PHOTOSYNTHETICA 6 (2): 169—176, 1972

The Reduction of Protochlorophyllide into Chlorophyllide

V. Demonstration of Energy Transfer inside the $P_{688-676}$ Molecular Units*

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Abstract

From measurements of low-temperature emission, absorption and action spectra of fluorescence of partly phototransformed bean leaves, evidences for *in vivo* energy transfer from protochlorophyllide to chlorophyllide are given. The number of pigment molecules per transfer unit is shown to be much greater *in vivo* than *in vitro*.

In its original forms, the protochlorophyllide-lipoprotein complex is found in the plastids of etiolated leaves. Red low-temperature absorption of an etiolated leaf shows a main peak at 647 nm, a shoulder at 638 nm and a shoulder at 628 nm. The low-temperature fluorescence bands are located at 657 and 630 nm. The 638 nm absorbing form does not fluoresce *in vivo*; it transfers energy to the 647 nm form. If a leaf is irradiated by a brief flash at room temperature and then frozen immediately afterwards in liquid nitrogen, the main red absorption peak is found to have shifted to 676—678 nm. The main fluorescence band shifts accordingly from 657 to 688 nm. We previously called this process the $P_{657-647} \rightarrow P_{688-676}$ spectral transformation (SIRONVAL and MICHEL 1967; SIRONVAL *et al.* 1967). The kinetics and other aspects of this *in vivo* transformation of low-temperature emission have been studied elsewhere (SIRONVAL *et al.* 1968; SIRONVAL and BROUERS 1970).

From their observations of the absorption and low-temperature emission spectra and of the fluorescence action spectra in preparations of the protochlorophyllide-lipoprotein complex, KAHN, BOARDMAN and THORNE (1970) concluded that, after irradiation, units are found containing four to five chromophores among which energy transfer may occur (at −196 °C). Other facts led SIRONVAL and KUYPER (1972) to the conclusion that the absorption of light by protochlorophyllide in the prolamellar bodies induces *in vivo* the formation of transfer units containing several pigment molecules.

In this paper, we discuss low-temperature absorption, emission and action spectra of fluorescence, and we demonstrate the transfer of energy from protochlorophyllide to chlorophyllide in intact bean leaves frozen at −196 °C at different stages of the phototransformation process.

* *Received* December 3, 1971.

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MATERIAL AND METHODS

Leaf material and fluorescence spectra: Primary bean leaves (*Phaseolus vulgaris* L. cv. Commodore) grown in complete darkness were used. Methods for growing the plants, for recording low-temperature emission and for estimating the extent of the $P_{657-647} \rightarrow P_{688-676}$ spectral transformation have been described elsewhere (SIRONVAL *et al.* 1968; SIRONVAL and KUYPER 1972).

Sample irradiation: A 1 ms photographic flash (*Multiblitz Report PORBA*) was used. About a 50% spectral transformation was obtained when the distance from the flash to the leaf was around 50 cm. The samples were irradiated, either at room temperature or at −10 °C.

Absorption spectra at liquid nitrogen temperature: The *in vivo* absorption spectra were recorded at liquid nitrogen temperature using a *Cary 14 R* spectrophotometer equipped with an attachment built at the Biophysical Laboratory, University of Utrecht, The Netherlands. Two superposed leaves were used for recording.

Fluorescence excitation spectra: The excitation beam was provided by a quartz iodine 500 W lamp. The light passed through a *Bausch and Lomb* diffraction grating monochromator. It was reflected inside a Dewar onto the sample which was maintained in liquid nitrogen as described in Figs. 1 and 2, SIRONVAL *et al.* (1968). The fluorescence was received at an angle of about 45°; it passed through a grating *Zeiss* monochromator *M20* set at 700 nm. The excitation spectra were corrected for energy variations of the exciting light between 600 and 700 nm. The energy was measured by means of a thermopile (type *E1*, special, *Kipp*, Delft, The Netherlands).

Acknowledgements: The authors wish to thank Prof. J. B. Thomas and Dr. J. C. Goedheer, Utrecht, the Netherlands, for the use of the *Cary 14R* spectrophotometer. They also thank the "Fonds National de la Recherche Fondamentale Collective, FRFC" and the "Institut pour l'Encouragement de la Recherche Scientifique appliquée à l'Industrie et à l'Agriculture, IRSIA", Brussels, Belgium, for their financial support.

