PHOTOSYNTHETICA 6 (3): 254—275, 1972

The Reduction of Protochlorophyllide into Chlorophyllide

IV. The Nature of the Intermediate $P_{688-676}$ Species*

C. SIRONVAL and Y. KUYPER**

Laboratory of Photobiology, Research Centre of Gorsem, Sint Truiden, and Department of Botany, The University, Liège, Belgium

Abstract

The intermediate $P_{688-676}$ species appearing in the leaf as a result of the $P_{657-647} \xrightarrow[(1\ ms)]{h\upsilon} P_{688-676}$ phototransformation is defined as a macromolecular entity having the property of emitting light at 688 nm at liquid nitrogen temperature. Consequently the proportion of $P_{688-676}$ found after irradiation of an etiolated leaf is measured at any fluence p by the extent of the change of the low temperature emission of the leaf (i_p). This extent differs from the extent of reduction of protochlorophyllide into chlorophyllide (c_p). This means that the photoproduction of $P_{688-676}$ (as defined in the text) and the photoreduction of protochlorophyllide into chlorophyllide are distinct processes. — The relation between i_p and c_p has the same form from +37 to −15 °C. In this temperature range i_p always exceeds c_p except at infinite fluence, when the extent of phototransformation of the emission reaches 100%. However, when a leaf is irradiated at a temperature below −90 to −100 °C, the extent of reduction c_p tends to be equal to the extent of change of low temperature emission i_p ($i_p \cong c_p$, whatever the fluence received). In this case a rise of temperature from below −90 to above −80°C after irradiation of the leaf provokes a dark transformation of the low temperature emission spectrum: emission increases at 688 nm and decreases at 657 nm in the dark, the rate of the process increasing with increasing temperature. The dark change tends to reproduce the relation between (i_p) and (c_p) as found between +37 °C and −15 °C. During this change a dark reduction of protochlorophyllide may be demonstrated. The reduction process is thus separable from the light absorption act. — The facts oblige us to distinguish between two kinds of reduction of protochlorophyllide in the leaf. The reductions of the first kind may apparently occur below −100 °C. Those of the second kind depend on the formation of $P_{688-676}$. The latter results from a light-triggered transconformation of the lipoprotein which takes place above −80 °C. — The $P_{688-676}$ macromolecular entity is described as a unit containing a mixture of protochlorophyllide and chlorophyllide molecules in variable proportions. Energy transfer from protochlorophyllide to chlorophyllide is permitted inside this unit. Hence the denomination "energy transfer unit".

* *Received* October 24, 1971; *accepted* January 17, 1972.
This work was supported financially by the "Institut pour l'Encouragement de la Recherche Scientifique appliquée à l'Industrie et à l'Agriculture", IRSIA, Brussels, Belgium.

** *Requests for reprints should be sent to:* Laboratory of Photobiology, Department of Botany, The University, Sart-Tilman, 4000, Liège, Belgium.

Fig. 1 illustrates apparatus used to enable a fresh sample from an etiolated primary bean leaf to fall into a Dewar containing nitrogen immediately after being exposed to a 1 ms flash of light. This apparatus has been used by SIRONVAL and MICHEL (1967) to show that, after brief illumination and then immediate trapping by freezing, the *in vivo* species differs from the species found by SHIBATA (1957) in the minutes following illumination. Indeed, according to the procedure illustrated in Fig. 1, if any light is absorbed by protochlorophyllide, the spectrum of the leaf undergoes a change which we have called the $P_{657-647} \xrightarrow{h\upsilon} P_{688-676}$ spectral transformation or phototransformation (reaction [I]) in which the first figures of the subscripts pertain to the emission bands and the second to the red absorption bands (SIRONVAL and MICHEL 1967, Fig. 1; SIRONVAL *et al.* 1967, 1968). The product of reaction [I] ($P_{688-676}$) is characterized empirically by a red absorption band around 676 nm, the corresponding emission being around 688 nm. Hitherto, in this series of articles we have made no definite judgment on its nature.

