark followed by dark; ○——○, leaves given 5 photoconversions of 2 min white/3 h dark followed by dark; △---△, leaves given 1 photoconversion of 2 min white followed by dark; ●—●, leaves kept in the dark. Each point is the average of 10 leaves. (b) Relative maximum leaf expansion A_m/A_1 during darkness at 25° following n initial photoconversions of 2 min white/ 3 h dark from the curves of (a). (c) Leaf expansion of 10-day-etiolated bean seedlings following 12 photoconversions in 2 min white light at 600 ft candles with various dark time spacing, followed by complete darkness 25°. ■– –■, 10-min dark spaces; ○———○, 20-min dark spaces; △---△, 1-h dark spaces; ●—●, 3-h dark spaces; □——·——□, 8-h dark spaces. The time zero is the commencement of the first photoconversion in each case.

phyll *b*, and as *n* increased chlorophyll *b* increased to about 15 % at Photoconversion 33. Extrapolation of the chlorophyll $b/(a+b)$ curve indicated that some 37 photoconversions would be necessary to give a chlorophyll a/b of 3.

Leaf expansion and the formation of chlorophylls a and b

In order to investigate further the relationship between leaf expansion and the formation of chlorophyll *a* and chlorophyll *b* two comparison experiments were made. In one experiment, 10-day-etiolated bean seedlings were given 30 photoconversions of 2 min white light/3 h dark, and then transferred to greening under continuous white light at 600 ft candles for a period of 72 h at 25°. Measurements of the chlorophyll *a* content per leaf at set times of continuous greening *t* hours were made and related the level of chlorophyll *a* per leaf at Photoconversion 30 and recorded as a_t/a_{30}. Any further leaf expansion was recorded as A_t/A_1, whilst the formation of chlorophyll *b* was recorded as the ratio $b/(a+b)$, as shown in Fig. 5a. Leaf expansion during the 30 photoconversions, A_{30}/A_1 was about 8.5. In the second experiment, seedlings were given 30 photoconversions of 2 min blue light/3 h dark and then greened under continuous white light at 600 ft candles as before. The results for this case are given in Fig. 5b. Leaf expansion during the 30 blue light photoconversions was only 2.5. The values of a_{30}/a_1 per leaf were almost the same for both the blue and the white light multiple photoconversions prior to the continuous greening. Comparison of the Figs. 5a and 5b shows that where the 8.5-fold leaf expansion has occurred prior to further greening, a massive linear increase in chlorophyll *a* per leaf occurs over a period of 48 h, with some 24 h being required for the chlorophyll a/b ratio to reach a value 3. In the case of Fig. 5b after a short linear increase in chlorophyll *a*, the chlorophyll *a* per leaf is constrained to follow the further leaf expansion curve, with the chlorophyll a/b ratio reaching a value of 3 in only about 4 h. Assuming 20 protochlorophyllide molecules per holochrome, calculations show that each holochrome may be cycled once every 4 min at 25° during the linear increase of chlorophyll *a* of Fig. 5a.

Leaf expansion and carotenoid formation

Treatment of 10-day-etiolated bean seedlings with multiple photoconversion cycles of 2 min white light/3 h dark gave a vast leaf expansion with very restricted chlorophyll formation, the expanded leaves showing the dense yellow of the carote-

TABLE II

RELATIONSHIP OF LEAF EXPANSION AND CAROTENOID FORMATION

Photoconversion of 10-day-etiolated bean leaves in 2 min white light at 600-ft candles/3 h dark cycles at 25°. n = number of light/dark cycles. A_{472}, absorbance/cm at 472 nm, of a solution of leaf extract in ethanol, at dilution 1 leaf to 12.5 ml ethanol. c_n/c_1 = ratio of carotenoid A_{472} at the n^{th} photoconversion cycle to that at photoconversion 1 of the ethanol extract on a per leaf basis. A_n/A_1 ratio of leaf areas under the same conditions. See appropriate curve of Fig. 6a.

n	A_{472}	c_n/c_1	A_n/A_1
1	0.07	1.0	1.0
8	0.11	1.55	1.42
16	0.21	3.0	2.6
24	0.47	6.6	6.3
32	0.60	8.6	8.5

noid pigments. Expansion also inhibited chlorophyll *b* formation, so this treatment appeared suitable to investigate the relation between leaf expansion and carotenoid synthesis.