RESULTS

(*1*) Low-temperature emission and absorption of partly phototransformed leaves

Different degrees of spectral transformation were obtained by varying the distance from the etiolated leaf samples to the flash (Fig. 1). The extents of transformation of the low-temperature emission (or extents of spectral transformation i_p; definition in SIRONVAL and KUYPER 1972) were respectively 0·00, 0·27, 0·44 and 0·74. The appearance of a 688 nm emission band corresponded to the appearance of an absorption band at 676—678 nm. Curves *4* show that although the emission was much more intense at 688 nm than at 657 nm, absorbance at 677 nm was about half as much as absorbance at 649 nm. A similar situation appears in curves *2* and *3*. Thus, in general, whatever the fluence p, the extent of change of the low-temperature emission (i_p) was much greater than the corresponding extent of change of the low-temperature absorption.

(2) *In vivo* energy transfer from protochlorophyllide to the 678 nm absorbing pigment in partly phototransformed leaves

Recordings were made at liquid nitrogen temperature of the fluorescence emission and action spectra of fluorescence of several leaves phototransformed to varying extents. The temperature of each leaf was then raised in darkness from −196 °C to −10 °C and each leaf was submitted at −10 °C to an intense polychromatic flash which achieved 100% spectral transformation ($i_p = 1$). After this irradiation each

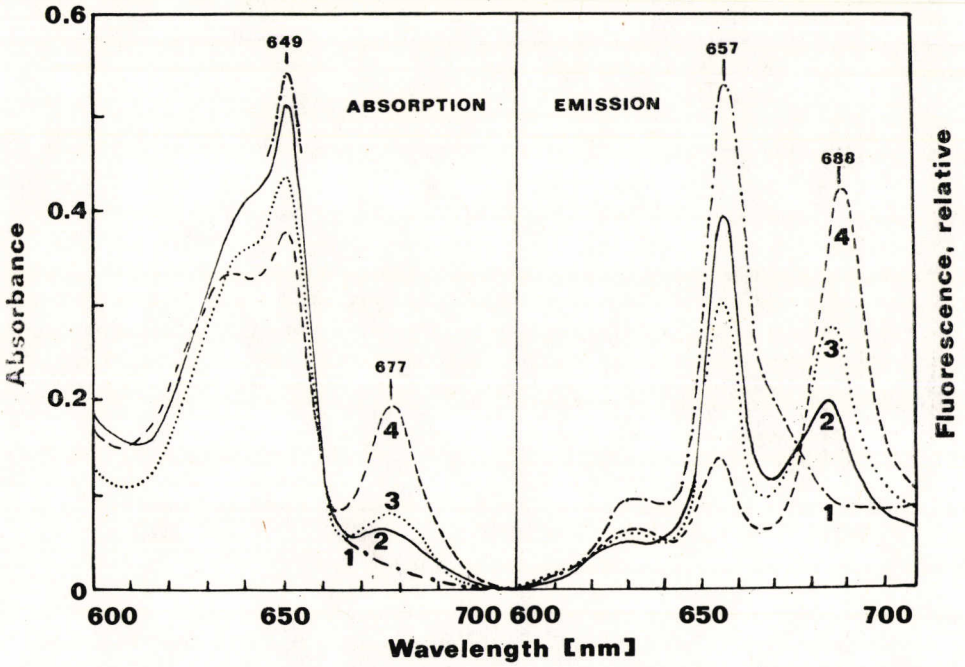

Fig. 1. Low-temperature (−196 °C) absorption and emission spectra during phototransformation of bean leaves. Values of i_p (extent of spectral transformation) from curve *1* to curve *4*: 0·00; 0·27; 0·44; 0·74.

leaf was immediately frozen in liquid nitrogen and low-temperature emission and action spectra were recorded again. The action spectra were normalized for an identical value of emission intensity at 700 nm in each 100% transformed leaves excited by means of 678 nm photons (Fig. 2, left). The fluorescence spectra were normalized for an identical value of the integral below the curves (Fig. 2, right).

The location of the emission band around 688 nm varied from 685 to 690 nm depending on the extent of spectral transformation. The corresponding action band was located at 678 nm. On the other hand the low-temperature fluorescence excited by 436 nm photons (Fig. 2, right) was emitted at the expense of light absorbed both by protochlorophyllide and by the 678 nm absorbing pigment, whereas the

172 fluorescence excited at 700 nm by 678 nm photons in Fig. 2 (left) came from light absorbed by the 678 nm pigment only.

