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THE GREENING OF ETIOLATED BEAN LEAVES

II. SECONDARY AND FURTHER PHOTOCONVERSION PROCESSES

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SUMMARY

1. Photoconversions in light–dark stages in etiolated bean leaves show that protochlorophyllide is resynthesized to the same level at each stage.

2. Photoconversions 2 and 3 follow the initial photoconversion process, but after Shift IV, chlorophyll (*E*674, *F*683) is formed.

3. At fractional or partial Photoconversions 2 or 3, protochlorophyllide transfers energy to chlorophyllides *F*687 and *F*694 but not to chlorophyll (*E*674, *F*683).

4. Resynthesized protochlorophyllide exists in discrete groups similar to the initial protochlorophyllide, with molecular transposition from these groups taking place at the shift to chlorophyll (*E*674, *F*683).

INTRODUCTION

AKOYUNOGLOU AND SIEGELMAN[1] studied the resynthesis of protochlorophyllide in dark-grown bean leaves, following initial photoconversion, by absorption spectroscopy. The resynthesis curves were sigmoidal, rising to a steady level in a manner depending on the leaf age.

Following the initial photoconversion studies of Part I of this series[2] the investigation was extended to include, the resynthesis of the secondary pool of protochlorophyllide with long dark periods, the secondary photoconversion processes, the resynthesis of the tertiary pool of protochlorophyllide with further long dark periods, and the tertiary photoconversion processes.

MATERIALS AND METHODS

The materials and methods used have already been described[2]. Whole plants were subjected to the light–dark cycles indicated in the later text, leaves only, being taken and mounted in the spectrofluorimeter cell immediately at the stage where fluorescence excitation and emission spectra were required. The terms, Photoconversion 1, Photoconversion 2 or Photoconversion 3 imply the leaf at the appropriate

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.

stage is photoconverted in white light at 800 ft-candles for a period of 2 min at room temperature, unless otherwise stated in the text.

The fluorescence excitation and emission spectra were recorded with the excitation monochromator set at a bandwidth of ± 1.5 nm and the fluorescence monochromator at a bandwidth of ± 1.0 nm.

RESULTS

Resynthesis of protochlorophyllide

14-day etiolated bean leaves, on the plant, were subjected to Photoconversion 1 and then returned to the dark growth room at 25°. After further fixed dark times, individual leaves were ground in the dark in 2.5 ml of ethanol. Aliquots of the dark ethanol extract were then subject to fluorescence analysis at 77°K, with excitation at 440 nm, to determine the protochlorophyllide resynthesis. Fig. 1 shows the protochlorophyllide *F*628 emission at various dark times. The protochlorophyllide resynthesis was subject to the expected lag phase of the order of 4 h, after Photoconversion 1, followed by a rapid rise at 5 h darkness, subsequent dark time leading to little further increase.

The work was extended to the resynthesis of protochlorophyllide, the tertiary pool, following Photoconversion 2. Leaves, on the plant, which had been given Photoconversion 1, were allowed a dark period of 6 h at 25° and then subjected to Photoconversion 2 at 25°, and returned to the dark for further periods, before dark ethanol extracts and measurements of *F*628 were made. The tertiary proto-

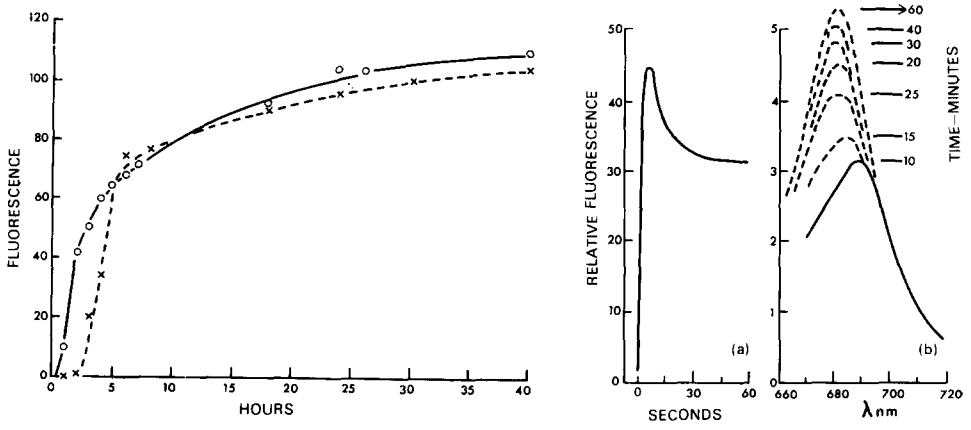

Fig. 1. 14-day etiolated bean leaves, showing the reformation of protochlorophyllide with dark time at 25°; ×---×, after Photoconversion 1; ○——○, after the sequence, Photoconversion 1 followed by a dark time of 6 h at 25° then Photoconversion 2. The ordinate shows the fluorescence amplitude at 628 nm, at 77°K with excitation at 440 nm for an ethanol extract of single leaves.

