BBA 46032

THE GREENING OF ETIOLATED BEAN LEAVES

I. THE INITIAL PHOTOCONVERSION PROCESS

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(Received March 31st, 1970)
(Revised manuscript received August 3rd, 1970)

SUMMARY

1. During the early stages of the initial photoconversion of the etiolated leaf at 20°, a dark stable intermediate photoproduct appears with a fluorescence peak at 674 nm at 77°K.

2. With E the excitation and F the emission peaks at 77°K, the sequence of fluorescence at photoconversion is protochlorophyllide ($E650, F655$) $\xrightarrow{I}$ Intermediate ($E668, F674$) $\xrightarrow{II}$ chlorophyllide ($E678, F687$) $\xrightarrow{III}$ chlorophyllide ($E682, F694$) $\xrightarrow{IV}$ chlorophyll ($E672, F680$). Shifts I and II are photoactivated, whilst Shifts III and IV occur in the dark and correspond to those described by absorption spectroscopy.

3. Protochlorophyllide is present in the leaf in molecular groups, and spectra show that at fractional (approx. 10 %), partial (approx. 40 %) or full photoconversion the same sequence of fluorescence is observed. Excitation spectra show that energy absorbed by protochlorophyllide is transferred to each of the forms of chlorophyll in the sequence with almost identical efficiency, indicating that transposition or diffusion of chlorophyll away from the protochlorophyllide complex is not the cause of the dark Shifts III or IV. Energy transfer to chlorophyll $F680$ after fractional or partial photoconversion does however cease after a long dark time at 20°.

4. From the appearance of the intermediate $F674$, it is determined that a fundamental group size of protochlorophyllide *in vivo* is of the order 20 molecules.

5. The intermediate $F674$ gives chlorophyll *a* after dark ethanol extraction from the leaf. The photoconversion rate for the intermediate $F674$ is however twice the rate for chlorophyllide $F687$, suggesting that photoconversion is a two-step, two-photon process *in vivo*.

INTRODUCTION

SHIBATA[1] studied the photoconversion process of etiolated bean leaves, and showed by absorption spectroscopy, that following photoconversion, the absorption peak of the chlorophyllide *a* formed, shifted from 682 to 672 nm in a time of the order of 30 min.

Abbreviations: peak wavelengths stated in nm, the method of measurement being indicated by the prefix A for absorbance, E for fluorescence excitation, and F for fluorescence emission.

BUTLER AND BRIGGS[2] investigated the influence of proplastid structure on the absorption bands of protochlorophyllide and the newly formed chlorophyllide. As a result of freeze-thawing and grinding they suggested that the protochlorophyllide $A650$ was aggregated in the prolamellar body and that chlorophyllide $A684$ was similarly aggregated, physical disruption leading to disaggregation and a shift of absorption maxima to shorter wavelengths. BUTLER[3] had earlier observed that the fluorescence yield more than doubled during the shift chlorophyllide $A684$ to chlorophyll $A673$ and suggested that this was consistent with disaggregation. MADSEN[4] has shown that protochlorophyllide may be photoconverted in less than $1 \cdot 10^{-3}$ sec.

SIRONVAL *et al.*[5] correlated phytylation with the $A684$ to $A673$ shift and postulated this as the cause. On the other hand, BOARDMAN[6] has shown that phytylation occurs over a longer time interval than this shift.

More recently GASSMAN *et al.*[7] reported a rapid transient form of chlorophyll, absorbing with a peak at 678 nm and shifting to 682 nm with a time of the order of 15 sec at 20° following photoconversion. In parallel work BONNER[8] also observed a rapid transient shift in absorbance and extended the work to show that it occurred in beans, barley, peas and corn. Both of these papers suggested that this rapid shift may be due to the movement of the newly formed chlorophyllide from the holochrome to a different membrane site.

Independently SIRONVAL *et al.*[9] observed the $A678$–$A682$ transient by photoconversion followed by very rapid transfer of the leaf sample to liquid nitrogen at 77°K to give an absorbance peak at 676 nm with a fluorescence emission peak at 688 nm.

LITVIN AND BELYAEV[10] also observed the $A678$, $F688$ transient form of chlorophyllide together with another fluorescence transient at 675 nm. Interpretation of their results was based on two methods of estimating percentage photoconversion. One method using the fall in fluorescence at 655 nm from the protochlorophyllide in the whole leaf gave much higher values of photoconversion, than their second method based on the analysis of ethanol extracts. They assumed that protochlorophyllide was present in the leaf as single molecules and explained their discrepancy by postulating a reverse reaction of chlorophyllide to protochlorophyllide on ethanol extraction.

KAHN *et al.*[11] investigated the photoconversion process of protochlorophyllide holochrome extracts from etiolated bean leaves by absorption and fluorescence emission and excitation spectroscopy. The excitation spectra revealed massive energy transfer from protochlorophyllide to chlorophyllide at partial photoconversion from which it was deduced that the protochlorophyllide was present in the holochrome in molecular groups with at least 4 molecules per group.

In Part I of this series a detailed study of the fluorescence properties of leaf samples at various stages of photoconversion of the initial protochlorophyllide to chlorophyll is presented.

MATERIALS AND METHODS

Brown Beauty beans (*Phaseolus vulgaris* L.) were surface sterilised, soaked for several hours in running tap water and planted in vermiculite that had been moistened with a dilute mineral nutrient solution. The plants were grown in darkness at $25 \pm 1°$ for 14 days with occasional watering. Primary leaves were excised and mounted in

1-cm quartz spectrofluorimeter cells in green safelight when needed for sight. The leaves were held gently between two thin clear sheets of perspex (0.5 mm thick) so sized to fit diagonally in the 1-cm quartz cells. Light from the monochromator was then incident on the leaf surface at 45° to the beam, and fluorescence was viewed from the rear side of the leaf at 90° to the beam, thus avoiding stray and scattered incident light.