We argue as follows: If energy is not transferred to 678 nm absorbing molecules the emission band around 688 nm should be due to light absorption by these molecules only, whatever the excitation wavelength. In this case, when comparing the

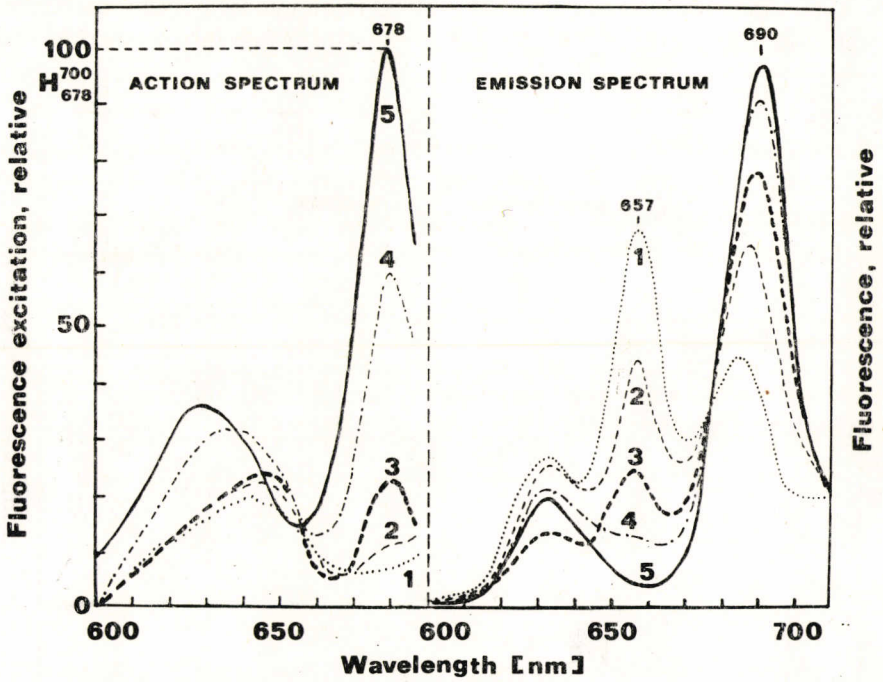

Fig. 2. Normalized low-temperature (−196 °C) action and emission spectra during photo-transformation of bean leaves. Values of i_p (extent of spectral transformation) from curve *1* to curve *5*: 0·33; 0·58; 0·78; 0·92; 1·00.

normalized emission and action spectra, we should find for a given leaf sample, whatever the extent of its spectral transformation, the same ratio between the amount of fluorescence at 700 nm excited with 678 nm photons, which necessarily reflects after normalization the concentration (among the pigments in the sample) of the 678 nm absorbing form, and the amount of fluorescence at 685—690 nm excited with 436 nm photons, since after normalization this should reflect the same concentration if no transfer occurs.

But this is not what we observed (Fig. 2). An increase of emission at 688 nm was seen when protochlorophyllide and the 678 nm absorbing pigment were excited simultaneously with blue radiation. This increase must be due to energy transfer from radiation absorbed by protochlorophyllide since there is no other energy source possible. On the other hand, the action spectra clearly showed excitation due to

protochlorophyllide absorption around 640—650 nm (compare curves *1*, *2* and *3* of Fig. 2).

(3) Chlorophyllide as the light-harvesting pigment

The normalized action spectra in Fig. 2 enable us to estimate the proportion of 678 nm pigment in partly phototransformed leaf samples. This proportion is given by

$$\frac{H^{700}_{678,p}}{H^{700}_{678,p=\infty}} = c_p$$

where (*1*) $H^{700}_{678,p}$ and (*2*) $H^{700}_{678,p=\infty}$ are the respective emission intensities observed at 700 nm when (*1*) a sample partly transformed at fluence p, and (*2*) the same sample 100% transformed at infinite fluence, are excited with 678 nm photons. The emission spectra in Fig. 2 give the extents of transformation of the low-temperature emission (i_p) for the same samples.