Fig. 2. a. The fluorescence emission amplitude at 691 nm, at 20° of a 14-day etiolated leaf, after the sequence photoconversion 1 and 18 h dark period at 25°, when the secondary photoconversion is carried out with excitation at 650 nm, bandwidth ± 5 nm, incident intensity 2000 ergs·cm^{-2}·sec^{-1}. b. The fluorescence emission spectra at 20° following the treatment in (a) showing the shift in fluorescence peak and increase in fluorescence quantum efficiency with time. Excitation at 650 nm, bandwidth ± 1.5 nm, and incident intensity of 200 ergs·cm^{-2}·sec^{-1}.

chlorophyllide increased without significant lag, in an exponential manner as shown in Fig. 1 to a level similar to that of the secondary protochlorophyllide.

Measurements were also made on whole leaves at 77°K using the protochlorophyllide *F*655 emission in an analogous sequence. The *F*655 emission for both the secondary and the tertiary resynthesis gave curves almost identical to Fig. 1. This not only indicated that the protochlorophyllide pools were similar in size, but also that energy transfer from protochlorophyllide to chlorophyll in the leaf in each case was improbable.

By both methods of measurement the secondary and tertiary protochlorophyllide pool sizes were of the same magnitude as the primary pool of the etiolated leaf.

Photoconversion 2 at 20°

A 14-day etiolated leaf which had been given Photoconversion 1 was maintained in the dark at 25° for 18 h, mounted in the spectrofluorimeter, and then photoconverted in light at 650 nm, at 20°, the fluorescence emission at 691 nm being recorded in Fig. 2a. Photoconversion 2 occurred during the initial 10-sec period, the fall in fluorescence at *F*691 being characteristic of Shift III. With the excitation still at 650 nm, but with a bandwidth of ± 1.5 nm, fluorescence emission spectra at 20° were recorded over the period of 1 h as shown in Fig. 2b. The dark Shift IV followed with the fluorescence peak shifting from 692 nm to the region of 680 nm. The time taken for Shift IV to occur was however one-half of that following Photoconversion 1.

Photoconversion 2 studied by fluorescence spectra at 77°K

Following Photoconversion 1, the protochlorophyllide resynthesized in selected dark periods of 5, 18, 24 and 66 h permitted studies of Photoconversion-2 processes. All the dark periods allowed similar conclusions. Figs. 3a and 3b compare the fluorescence emission and excitation spectra of whole leaves at 77°K. The solid curve relates to the leaf after 66 h darkness, the fluorescence peaks being at 655 and 680 nm. The excitation spectrum with the fluorescence monochromator set at 690 nm, *i.e.* within the chlorophyll *F*680 band, gave an excitation peak at 672 nm, but no peak at 650 nm, indicating that energy absorbed by the protochlorophyllide was not transferred to the primary chlorophyll. The dashed spectra of Figs. 3a and 3b relate to the leaf at fractional Photoconversion 2, the emission peaking at 687 nm. The excitation spectrum, with the fluorescence monochromator set at 700 nm, *i.e.* within the 687-nm band, gave an excitation peak at 680 nm with energy transfer peaks at 650 and 637 nm, characteristic of the protochlorophyllide complex. Energy absorbed by the unconverted protochlorophyllide was then being transferred to the newly converted chlorophyllide *F*687 but not to the primary chlorophyll *F*680. The excitation spectrum with the fluorescence monochromator set at 680 nm, indeed showed little excitation at 650 nm, indicating that the fluorescence is due to a mixture of two species. The spectra of Figs. 3a and 3b indicate that the secondary protochlorophyllide is present in molecular groups, spaced apart from the chlorophyll photon transfer within individual groups taking place, with no transfer between groups.