Two of the curves of Fig. 6a compare leaf expansion under continuous white light with that under 2 min white/3 h dark cycles over a period of 150 h. It is clear that the leaf expansion, although slightly lower and delayed is of the same order for both cases. Table II shows the relation between carotenoid per leaf measured as c_n/c_1 with leaf expansion A_n/A_1 against the number of photoconversions. In the leaf extract in ethanol, the absorbance at 472 nm is taken as a measure of the carotenoid, corrections for chlorophyll *a* and *b* at this wavelength being negligible under these conditions. Carotenoid formation and leaf expansion are linear with respect to each other and are both light triggered but dark synthesis processes. In a similar measurement after 25 photoconversions in 2 min blue light/3 h dark, $A_{25}/A_1 = 1.7$ with $c_{25}/c_1 = 1.6$ confirming the linear relationship, and showing independence of chlorophyll synthesis.

The curves of Fig. 6a also show the manner of leaf expansion of seedlings given 1, 5, 9 or 13 photoconversions of 2 min white light/3 h dark followed by continuous dark at 25° in each case. The case of 13 photoconversions only is almost indistinguishable from the curve for multiple photoconversions of Fig. 6a. Each expansion curve is really a combination of two curves, one which shows an exponential rate of increase and the other an exponential rate of decrease, the general form of which may be expressed as

$$A(t) = A_m/(1 + e^{-k(t-t_0)}) \qquad (1)$$

with k and t_0 constants, $A(t)$ the area at time t, A_m the maximum area under the given conditions, with an inflection point at $t = t_0$ where $A(t_0) = A_m/2$. Appropriate choice of the constants A_m, k and t_0 gives an approximate expression for each of the curves of Fig. 6a. The relation $A(t_0) = A_m/2$ gives a convenient way of estimating the value of A_m for each condition of Fig. 6a values of which are plotted in Fig. 6b against the number of photoconversions. The relative values of A_m are linear with n up to about 10 photoconversions and beyond Photoconversion 12, there is no effect on the following leaf expansion. The relation of carotenoid per leaf and leaf expansion was also measured under these conditions, the results of which in Table III show a linear relation between c_n/c_1 and A_n/A_1.

TABLE III

FURTHER RELATIONSHIP OF LEAF EXPANSION AND CAROTENOID FORMATION

Photoconversion of 10-day-etiolated bean leaves in 2 min white/3 h dark for n cycles followed by long dark period at 25°. Details for Table II and appropriate curves of Fig. 6a.

n	*Dark time(h)*	A_{472}	c_n/c_1	A_n/A_1
1	0	0.07	1.0	1.0
1	160	0.18	2.6	2.6
5	144	0.44	6.3	5.9
9	144	0.56	8.0	8.6
12	120	0.78	11.1	11.0

The requirement of between 10 and 12 photoconversions was determined using 3-h dark periods, the latter being chosen originally to allow protochlorophyllide resynthesis, but to effect leaf expansion is an arbitrary choice. To investigate the effect of the dark spaces on leaf expansion, 10-day seedlings were treated with 12 photoconversions with various dark spaces as shown in Fig. 6c where the subsequent dark expansion is plotted. These results suggest that a 3 h dark spacing would seem about optimum for a maximum leaf expansion. It was also found that the light period of 2 min could be reduced to as low as 15 sec with the same light intensity, without change in the results but at 5-sec exposures the value of A_{30}/A_1 was 6.7. The results then indicate that at 25°, between 10 and 12 exposures to white light at 600 ft candles for at least 15 sec with 3-h dark spaces, give rise to a near maximum leaf expansion for a minimum of light energy incident.