Absorption spectra of single leaves were recorded with a Cary Model 14R spectrophotometer fitted with a Cary Model 1462 scattered transmission attachment. Spectra could also be measured at 77°K with an assembly described previously by BOARDMAN AND HIGHKIN[12].

Fluorescence emission and excitation spectra were recorded on an instrument which automatically corrected the spectra for photomultiplier and monochromator responses, and variation in energy output of the light source as described by BOARDMAN AND THORNE[13]. The excitation monochromator was normally operated with a bandwidth of ± 1.5 nm and the fluorescence monochromator with a bandwidth of ± 1.0 nm.

The quartz cell holder could be maintained at any fixed temperature within the range + 40 to − 10° to an accuracy of ± 0.1°. Alternatively the holder could be held at 20° for photoconversion of leaves, and then rapidly cooled and held at 77°K for long periods.

Light incident on the leaf was uniform over the whole of its area, and was measured by means of a Zeiss vacuum thermocouple Type V.Th.8. together with a Keithley microvoltmeter Type 150B. This arrangement was stable to ± 0.1 μV, with a calibration sensitivity of 1.0 μV for an incident energy of 36 ergs·cm^{-2}·sec^{-1}.

The method of investigation adopted was that of photoconversion to a selected degree at 20°, followed by rapid cooling in the dark to 77°K by means of liquid N_2. At 77°K further photoconversion is completely stopped, allowing fluorescence analysis to be made. It is convenient to define the degree of photoconversion, as fractional where approx. 10% of the protochlorophyllide in the leaf is photoconverted, and partial, where 30–40% is photoconverted.

RESULTS

Effect of leaf age on fluorescence at 77°K

KAHN *et al.*[11] have given the fluorescence emission and excitation spectra of 14-day etiolated bean leaves at 77°K, the protochlorophyllide emission giving sharp peaks at 630 and 655 nm, only the latter being photoconvertible at room temperature. The excitation spectrum for *F*655 gave a peak at 650 nm with a shoulder at 637 nm, whilst that for *F*630 gave a peak at 628 nm. Excitation spectra over the range 600–700 nm only were investigated since this was free of the attenuating effect of the carotenoids.

To assess the effect of leaf age on the fluorescence emission at 77°K, the ratio *F*655/*F*630 was measured for various ages of etiolated bean leaves, the results being shown in Table I. It is significant that the *F*630 form is present in substantial degree even in the 4-day leaf, which suggests it may be the precursor for the *F*655 form, and that increase in the *F*655 form takes place in the dark up to 16 days and from then on some reversion or deterioration occurs. In most of the subsequent investigations then,

we standardised on the use of 14-day etiolated bean leaves, since at this stage they were sufficiently developed to enable spectra to be determined on single leaves.

Photoconversion Shifts I and II

Whilst investigating the photoconversion of protochlorophyllide, interest fell on the low fractional conversion (approx. 5 %). Percentage photoconversions were determined from ethanol extracts of the whole leaves with fluorescence analysis at 77°K, details of which appear later. Single leaves were mounted in the cell holder of the spectrofluorimeter. Fractional conversion was carried out at 20° by exposure to light from the excitation monochromator at 622 nm with a bandwidth of ± 5 nm, and incident light intensity on the whole leaf area of 2000 ergs·cm^{-2}·sec^{-1}, for fixed periods. The excitation at 622 nm was chosen so as to include absorbance by all forms of protochlorophyllide. A time of up to 200 sec at this wavelength and incident intensity was required to approach full conversion. Following this conversion the leaf was rapidly cooled to 77°K in the dark, and fluorescence emission and excitation spectra were recorded as shown in Figs. 1a–1d. An intermediate fluorescence occurred at 674 nm with the 2-sec fractional conversion, which appeared as a shoulder on the emissions at 687 nm following the 5- and 10-sec fractional conversions. The terminal photoconversion stage was chlorophyllide *F*687. The intermediate *F*674 persisted as a shoulder in the emission spectra after various degrees of conversion. Etiolated leaves on the other hand exhibit no emission peak at 674 nm. After photoconversion in 2 min of white light at 800 ft-candles the emission at 674 nm appeared as a vestigial shoulder on the main *F*694 band, but disappeared after some 10 min photoconversion in white light, indicating a greatly reduced rate of conversion approaching the 100 % level.

Under the conditions of measurement relating to Fig. 1, the etiolated leaf, with the fluorescence monochromator set at 675 nm or longer, gives less than 2.5 units on

TABLE I

THE RATIO OF FLUORESCENCE EMISSION PEAKS, *F*655/*F*630 AT 77°K WITH EXCITATION AT 440 nm FOR SINGLE ETIOLATED BEAN LEAVES OF INCREASING AGE

	Age (days)							
	4	*6*	*10*	*14*	*16*	*21*	*24*	*27*
*F*655/*F*630	0.9	1.7	2.0	2.3	2.7	2.5	2.1	1.9

TABLE II

THE DARK STABILITY OF THE INTERMEDIATE *F*674

14-day etiolated bean leaves were fractionally photoconverted at 20° for 2 sec in 622-nm light, intensity 2000 ergs·cm^{-2}·sec^{-1}, and kept in the dark, on the plant, for various periods at 20°. The figures give the average ratios for three leaves of *F*655/*F*674 and *F*655/*F*630 taken from the subsequent 77°K emission spectra with excitation at 440 nm.