Table 1

Extent of transformation of low-temperature emission (i_p) and extent of protochlorophyllide reduction (c_p; $c_{p,c}$) during the phototransformation of bean leaves at room temperature (explanations in the text).

i_p	c_p	$c_{p,c}$
0·22	0·06	0·03
0·33	0·07	0·05
0·39	0·03	0·07
0·53	0·09	0·12
0·55	0·09	0·13
0·58	0·11	0·14
0·68	0·18	0·20
0·72	0·23	0·23
0·75	0·23	0·26
0·78	0·22	0·29
0·88	0·50	0·46
0·92	0·61	0·57

Values of (i_p) and of (c_p) (Table 1) show that (i_p) did not vary in the same proportion as (c_p). The proportion of chlorophyllide in the total pigment content ($c_{p,c}$) was calculated from the (i_p)'s using the rule given by SIRONVAL and KUYPER (1972; expression *2*), the values of the constants being those at +23 °C (Table 3 in SIRONVAL and KUYPER 1972). The good agreement between (c_p) and ($c_{p,c}$) shows that the 678 nm pigment responsible for the 688 nm emission — the light-harvesting pigment — was a pigment which appeared as being chlorophyllide when extracted from the lipoprotein.

DISCUSSION

Using mostly preparations of an active protochlorophyllide-lipoprotein complex KAHN, BOARDMAN and THORNE (1970) demonstrated energy transfer from protochlorophyllide to chlorophyllide in partly transformed samples at liquid nitrogen temperature. They introduced a parameter E which they called "efficiency of the energy transfer", which appears to be in our terminology the ratio R_p of the concentration of protochlorophyllide molecules belonging to the transfer units to the concentration of all protochlorophyllide molecules (protochlorophyllide inside + protochlorophyllide outside units):

$$R_p = \frac{i_p - c_p}{1 - c_p}$$

in which the subscript p refers to the fluence (see Appendix). From a comparison of values obtained experimentally for this ratio with values calculated from a statistical model which assumes a binomial distribution of reduced molecules inside the units (see THORNE 1971), the authors concluded that *in vitro* the units contained four to five pigments each.

Working in another way, SIRONVAL and KUYPER (1972) showed that energy transfer units were found in phototransformed leaves. They described one transfer unit as containing (n) pigment molecules linked to a lipoprotein. ($n - m$) of these molecules absorb in the red at 637—647 nm and transfer energy inside the unit to (m) molecules having a red absorption band at 676—678 nm. Any given 676—678 nm pigment molecule emits a 688 nm fluorescence from energy collected inside the unit to which it belongs (at liquid nitrogen temperature).

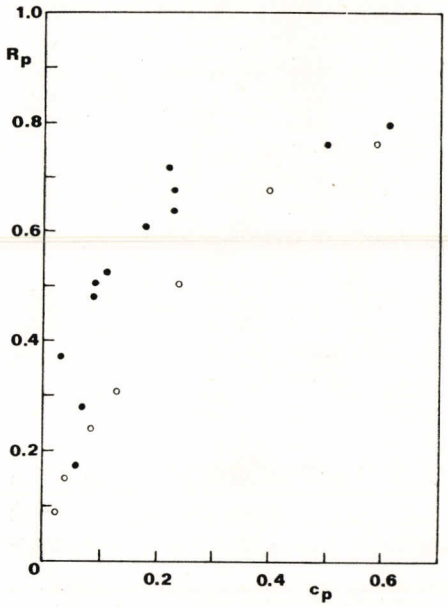

Fig. 3. Plot of values of R_p against c_p (see the text). *In vitro* values of KAHN, BOARDMAN and THORNE (1970, Fig. 6) (○) are compared with *in vivo* values (●) taken from Table 1.

Values for R_p were calculated from the *in vivo* (c_p)'s and (i_p)'s found in Table 1. They are plotted against (c_p) in Fig. 3 and compared to the *in vitro* values of KAHN, BOARDMAN and THORNE (1970). The *in vivo* and *in vitro* values are not superposable. This is due to the fact that the number of pigment molecules per transfer unit is higher than ten *in vivo*. The great number of pigment

molecules per transfer unit *in vivo* explains the high quantum yield observed for the transformation of low-temperature emission of protochlorophyllide in the leaf (SIRONVAL and BROUERS 1970).

On the other hand, the agreement between (c_p) and ($c_{p,c}$) values in Table 1 points to the pigment responsible for absorption at 678 nm as being chlorophyllide. It is also clear from the table that the quantity of fluorescence emitted per chlorophyllide molecule decreases (relatively) as photo-transformation proceeds.