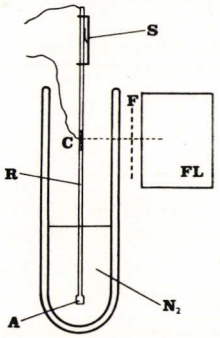

Fig. 1. Apparatus used for freezing in liquid nitrogen immediately after a 1 ms flash. The sample is placed in the holder (*S*) which is made to fall freely down a slide (*R*) into liquid nitrogen (N_2). At (*C*) a contact triggers the flash (*FL*). The light reaches the sample through a window in the Dewar; a filter (*F*) is interposed if desired. A stop (*A*) prevents the holder from hitting the bottom of the Dewar.

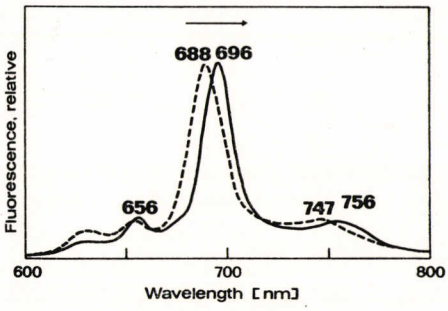

Fig. 2. The dark, 30 s emission shift from 688 to 696 nm. Dashed line: −196 °C emission of an etiolated leaf which received a non-saturating, 1 ms flash in the apparatus of Fig. 1. Continuous line: emission of an identical leaf which received an identical flash, but was kept in darkness at room temperature for 30 s before being frozen in liquid nitrogen.

The Shibata species has been called C_{684} (SHIBATA 1957) because it was thought to contain chlorophyllide and because it absorbs around 682–684 nm (emission around 697 nm). It is known now that it is formed in darkness after a 1 ms flash as the result of a rapid dark shift: $P_{688-676} \xrightarrow[20-30\,s]{darkness} C_{697-682}$ (reaction [II]). The shift has been demonstrated by illuminating the leaf as in Fig. 1 and then preventing it from falling directly into liquid nitrogen (Fig. 2). It is completed at room temperature within some 30 s (SIRONVAL and MICHEL 1967; GASSMAN, GRANICK and MAUZERALL 1968; BONNER 1969).

Preliminary studies of the relationship between the extent of the $P_{657-647} \xrightarrow{h\upsilon} P_{688-676}$ spectral transformation (reaction [I]) and the corresponding extent of reduction of protochlorophyllide into chlorophyllide led us to conclude that the disappearance of $P_{657-647}$ and the appearance of $P_{688-676}$ could not merely result from the reduction of protochlorophyllide (see SIRONVAL and MICHEL 1967, in particular Fig. 3). Hence the rapid dark shift (reaction [II]) was tentatively described in 1967 as corresponding to the completion of the reduction of protochlorophyllide. Similarly LITVIN and BELYAEVA (1968) observed that the extent of the decrease

256 of 657 nm low temperature emission after brief irradiation (some ms) appreciably exceeded the corresponding extent of reduction (as measured by extraction in methanol). They thought that this could indicate some instability of intermediate pigment form(s) produced by brief light flashes.

The rapid shift was also observed and studied by GASSMAN, GRANICK and MAUZERALL (1968). These authors stated that "the dark grown leaf, after an actinic flash, forms nascent chlorophyllide with an absorption maximum at 678 nm". This 678 nm "nascent chlorophyllide" is easily proved to be identical to the $P_{688-676}$ species: (*1*) it appears after irradiation with a 1 ms flash of light (reaction [I]); (*2*) its appearance is followed by the rapid shift (reaction [II]). GASSMAN, GRANICK and MAUZERALL (1968) showed that the rate of the dark shift decreased as the temperature was reduced from room temperature to 0 °C. The shift did not occur at −70 °C. It did not possess any isosbestic point, which could imply that its nature is complex. GASSMAN, GRANICK and MAUZERALL (1968) therefore suggested that the shift was due to environmental changes occurring in the dark after the production of "nascent chlorophyllide". BONNER (1969) also reported the occurrence of a "short-lived intermediate form in the *in vivo* conversion of protochlorophyllide 650 to chlorophyllide 684". He studied the temperature dependence of the shift and agreed essentially with the data of SIRONVAL and MICHEL (1967) and GASSMAN, GRANICK and MAUZERALL (1968).