	Dark (h)				
	0	*0.5*	*1*	*5*	*16*
*F*655/*F*674	1.4	1.5	1.6	1.55	1.8
*F*655/*F*630	2.6	2.4	2.8	2.9	1.5

the excitation scale at 650 nm. The excitation spectra of Fig. 1. then show that the protochlorophyllide complex, at fractional photoconversion, transfers some energy to the intermediate *F*674 and substantial energy to each of the chlorophyllides *F*687 or *F*694.

Fractional conversion with wavelengths of 440, 637, 650 and 655 nm was next investigated. In each case the same intermediate *F*674 appeared first, the spectra following the sequence of Fig. 1. The intermediate *F*674 is then not peculiar to any one form, but a property of the total protochlorophyllide complex.

The dark stability of the intermediate *F*674 was also investigated. After fractional conversion for 2 sec at 622 nm, leaves on the plant were kept in the dark at 20° for various periods, the results of 77°K spectra being given in Table II. The intermediate fluorescence still peaked at 674 nm in each case, the ratios *F*655/*F*674 and *F*655/*F*630 of the 77°K spectra showing little significant change in up to 5 h darkness. At 16 h the ratio *F*655/*F*674 had increased, whilst *F*655/*F*630 had decreased, suggesting that in the dark some of the *F*674 may be reverting to *F*630. Denaturation of an etiolated leaf, by heating to 60° for 5 min in the dark, for example, causes all the *F*655 to revert to *F*630 emission, with a complete loss of photoconversion.

An attempt was also made to enhance the intermediate *F*674 by carrying out fractional photoconversions at temperatures down to −60° but no significant change in the nature of the process was found, aside from reduced photoconversion.

Shift IV, the slow dark shift

A comparison was made of the emission and excitation spectra at 77°K of a converted leaf, with a converted leaf allowed a following 90-min dark period at 20°. The

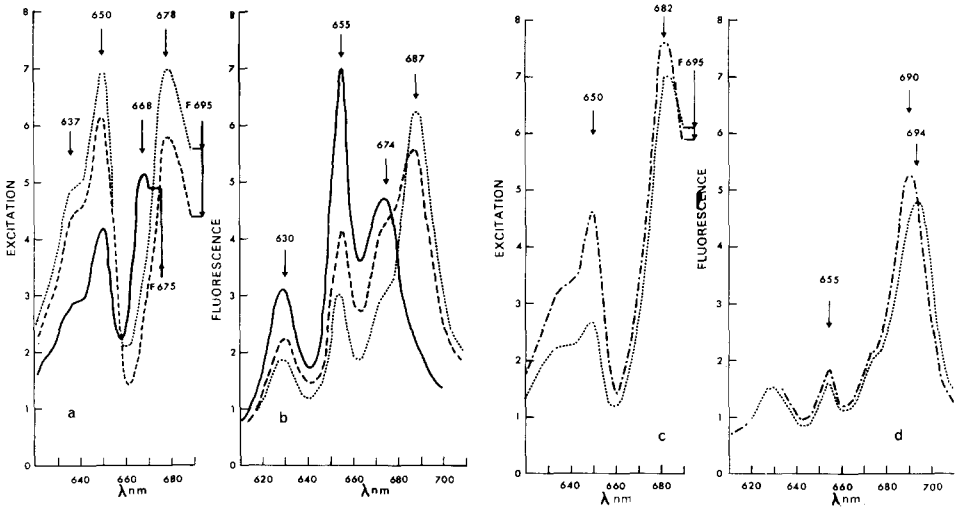

Fig. 1. a, fluorescence excitation spectra; b, fluorescence emission spectra for 14-day etiolated leaves at 77°K, after various times of photoconversion at 20° in light at 622 nm, bandwidth ± 5 nm, intensity 2000 ergs·cm^{-2}·sec^{-1}. ———, 2 sec; – – – – –, 5 sec; and · · · · · ·, 10 sec, respectively, 5, 10, and 16% fractional photoconversion. c, fluorescence excitation spectra; d, fluorescence emission spectra, conditions as before. —·—, 20 sec; and – – – – –, 60 sec, respectively, 30 and 50% partial photoconversion. All emission spectra were for excitation at 440 ± 1.5 nm. Excitation spectra with fluorescence monochromator at the *F*λ point shown.

dark period showed the chlorophyllide $F694$ shifted to $F680$ in accord with the absorption shift recorded by SHIBATA[1]. The fluorescence quantum efficiency also increased 2-fold as observed by BUTLER[3].

At 20°, following photoconversion, a leaf gave the emission spectra recorded superimposed in a time sequence as shown in Fig. 2. A study of the spectra taken at the various temperatures showed that the shift involved, first the change in energy level from $F694$ to $F680$ followed by a doubling of fluorescence emission. Shift IV stopped completely at 0° and was very slow moving at 5°, and completes the shift $F694$ to $F680$ in about 5 h with little increase in fluorescence. The time to half com-

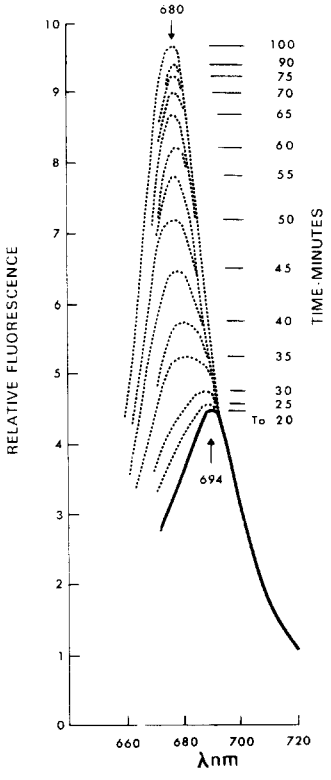

Fig. 2. The dark Shift IV. SHIBATA[1] $A682 \rightarrow A672$ followed by fluorescence emission change from $F694$ to $F680$ at 20° for a 14-day etiolated bean leaf. Excitation at 650 ± 1.5 nm, following photoconversion, shows the doubling of the fluorescence emission together with the wavelength change.