Figs. 3c and 3d give the fluorescence emission and excitation spectra of leaves with a fractional Photoconversion 2, followed either by a dark period at 20° for

17 min, or by a dark period at 20° for 1 h, before rapid cooling to 77°K. At the fractional Photoconversion 2, leaves exhibited a dark time-course phenomenon similar to that found for fractional Photoconversion 1. The Shift III, was slow, taking at least 15 min at 20°, but the subsequent Shift IV was fast being complete within a further few minutes. However at fractional Photoconversion 1 the Shift IV gave chlorophyll *F*680 whereas at fractional Photoconversion 2 the shift gave chlorophyll *F*683. The 17-min dark period shows the three fluorescence species at 683, 687 and 694 nm, and energy transfer from protochlorophyllide. However within the 1-h dark period, the dashed curves of Figs. 3c and 3d show that Shift IV completes to give chlorophyll *F*683 with no energy transfer from the unconverted protochlorophyllide. This is interpreted as indicating that the newly formed chlorophyll suffers a molecular transposition from the protochlorophyllide groups to the primary chlorophyll. The transposition is away from the protochlorophyllide groups, since excitation at 650 nm is no longer apparent. The primary chlorophyll *F*680 now appears as chlorophyll *F*683. It is then inferred that the transposition is to the matrix primary chlorophyll *F*680 for only a fractional Photoconversion 2 is sufficient to cause the energy shift to this main body of chlorophyll. If the new fractionally formed chlorophyll was independently located, it would be in insufficient

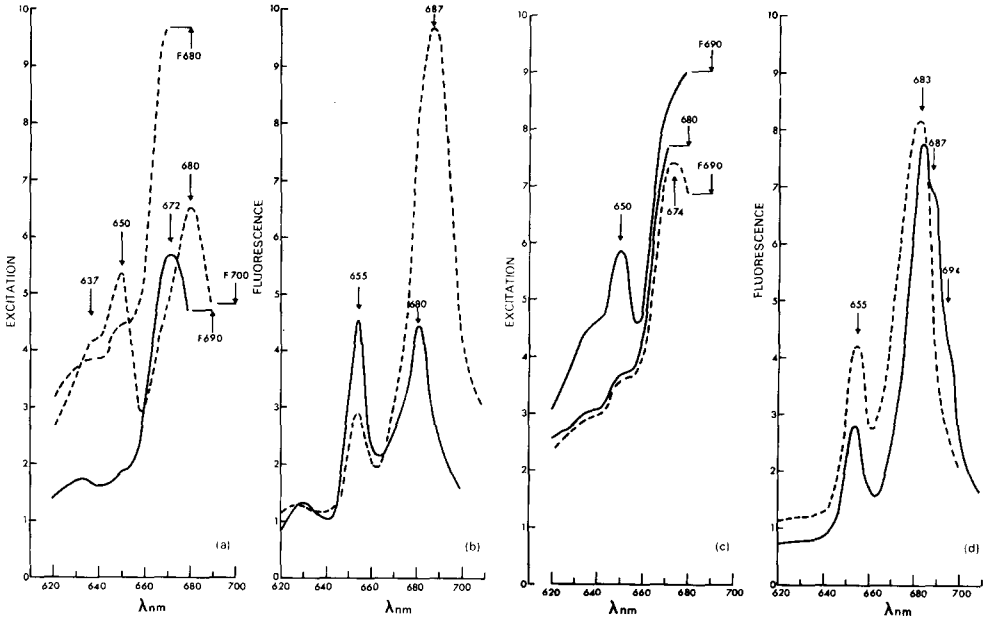

Fig. 3. Fluorescence excitation and emission spectra at 77°K of 14-day etiolated bean leaves. Fluorescence emission with excitation at 440 nm. Excitation spectra taken with the fluorescence monochromator set at the *F* wavelength in nm as indicated. a and b: ——, after the sequence Photoconversion 1 and 66 h dark at 25°; - - - - -, the previous sequence then fractional (approx. 15%) secondary photoconversion in light at 622 nm, bandwidth ± 5 nm, for 10 sec with incident intensity 2000 ergs·cm^{-2}·sec^{-1} at 20° and rapid transfer to 77°K. c and d: ——, after the sequence Photoconversion 1, 66 h dark at 25°, fractional (approx. 15%) secondary photoconversion in light at 622 nm, bandwitdh ± 5 nm, for 10 sec with incident intensity 2000 ergs·cm^{-2}·sec^{-1}, and 17 min dark at 25° before rapid transfer to 77°K; - - - - -, the previous sequence but with a final dark period of 1 h at 25°.

quantity to cause a shift in *F*680. However if it was transposed to a close configuration with respect to a few of the primary chlorophyll molecules, to cause a small energy shift with these, then energy transfer within the primary chlorophyll matrix will occur to give emission from the lowest energy level at *F*683.