DISCUSSION

Formation of chlorophyll b

The extrapolation of the results of Fig. 1 shows that some 25 photoconversions are necessary to give a chlorophyll *a*/*b* ratio of 3. If this is taken as a criterion for the formation of the first complete units of Photosystems I and II, and it is assumed that each holochrome may be regarded as a protein carrier of capacity of some 20 protochlorophyllide molecules per photoconversion, then the synthesis of about 500 molecules of chlorophyll per holochrome is required. Some confirmation of this is given under quite different conditions, where the results for 21-day leaves indicate that some 37 photoconversions are necessary to give a chlorophyll *a*/*b* ratio of 3 which with the assumption of 17 molecules of protochlorophyllide per holochrome at this leaf age[1], suggests that the synthesis of some 600 molecules of chlorophyll per holochrome is required.

This elementary size of the photosynthetic unit is in approximate agreement with the values obtained from the measurements of EMERSON AND ARNOLD[14] or of JOLIOT [15], from stoichiometric determinations as summarized by BOARDMAN[16], or from CO_2 reduction as calculated by WILD[17]. It may then be deduced that each holochrome has a capacity of some 20 photoactive protochlorophyllide molecules and not a multiple of this (see ref. 1).

The changeover of Φ_{680}/Φ_{Tot} in the emission spectra of Fig. 2a occurs quite sharply as a function of photoconversion number as in Fig. 1a, and correlates with the formation of chlorophyll *b* between 2 and 7 % of the total chlorophyll. The sudden nature of the changeover indicates that the bulk of the chlorophylls follow a homogeneous pattern of development under the light/dark regime imposed, although a degree of heterogeneity is admitted. After the changeover, the ratio Φ_{680}/Φ_{Tot} maintains a value of about 25 %, even when chlorophyll *b* later increases rapidly as in Fig. 1a. Mature chloroplasts[5] give a value of $\Phi_{680}/\Phi_{Tot} = 25$ %. Since it is generally held[16] that Photosystems I and II occur in about equal molecular quantities in chloroplasts, it is reasonable to conclude that under this light/dark regime, Photosystems I and II are forming in parallel, with about equal distribution of chlorophylls. Also consistent with this, is the halving of the fluorescence emission at 693 nm with respect to that at 686 nm at the changeover if one assumes no change in quantum efficiency, together with the increase in F_∞/F_0 from 1.3 to 2.1, if one assumes that the kinetic effect

arises from Photosystem II fluorescing centres only, as established by VREDENBERG AND SLOOTEN[18].

In the excitation spectra of Fig. 2b additional peaks at 473 nm and 493 nm arise as chlorophyll *b* forms, the peak at 473 nm being due to chlorophyll *b* and that at 493 nm to the carotenoids, with energy transfer to chlorophyll *a*. SHLYK AND GODNEV[19] concluded that chlorophyll *b* is formed from newly formed chlorophyll *a* molecules and SHLYK *et al.*[20] have reviewed the evidence for this. MICHEL-WOLWERTZ[21] also investigated this and reached a similar conclusion. AKOYUNOGLOU *et al.*[22] have provided evidence to show that chlorophyll *b* is formed directly from chlorophyll *a*. It is also a matter of observation that carotenoids are present in abundance in the etiolated leaf prior to the formation of chlorophyll *a*. THORNE AND BOARDMAN[7] have also observed that light is necessary for the formation of chlorophyll *b*. It is then suggested that chlorophyll *b* may be formed at sites in the membranes where a carotenoid molecule lies adjacent to a newly formed chlorophyll *a* molecule by a light stimulated reaction.

BOARDMAN and THORNE[9] have compared the fluorescence properties of a barley mutant with no chlorophyll *b* with that of normal barley. The mutant required much higher light intensity for growth and at low light gave only 1/3 to 1/10 of the fluorescence emission of normal barley, indicating that whilst chlorophyll *b* is not absolutely essential for photosynthesis, it appears in the role of an efficiency factor in energy transfer.

The results also suggest that the fluorescing centres of Photosystems I and II are completely synthesised when chlorophyll *b* is within 2–7 % of total chlorophyll. This coincides with the complete changeover of $\Phi_{680}/\Phi_{\mathrm{Tot}}$, and with the appearance of emissions at 693 nm and 735 nm which are associated with Photosystem II and I respectively in mature chloroplasts[5]. The excitation spectra of Figs. 2b and 1b show that at the 2–7 % level, chlorophyll *b* is present in each forming molecular array of the Photosystems I and II.