TABLE III

TIME TAKEN TO HALF COMPLETE THE SHIFT $F694$ TO $F680$ FOR 14-DAY ETIOLATED BEAN LEAVES AT VARIOUS TEMPERATURES

	Temperature (°)					
	5	*10*	*15*	*20*	*27*	*35*
$t_{1/2}$ (min)	160	54	32	23	18	12

plete the shift $F694$ to $F680$ is shown in Table III, further time being necessary for the doubling of fluorescence at $F680$ to occur. The dark Shift IV then seems to involve two distinct processes in sequence, which implies that two causes are involved.

It was of interest to determine whether it was a requirement for full conversion to occur before the Shift IV took place, and whether energy transfer occurred. A partial conversion (approx. 40 %) was made at 20°, the excitation and emission spectra of leaves at 77 °K some 2 min after partial conversion, and following a dark time of 90 min at 20°, being given in Fig. 3. It was clear firstly, that the Shift IV still occurred at partial conversion, and secondly that the unconverted protochlorophyllide transferred some of its absorbed energy before and after the shift.

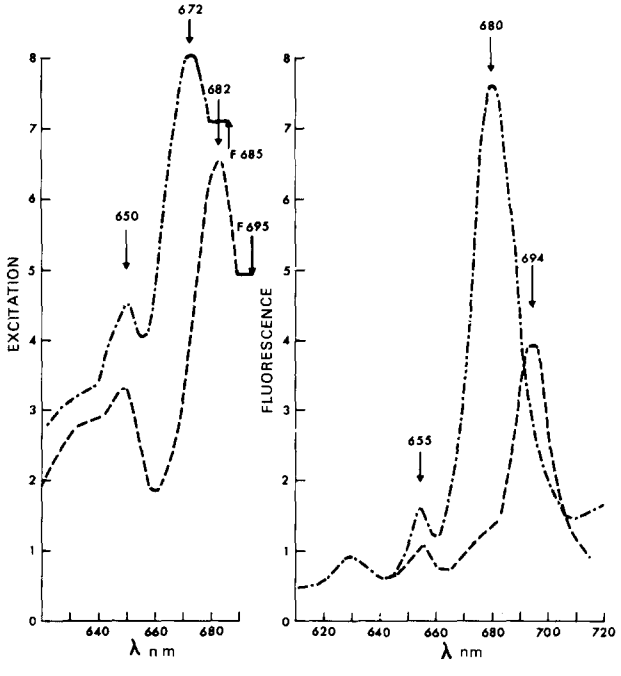

Fig. 3. Fluorescence excitation and emission spectra at 77 °K of a 14-day etiolated bean leaf after partial photoconversion (approx. 40%) at 20°. – – – – –, to 77 °K immediately following partial conversion; —·—, partial photoconversion followed by 90-min dark time at 20°, then taken to 77 °K, showing the full Shift IV. Emission spectra excited at 440 ± 1.5 nm. Excitation spectra with fluorescence monochromator set at the $F\lambda$ point shown.

Shift III, the rapid dark shift

The fractional conversion studies showed that the intermediate $F674$ gave rise to chlorophyllide $F687$, while full conversion studies gave chlorophyllide $F694$. As has been noted earlier, several observers have recently reported the rapid absorbance change from 678 to 682 nm after conversion. The nature of this change was studied by means of fluorescence kinetics. The fluorescence monochromator was set at 691 ± 1 nm, midway between $F687$ and $F694$, and etiolated leaves were converted in the spectrofluorimeter using the excitation light as conversion light at 650 nm. Conversions were recorded at various temperatures giving the results shown in Fig. 4a. Conversion was almost completed during the rise time of these curves within a period of some 15

sec, followed by a reducing in fluorescence at 691 nm, because chlorophyllide $F687$ is twice as fluorescent as chlorophyllide $F694$. The shift from $F687$ to $F694$ is very rapid at 35° and takes place well within the conversion time but is much slower at 0°. Excitation at 650 nm was used since the two forms of chlorophyllide have similar absorbance. In order to record emission spectra of Shift III in a manner analogous to Fig. 2 for Shift IV, it is necessary to slow the shift by the selection of an appropriate low temperature. Spectra for Shift III following conversion are shown in Fig. 4b at −5°, where it is clear that a halving of fluorescence accompanies the shift from $F687$ to $F694$. Shift III occurs in the dark, and also at fractional or partial conversion.