Figs. 4a and 4b give the fluorescence emission and excitation spectra at 77°K for a partial Photoconversion 2 with or without a following 1-h dark period at 20°. The solid curves of Figs. 4a and 4b show the form chlorophyllide *F*694 with the appropriate excitation spectrum showing energy transfer from the unconverted protochlorophyllide. Following the 1-h dark period at 20°, Shift IV has occurred to give chlorophyll *F*683 with no energy transfer from the remaining protochlorophyllide. The results of both fractional and partial Photoconversion 2 are in agreement, with the additional information that energy transfer to chlorophyllide *F*694 occurs.

Finally, to complete the picture of the process of Photoconversion 2, Figs. 4c and 4d give the fluorescence emission and excitation spectra of leaves given Photoconversion 1, with a 66-h dark period at 25° followed by a full Photoconversion 2 at 20° with and without a 45-min dark period, where the shift chlorophyllide *F*694 to chlorophyll *F*683 is shown.

The photoactivated Shifts I and II at Photoconversion 2 were not investigated separately due to the difficulty of observing the low-level intermediate *F*674 in the presence of the chlorophyll *F*680.

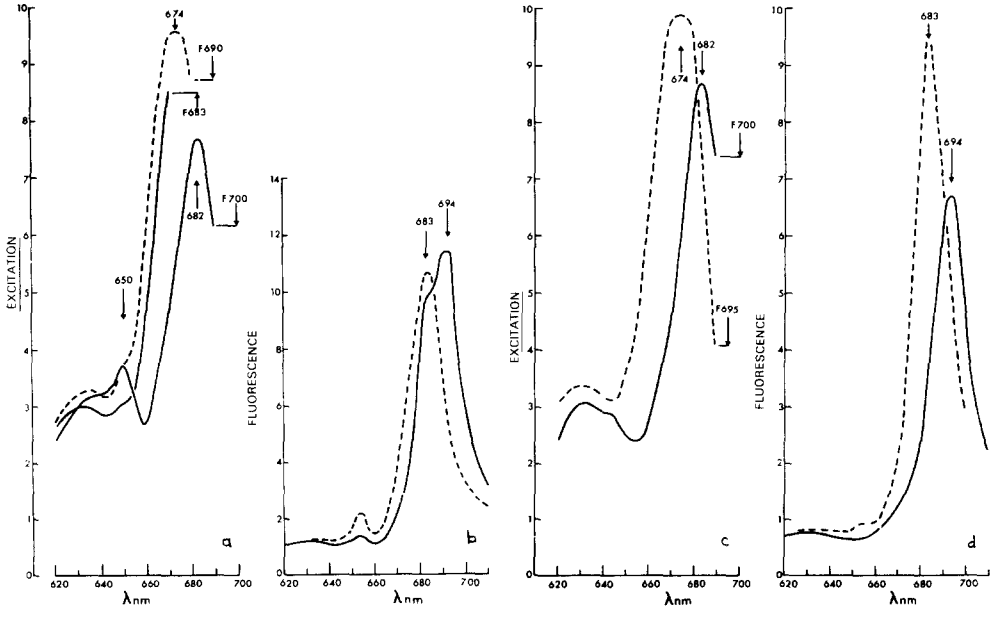

Fig. 4. Fluorescence emission and excitation spectra at 77°K of 14-day etiolated bean leaves. Conditions as Fig. 3. a and b: ——, after the sequence Photoconversion 1, 66 h dark at 25°, partial (approx. 40%) secondary photoconversion in light at 622 nm, bandwidth ± 5 nm, for 40 sec with incident intensity 2000 ergs·cm^{-2}·sec^{-1}; - - - - -, the previous sequence with a 1-h dark period at 25° before transfer to 77°K. c and d: ——, after the sequence Photoconversion 1. 66 h dark at 25°, Photoconversion 2 and rapid transfer to 77°K; - - - - -, the previous sequence with a 45-min dark period at 25° before transfer to 77°K.