APPENDIX

Let i_p be the degree of spectral transformation at the end of fluence p, measured by recording the emission spectrum at $-196\,°C$, and c_p the degree of reduction determined by measuring the proportion of reduced pigment in the total content of reducible pigment (see SIRONVAL and KUYPER 1972). Let $H_{p,657}$ be the intensity of emission at liquid nitrogen temperature at 657 nm after fluence p, having deduced the contribution of the 630 nm emitting pigment at 657 nm. Then in our experimental conditions:

$$H_{p=o,657} = H_{p,657} + H_{p,688} \tag{1}$$

in which $H_{p=o,657}$ is the intensity of initial fluorescence of the etiolated leaf at 657 nm, and $H_{p,688}$ the intensity of the leaf's emission at 688 nm after fluence p, as measured at liquid nitrogen temperature.

Finally, let R_p be the proportion, among the protochlorophyllide molecules not reduced after fluence p, of molecules likely to transfer energy to the 688 nm emitting pigment at the temperature of liquid nitrogen (and after fluence p). R_p may be expressed in the following way:

$$R_p = \frac{[H_{p=o,657} \times (1 - c_p)] - H_{p,657}}{[H_{p=o,657} \times (1 - c_p)]} \tag{2}$$

which may also be written:

$$H_{p,657} = H_{p=o,657}\,(1 - c_p)\,(1 - R_p) \tag{3}$$

In Eq. (3) the factor $[(1 - c_p)\,(1 - R_p)]$ represents the proportion of pigment molecules emitting at 657 nm after fluence p; these are the ones which, among the non-reduced molecules, are not likely to transfer energy to the reduced molecules. Knowing that

$$i_p = \frac{H_{p,688}}{H_{p=o,657}}$$

(SIRONVAL and KUYPER 1972, Appendix 2), it is easy to write R_p in terms of i_p and c_p from Eq. (1) and (3):

$$R_p = \frac{i_p - c_p}{1 - c_p} \tag{4}$$

Expression (3) has the same form as expression (2) of KAHN, BOARDMAN and THORNE (1970). It is identical to this expression if we admit that the corrected area of the emission band at 657 nm

176 is Gaussian, since in this case the areas $(A - A_{pi})$ and (A_{pa}) in expression (2) of KAHN, BOARDMAN and THORNE (1970) are respectively proportional to $H_{p,657}$ and $H_{p=o,657}$. In a first approximation, it may indeed be admitted that the area in question is Gaussian.

REFERENCES

KAHN, A., BOARDMAN, N. K., THORNE, S. W.: Energy transfer between protochlorophyllide molecules: Evidence for multiple chromophores in the photoactive protochlorophyllide-protein complex *in vivo* and *in vitro*. — J. mol. Biol. **48**: 85—101, 1970.

SIRONVAL, C., BROUERS, M.: The reduction of protochlorophyllide into chlorophyllide. II. The temperature dependence of the $P_{657-647} \rightarrow P_{688-676}$ phototransformation. — Photosynthetica **4**: 38—47, 1970.

SIRONVAL, C., KUYPER, Y.: The reduction of protochlorophyllide into chlorophyllide. IV. The nature of the intermediate $P_{688-676}$ species. — Photosynthetica **6** (3): in press, 1972.

SIRONVAL, C., MICHEL, J.-M.: On a "photoenzyme" or, the mechanism of the protochlorophyllide-chlorophyllide photoconversion. — In: Book of Abstracts, Europ. Photobiol. Symp. Pp. 105—108. Hvar, Yugoslavija 1967.

SIRONVAL, C., BROUERS, M., MICHEL, J.-M., KUIPER, Y.: The reduction of protochlorophyllide into chlorophyllide. I. The kinetics of the $P_{657-647} \rightarrow P_{688-676}$ phototransformation. — Photosynthetica **2**: 268—287, 1968.

SIRONVAL, C., KUYPER, Y., MICHEL, J.-M., BROUERS, M.: On the primary photoact in the conversion of protochlorophyllide into chlorophyllide. — Studia biophys. **5**: 43—50, 1967.

THORNE, S. W.: The greening of etiolated bean leaves. I. The initial photoconversion process. — Biochim. biophys. Acta **226**: 113—127, 1971.