Dark shift kinetics at fractional conversion

Following the investigations of the photoactivated Shifts I and II it was observed that the shift $F687$ to $F694$ did not appear to be as fast at 20° for the fractional conversion compared with full conversion. Following fractional conversion, leaves

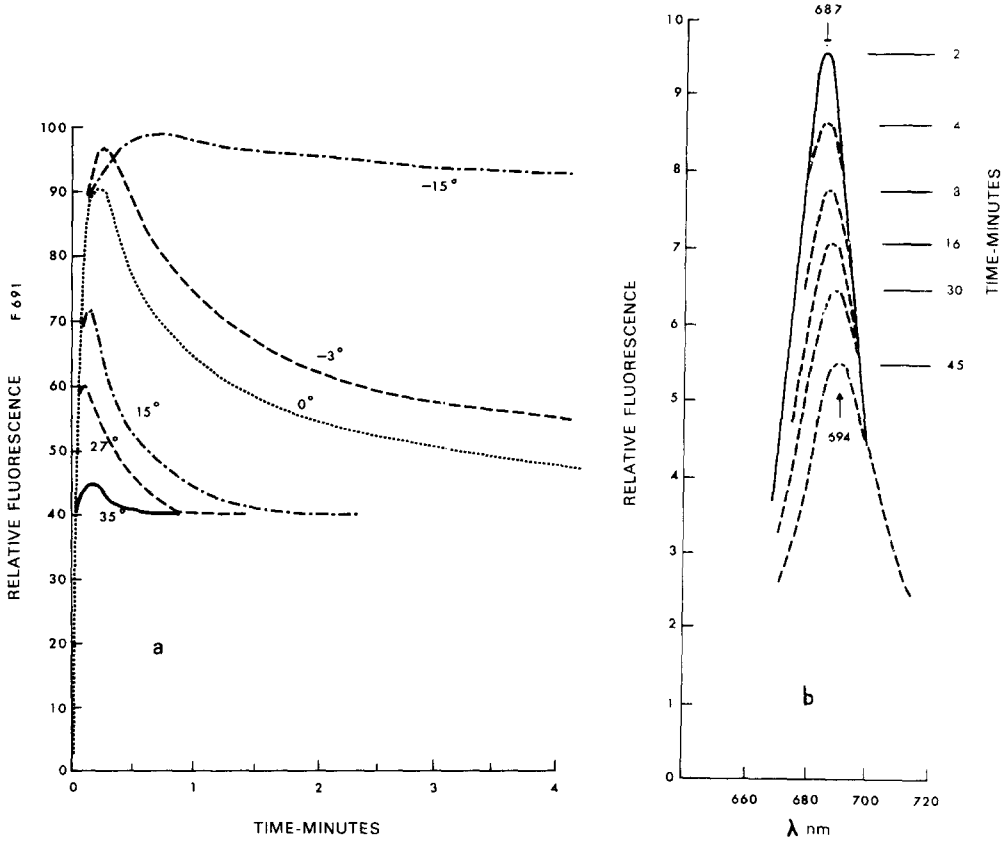

Fig. 4. a. Measurement of dark Shift III as a function of time at the fixed temperatures 35, 27, 15, 0, −3 and −15° for 14-day etiolated bean leaf. Photoconversion, and subsequent excitation at 650 nm, bandwidth ± 5 nm, intensity 2000 ergs·cm^{-2}·sec^{-1}. Leaf fluorescence emission at 691 nm, bandwidth ± 1.0 nm. Photoconversion occurs during the first 10–15 sec of the curves. b. Shift III, following photoconversion, at −5°, as a function of time showing change from $F687$ to $F694$ with the fluorescence quantum efficiency dropping from ϕ to $\phi/2$.

were kept in the dark at 20° for periods of 5, 10, 15, 16 and 20 min before the sample was cooled to 77°K. The 5- and 10-min dark periods at 20° showed a curve identical to the first curve of Fig. 5, with no shift from $F687$ to $F694$ taking place, the curve still peaking at $F687$. In 15–17-min dark periods at 20° the curve showed some change and at 20 min the fluorescence emission at 77°K had changed to 680 nm. This effect was quite repeatable with a series of leaves. To reduce physiological variables, one leaf of a pair from a 14-day etiolated bean plant was subjected to fractional conversion with a 20-min dark period at 20°, which at 77°K gave a full shift to $F680$, while the other leaf of a pair was subjected to full conversion at 20°, the shift $F694$ to $F680$ following the curves of Fig. 2.

Thus we have the anomaly that at 20° the shift $F687$ to $F694$ which is fast and occurs within a half shift of approx. 25 sec following full conversion is now slow at fractional (approx. 10 %) conversion with a time for shift of approx. 15 min, whereas the shift $F694$ to $F680$ which is slow with a half shift time of approx. 25 min at full conversion becomes fast at fractional conversion with a half shift time of the order of 3 min. It is significant that the dark shifts still occur even at fractional conversion, and

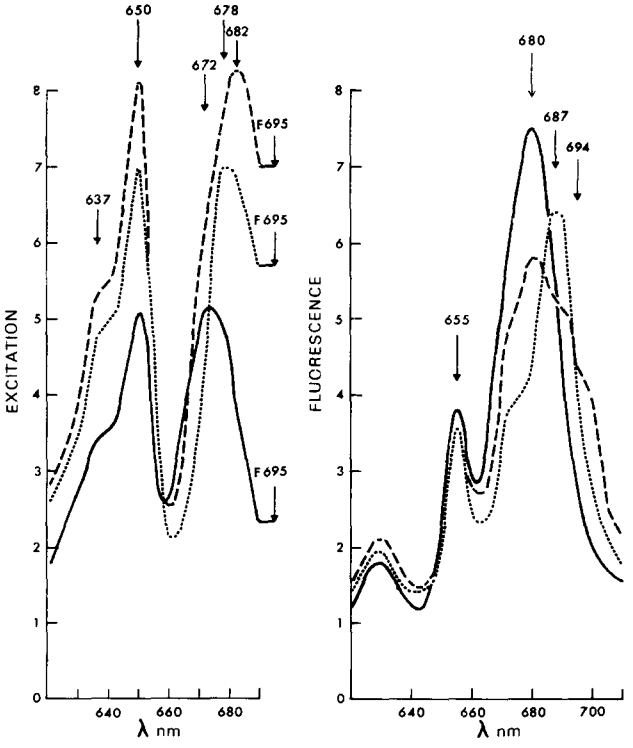

Fig. 5. The nature of Shifts III and IV for 14-day etiolated bean leaves at fractional photoconversion (approx. 10%) in light at 650 nm, bandwidth ± 5 nm, intensity 2000 ergs·cm^{-2}·sec^{-1}. Fluorescence emission and excitation spectra recorded at 77°K after various periods of dark time at 20°; ·····, after 2, 5 or 10 min dark time at 20° following fractional photoconversion; – – – – –, after 16 min dark time at 20°; ———, after 20 min dark time at 20°. Excitation at 440 ± 1.5 nm for fluorescence emission. Excitation spectra with fluorescence monochromator set at the $F\lambda$ point shown.

the reversal of the time of the shifts suggests the causes of the shifts are entirely different.