Further observations

Photoconversion 3 processes, following Photoconversions 1 and 2 with intervening dark times of 24 h at 25°, were also investigated. At 20°, shifts analogous to Figs. 2a and 2b occurred. At 77°K the spectra showed that the tertiary protochlorophyllide did not transfer energy to the chlorophyll *F*683 present in the leaf. At fractional (approx. 10%) or partial (approx. 40%) Photoconversion 3, the shifts followed the pattern of Photoconversion 2, with energy transfer from protochlorophyllide to chlorophyllides *F*687 and *F*694, but not to the final chlorophyll *F*683. The rates of Photoconversions 2 and 3 under identical light exposures were similar.

Photoconversion-4 processes, following Photoconversions 1, 2 and 3 with intervening 24-h dark periods at 25° were also studied and found to follow the pattern set by Photoconversion 2, both at fractional or partial conversion.

Fluorescence spectra were also run to include the upper vibrational level of chlorophyll at 735 nm, but no significant change in the relation of the 683- to the 735-nm band was found up to the Photoconversion-4 stage, at least 75% of the fluorescence occurring in the 683-nm band.

Ethanol extracts of leaves at the Photoconversion-4 stage were also made and submitted to fluorescence analysis at 77°K to determine the amount of chlorophyll *b* formed. With excitation at 474 nm, the 'Soret' peak of chlorophyll *b* in ethanol at 77°K, measurements showed that the ratio chlorophyll *a*/chlorophyll *b* > 100/1, indicating that either continuous illumination or many more photoconversion stages were necessary for the development of chlorophyll *b*.

DISCUSSION

Photoconversions 2 and 3 follow the same general sequence of shifts found for the initial photoconversion, with two important differences. Firstly, the terminal chlorophyll appears with the fluorescence peak *F*683. Secondly, at fractional or partial Photoconversions 2 and 3, whilst energy transfer from protochlorophyllide to chlorophyllides *F*687 and *F*694 takes place, after Shift IV energy transfer to chlorophyll *F*683 does not occur. Also the resynthesized secondary or tertiary protochlorophyllide does not transfer energy either to chlorophyll *F*680 or to chlorophyll *F*683, respectively. These differences are perhaps not so surprising in view of the structural changes which take place during the lag phase as described by VIRGIN *et al.*[3]. Photoconversion 1 must be regarded as a unique case, where lack of structural development accounts for the differences observed.

In Part 1 of this series[2], the group size of protochlorophyllide *in vivo* was found to be of the order of 20 molecules. Quantitative determination of the group size of the secondary and tertiary group size is more difficult, due to the presence of the primary chlorophyll. However the fluoresence excitation spectra indicate that energy transfer occurs from protochlorophyllide to chlorophyllides *F*687 and *F*694 from which it is clear that resynthesis occurs in molecular groups. The pool size of both the secondary and tertiary protochlorophyllide as indicated by Fig. 1, are of the same magnitude. It is also evident that the Photoconversion-2 process closely parallels that of Photoconversion 1 as determined by the exposure–time relationship. It may then be tentatively concluded that the secondary and tertiary protochlorophyllide

groups are also of the order of 20 molecules. GASSMAN AND BOGORAD[4] have suggested that the protochlorophyllide–holochrome could be regarded as an enzyme, capable of repeated resynthesis. GASSMAN *et al.*[5] suggested that Shift III, as observed by absorbance spectroscopy, may be the point at which a molecular transposition takes place. The evidence of fluorescence spectroscopy at Photoconversions 1, 2 and 3 denies this function at Shift III. However Photoconversions 2 and 3 indicate that molecular transposition does take place following Shift IV. In view of the similarity of group size at the primary, secondary and tertiary stages, and the indicated molecular transposition at Shift IV, the suggestion due to GASSMAN AND BOGORAD[4] is highly probable.

Up to the Photoconversion-4 stage, it has been observed that the 77°K fluorescence in the *F*683 band is 75 % of the total emission, and that no chlorophyll *b* has formed. BOARDMAN *et al.*[6] have detailed the fluorescence properties of particles of Photosystems I and II. A comparison with these shows that up to Photoconversion 4, the fluorescing centres of the photosystems have not been formed. This suggests that many further photoconversions, or continuous light, or both, are necessary for the formation of chlorophyll *b* and for the synthesis of the chlorophyll arrays of the two photosystems.

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