In old leaves, following the changeover in $\Phi_{680}/_{\mathrm{Tot}}$, the chlorophyll *b* is steady as further chlorophyll *a* is formed, followed by a later rapid increase of some further 18 % of the total chlorophyll during which no change in $\emptyset_{680}/\emptyset_{\mathrm{Tot}}$ or in fluorescing energy levels takes place.

Under these step greening conditions, then, about 1/4 of the chlorophyll *b* has a profound effect on the full formation of the fluorescing centres of the developing photosystems, whilst the time delayed balance of some 3/4 of the chlorophyll *b* is without effect on these. THORNE and BOARDMAN[7] have also observed that under white light, the greening of etiolated peas, shows, at a chlorophyll $a/b = 14:1$, an analogous time delay of chlorophyll *b* formation with a rapid changeover in $\Phi_{680}/\Phi_{\mathrm{Tot}}$. The results indicate then, that in each developing photosystem, two different sites for the formation of chlorophyll *b* exist. One site, corresponding to between 2 and 7 % of the total chlorophyll, is associated with chlorophyll *a* in the establishment of the F_{693} and F_{735} fluorescing centres. These are the lowest emission energy levels of Photosystems II and I, respectively, and serve as an inner trap to retain transferred energy immediately adjacent to the central sink molecules chlorophyll a_{II} and chlorophyll a_{I}. The other site for chlorophyll *b* corresponding to some 18 % of the total chlorophyll, with delayed formation, appears remote from the fluorescing centres, with a possible peripheral or outer function in the forming molecular arrays.

The relation of the forming pigments and leaf expansion

Under the various multiple light/dark photoconversion cycles described, the initial development of the photoactive pigments may be expressed as follows:

$$a_n = na_1 \tag{2}$$

$$c_n = (A_n/A_1)c_1 \tag{3}$$

$$b_n = Ka_n f(S) \tag{4}$$

where a, b, and c are the chlorophyll a chlorophyll b and total carotenoid content per average leaf respectively, A the leaf area, with the subscript n and 1 relating to photoconversion cycle number, and K a constant. $f(S)$ is a function of S with $S = (\Delta\bar{a}/\Delta t)/(\Delta\bar{A}/\Delta t)$ were $\Delta\bar{a}/\Delta t$ is the average rate of chlorophyll a formation and $\Delta\bar{A}/\Delta t$ is the average rate of leaf expansion. Chlorophyll b is then formed only when chlorophyll a is formed at a substantial rate, with low leaf expansion. Leaf expansion follows the approximate general relation of Eqn. 1. Tables II and III show that the total carotenoid content per leaf increases in proportion to leaf expansion, so that the carotenoid at anytime t, $c(t)$, relates to the initial value $c(o)$, by $c(t) = \{(1+e^{+kt_0})/(1+e^{-k(t-t_0)})\}c(o)$ with an identical relation between $A(t)$ and $A(o)$ the corresponding leaf areas.

It is generally accepted that leaf expansion is phytochrome mediated[12]. COHEN AND GOODWIN[23] investigated the effects of red and far-red light on carotenoid synthesis in young etiolated maize seedlings. They concluded that the effects observed due to single irradiations were mediated by a phytochrome system, but that stimulation of carotenoid synthesis was not a primary site of action. CLAES[24] studied carotenoid formation in light dependent mutant of Chlorella and concluded since far-red reversibility did not occur that chlorophyll was involved in carotenoid formation despite the fact that the action spectrum resembled that of phytochrome. However she warned against generalising this result to higher plants. On the other hand, SCHNARRENBERGER and MOHR[25], studied carotenoid formation in mustard seedlings using far-red light (720 nm) to give a stationary state $P_{fr}/P_r = 0.03$ to avoid the interaction of photosynthesis. They found a correlation between plastid size and carotenoid synthesis. In the severely restricted greening measurements, presented here, it is clear that gross leaf expansion and carotenoid synthesis follow a parallel development,whether white or blue light is used independent of chlorophyll a synthesis.

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