Effect of extended dark time after Shift IV

Following fractional or partial conversion, it has been shown that Shifts III and IV occur in each case, with energy transfer from protochlorophyllide to each form of chlorophyll, the shifts being completed within 1 h. The effect of prolonged dark time following both degrees of conversion was then investigated, leaves being maintained on the plant for 20 h darkness at 20°. Excitation spectra showed that protochlorophyllide no longer transfers energy to chlorophyll *F*680 but still transfers to the intermediate *F*674. At 10 % conversion the emission spectrum of the 20-min dark time of Fig. 5 reverts to the 2-sec-exposure form of Fig. 1. The reduction of the transfer excitation at 650 nm to chlorophyll after 20 h darkness is at least 10-fold indicating that the chlorophyll and the unconverted protochlorophyllide have moved apart by at least 10 Å, if we assume an initial spacing of the order 25 Å in the group. The intermediate *F*674 appears to be bound to the protochlorophyllide group.

Group size of protochlorophyllide in vivo

The appearance of the intermediate *F*674 at the early stage of photoconversion gave a method of determining the grouping of the protochlorophyllide molecules in the etiolated leaf.

Fractional photoconversions were made on single leaves in incident light of 622 nm at 2000 ergs·cm^{-2}·sec^{-1} for periods of 1, 2, 3, 5, 10 or 15 sec. Rapid cooling to 77°K gave emission spectra similar to those of Fig. 1. From three independent photoconversions and spectra, average values of the amplitudes of fluorescence emission at 674 and 687 nm were determined and plotted as shown in Fig. 6a.

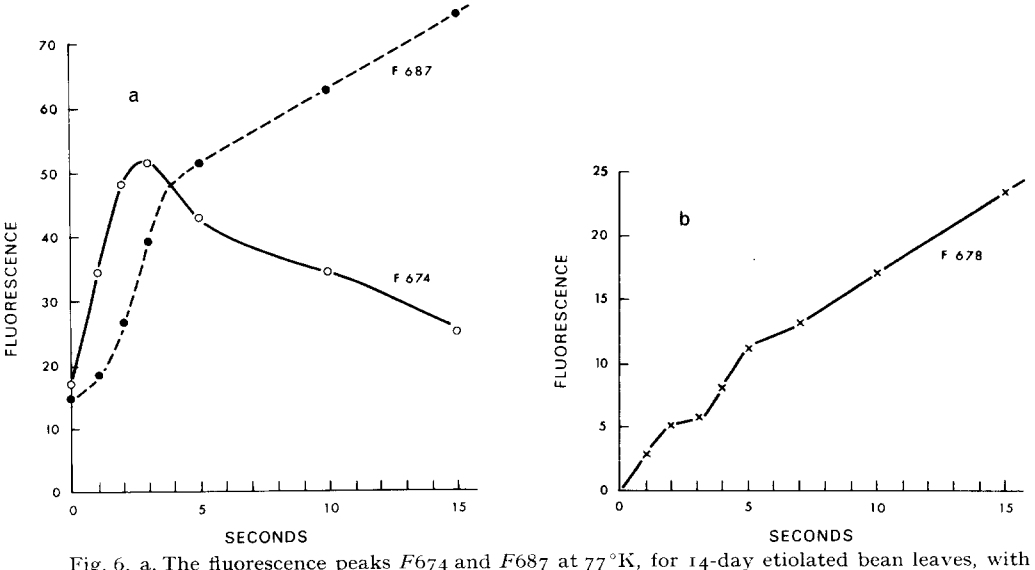

Fig. 6. a. The fluorescence peaks *F*674 and *F*687 at 77°K, for 14-day etiolated bean leaves, with photoconversion conditions as in Fig. 1. Values at each point are the average of three independent measurements. b. Fluorescence emission peaks at 678 nm, at 77°K and excitation at 440 nm, for dark ethanol extracts of fractionally photoconverted leaves corresponding to Curve a; each point being the average value of three independent experiments.

Measurements were also made of the amount of chlorophyll *a*, by fluorescence analysis at 77 °K on dark ethanol extracts of leaves at each fractional photoconversion. Excitation at 440 nm at 77 °K in ethanol, gives a chlorophyll *a* peak at 678 nm, superimposed in the protochlorophyllide spectrum which has a main peak at 628 nm together with upper vibrational levels with a minor peak at 685 nm. The increment in *F*678, above the zero conversion level is a measure of the amount of chlorophyll *a*, the amplitude of the protochlorophyllide peak at 628 nm allowing a standardisation correction for physiological variations between leaves. Average values of chlorophyll *a* *F*678 for each photoconversion period are shown in Fig. 6b. Chlorophyll *a* at full photoconversion was also determined, the average value of *F*678 for 10 extracts, on the same scale, was 104 ± 9 units.

A comparison of the curves of Figs. 6a and 6b shows that the intermediate *F*674 dark extracts into ethanol to give normal chlorophyll, and the slope over the first 2 sec of Fig. 6b compares with the average slope over the 5–15-sec range in the ratio 2.1/1. Since the bulk of protochlorophyllide is as yet unconverted, it is clear that chlorophyll is obtained in the extract from the intermediate *F*674 at a photoconversion rate 2-fold greater than that from chlorophyllide. This indicates a requirement in the ratio of at least two quanta to give a molecule of chlorophyllide *F*687 compared with one quantum for a molecule of the intermediate *F*674.

An estimate of the molecular group size *in vivo* may be made from the fractional level of photoconversion at which the intermediate *F*674 appears as maximal. The random nature of photon absorbance in the large number of such molecular groups, involves a progressive binomial distribution with the time of photoconversion amongst the molecular groups. However since the group size is not known, the estimate is made by an analogue method of analysis. The value of the photoconversion level at the maximum of the intermediate *F*674 was not taken from Fig. 6a due not only to a lack of statistical distribution information but also to a variable Gaussian overlap of the values of *F*674 and *F*687 in emission and unknown energy transfer parameters, but from the ethanol extract curve of Fig. 6b. Since the intermediate *F*674 and chlorophyllide *F*687 both yield chlorophyll *a* on extraction into ethanol, the point of inflection on the curve of Fig. 6b corresponds to the point at which one molecule of each group on average is in the intermediate state. At this point the value of *F*678 was 5.3 units, and so the size of the groups of protochlorophyllide *in vivo* was 104/5.3 = 20 molecules.

To confirm the course of chlorophyll *a* formation with photoconversion, fluorescence analysis at 77 °K was made by excitation at 660 nm. This avoided the emission of protochlorophyllide, gave an increase in sensitivity, but allowed no correction for physiological variations. The average values of *F*678 for 10 dark ethanol extracts at each photoconversion period is shown in Fig. 7. This method gave a slope ratio of 2.2/1. A full photoconversion value of *F*678 of 623 units and inflection point of *F*678 of 33 units, gave a protochlorophyllide group size *in vivo* of 19 molecules.

8- and 21-day etiolated bean leaves

Following the method of the previous section, measurements were also made on 8- and 21-day etiolated bean leaves. The object was to ascertain whether the group size was a function of leaf age or whether it was a constant and hence a more fundamental unit in the developing leaf. The results followed the same pattern as in Figs. 6a

and 6b. The 8-day leaf gave a group size of 19 molecules, and the 21-day leaf gave 17.5 molecules of protochlorophyllide.

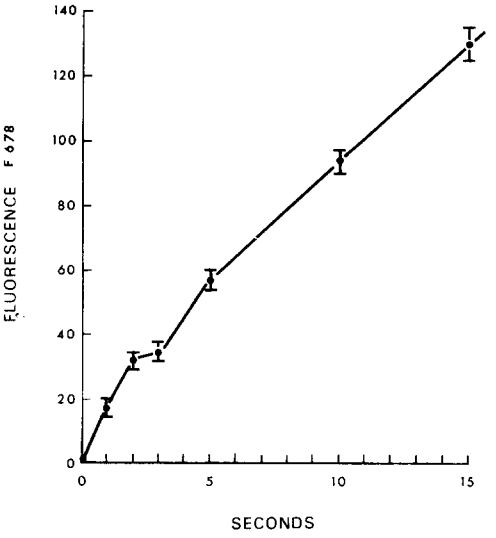

Fig. 7. Ethanol extracts of 14-day etiolated bean leaves after various photoconversions (as in Fig. 1). The values of fluorescence emission at 678 nm at 77 °K, with excitation at 660 nm. The average value and standard error of 10 independent measurements at each time are shown.

DISCUSSION

Table IV summarises the various stages observable during the primary photoconversion process for etiolated bean leaves. The intermediate *F*674 is not present in etiolated leaves. The excitation spectra of Figs. 1 and 5 show that energy transfer occurs from unconverted protochlorophyllide to each of the forms shown in Table IV. The ratio of the excitation peaks *E*650 to *E*672, *E*678 or *E*682 of Fig. 5 is almost identical in each case. The transposition or diffusion of chlorophyll away from the protochlorophyllide complex is not the cause of the dark Shifts III or IV.

FÖRSTER[14] has shown that the probability of energy transfer from a donor to an acceptor molecule is proportional to $\sigma^2(1/R^6\tau_e)\int\varepsilon_A f_D d\gamma$, where σ^2 is an orientation factor, R is the distance between the molecules, τ_e is the intrinsic emission lifetime of the donor in the abscence of other radiationless processes, with the integral of the overlap product of the emission spectrum f_D of the donor and the ε_A absorbance spectrum of the acceptor. The quantity $1/\tau_e$ is proportional to $\int\varepsilon_D d\gamma$ with ε_D the absorbance of the donor molecule over the first singlet level. It is desired to evaluate the distance at which a transfer of energy from protochlorophyllide to chlorophyllide has equal probability with fluorescence from protochlorophyllide, from a known value of 40–45 Å for a chlorophyll to chlorophyll transfer as measured by WEBER[15] and also by TWEET *et al.*[16]. An approximate estimate shows for equal probability the molecular spacing is no greater than 20–25 Å. This emphasises the importance of molecular spacing in energy transfer, and explains the conclusions based on the excitation spectra, described in the present results.

The intermediate arises first in the early stages of conversion, yet only a small

TABLE IV

THE SEQUENCE OF PHOTOCONVERSION OF THE PRIMARY POOL OF PROTOCHLOROPHYLLIDE OF 14-DAY ETIOLATED BEAN LEAVES

Excitation (E) and fluorescence (F) emission peaks in nm at 77 °K.

Shift	*Activation*	*Pigment*	*Dark stability*
		Protochlorophyllide complex ($E628$, $F630$) ($E637$, $F655$)	Stable
I	Photo	↓ ($E651$, $F655$)	
		Intermediate ($E668$, $F674$)	Stable
II	Photo	↓	
		Chlorophyllide ($E678$, $F687$)	Unstable
III	Dark	↓	
		Chlorophyllide ($E683$, $F694$)	Unstable
IV	Dark	↓	
		Chlorophyll ($E672$, $F680$)	Stable

fraction of the group of molecules is converted to the intermediate state, the great majority being converted to chlorophyllide $F687$ as is evidenced by both absorption and excitation spectra. The photoactive sequence of Table IV must be interpreted on the basis of groups of molecules subjected to random absorbance by individual molecules. Within each group at room temperature the probability of energy transfer between like molecules is greater than the probability of photoconversion which in turn is greater than the probability of fluorescence emission. Then, either the intermediate $F674$ and chlorophyllide $F687$ are each phototerminal products within the groups, or the intermediate $F674$ is the first step of a two-step process to give chlorophyllide $F687$ for each molecule in a series sequence.

The two phototerminal molecules of a parallel formation tend to imply different precursors in the protochlorophyllide complex, and hence it should be possible to enhance or retard the formation of the intermediate by selective wavelength conversion, but the experimental evidence denies this.

The intermediate is dark-stable for at least 5 h, while chlorophyllide $F687$ undergoes two dark shifts within 1 h, and one might expect that if the intermediate were a phototerminal pigment that it also may undergo some form of analogous dark shift.

The vestigal appearance of the $F674$ emission approaching full conversion has been noted. This may imply a phototerminal product or alternatively a greatly reduced rate of conversion approaching the 100 % level. With a long dark time after Shift IV at fractional or partial conversion, protochlorophyllide transfers energy to the intermediate but not to chlorophyll $F680$. One might expect that the first formed intermediate would be the first to undergo diffusion away from the group, but in fact it seems bound even when the chlorophyll $F680$ has undergone such a diffusion.

The series sequence on the other hand, which also follows the course of Table IV needs no assumption to explain the early appearance of the intermediate, and would follow the same sequence irrespective of the wavelength of conversion. As a two-step photoprocess, light is necessary to convert the intermediate to chlorophyllide $F687$ and as such the intermediate would be dark stable, and more closely bound to the parent group.

The suggestion that Shift III may represent a diffusion of chlorophyllide *F*687 away from the holochrome, is not in accord with energy transfer at fractional or partial conversion. Excitation spectra indicate an essential group character at Shift III. Since chlorophyllide *F*687 has derived two hydrogen atoms from its environment during photoconversion, the energy level fall at Shift III seems to represent a simple relaxation effect of the environment on the newly formed chlorophyllide.

Shift IV which appears to involve two processes in sequence, is slow following full conversion and ceases at 0°, represents an increase in energy level, with energy transfer to both chlorophyllides *F*694 and chlorophyll *F*680 at 77°K. The energy change indicates a metabolic chemical change in the molecule, followed by possible orientation changes to give increased fluorescence. The doubling of fluorescence at Shift IV would seem to be in some way causally related to the halving of fluorescence at Shift III.

The results indicate that protochlorophyllide is present in the etiolated leaf in groups of the order of 20 molecules, or possibly multiples of this, and as such is a fundamental unit. This value is subject to correction due to the random nature of photon absorption.

Both the intermediate *F*674 and chlorophyllide *F*687 yield chlorophyll *a* on dark ethanol extraction, the photoconversion rate for the intermediate however, being twice the rate for that of chlorophyllide *F*687 *in vivo*.

The fall in protochlorophyllide fluorescence at *F*655 in the leaf is not to be taken as a measure of percentage photoconversion, since energy transfer to the first formed chlorophyllide molecule of each group is dominant, giving a rapid fall at *F*655 as shown in Fig. 1.

SMITH AND FRENCH[17] studied the quantum yield for photoconversion using protochlorophyllide–holochrome extracts. Photoconversion of whole leaves and of the extracts was shown to follow the second-order rate law, with a quantum yield for the holochrome extracts of 0.60 molecule/quantum, or 1.6 quanta/molecule. Allowing for some inefficiency it would seem most probable that 1 quantum/molecule was sufficient to convert protochlorophyllide to chlorophyllide in the holochrome.

KAHN *et al.*[11] studied the holochrome *in vitro*, by the fluorescence method, and found that at fractional photoconversion (approx. 4 or 8 %) no sign of the whole leaf intermediate *F*674 appeared. This suggests that photoconversion *in vitro* requires only 1 quantum, whereas 2 sequential quanta may be required *in vivo*.

Photoconversion exhibits both photochemical and thermochemical properties. The rate of photoconversion is then dependent on the absorbance of the sample at any instant, and hence to the concentration of protochlorophyllide, and also on the concentration of hydrogen atoms, which must also be considered to be proportional to the concentration of protochlorophyllide at any instant, and so $-dc/dt = kc^2$, such a law holding whether 1 or 2 quanta/molecule are required to complete conversion, but differing in the proportional constant k. Unfortunately the determination of a value for k *in vivo* is difficult due to absorbance and scattering uncertainties. A simple mathematical analysis shows that with the protochlorophyllide present in discrete groups *in vivo*, the same photoconversion law holds good over the main range of conversion, provided the energy-transfer efficiency remains constant, with a possible exception beyond the 95 % conversion level, where absorbance of the sample may no longer be considered of constant form.

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