Following up earlier observations (LITVIN and BELYAEVA 1968), LITVIN and BELYAEVA (1971) suggested the existence of two successive light steps in protochlorophyllide reduction. They claimed to find two "nascent chlorophyllide forms", "$Chl_{684-676}$" and "$Chl_{690-680}$", after brief irradiations of an etiolated leaf. According to them light first produces "$Chl_{684-676}$", "$Chl_{690-680}$" being the product of a second photoreaction of which "$Chl_{684-676}$" is the substrate. They concluded that the $P_{688-676}$ species is a mixture of "$Chl_{684-676}$" and "$Chl_{690-680}$".

These data are related to those published by THORNE (1971). THORNE also proposed a sequence of two light reactions: the product to the first reaction is a pigment emitting at 674 nm and absorbing at 668 nm. The second reaction transforms this pigment into another emitting at 687 nm and absorbing at 678 nm. In fact LITVIN and BELYAEVA (1971) found that their "$Chl_{684-676}$" was transformed in the dark into "$Chl_{675-670}$". Presumably THORNE did not distinguish this dark step.

In the present paper, we hope to contribute to a better definition of the nature of the intermediate $P_{688-676}$ ("nascent chlorophyllide") and at the same time provide some new insight into the process of protochlorophyllide reduction.

MATERIAL AND METHODS

Leaf material: Primary leaves of the bean cv. "Commodore" were used, cultivated in darkness as described by SIRONVAL *et al.* (1968). The expression "a homogeneous set of leaves" in the present text means a set of etiolated, primary leaves of: (*1*) the same age (generally collected 20 d after germination); (*2*) the same weight (about 30 mg per leaf); (*3*) the same intensity of the low temperature emission at 630 nm relatively to the 657 nm emission. The relative intensity of low temperature emission at 630 nm may vary, from one set of leaves to another, between the two extremes shown by Figs. 2 and 3 of this paper.

Leaf irradiation at a given temperature: Apart from actual irradiation, the only light used during the manipulations came from an *OSRAM 4543* lamp surrounded by a neutral filter plus a green cellophane sheet (maximum transmission at 520 nm). This light had no effect on the leaf properties being studied (= safe light). Before irradiation, the sample was introduced in the holder shown in SIRONVAL and BROUERS, Fig. 1 (1970).

(*a*) For irradiations at temperatures below −60 °C, the sample was placed in its holder inside a Dewar as shown in SIRONVAL and BROUERS, Fig. 2 (1970), or sometimes simply dipped into liquid nitrogen. The irradiation took place inside the Dewar, after temperature equilibration, through an unsilvered area using polychromatic light from a xenon source (*OSRAM XBO*, 450 W/P).

(*b*) For brief irradiations (1 ms) at room temperature, an electronic flash (*Multiblitz 50, Gesellschaft für Multiblitzgeräte*, Porz-Westhoven, FR Germany) was used. The quantity of radiation received by the sample was varied by altering the distance between the flash and the sample. When using the 660 nm filter, the electronic flash equipment was placed inside a closed box, the radiation being transmitted to the sample through the filter.

Characteristics of the 660 nm filter: The filter was constructed by *Baird Atomic* (The Hague, The Netherlands). The transmission band was centred at 660 nm; the bandwidth at half-peak was 6 nm and transmission at maximum, 57%.

Estimation of the amount of spectral transformation (T %): T % was estimated from the fluorescence emission recorded at −196 °C as described in SIRONVAL *et al.* (1968). He repeatedly found that the amount of spectral transformation never changes when recording low temperature emission.

Pigment extraction from one leaf sample: A 100 (or 50) ml mortar was filled several times with liquid nitrogen until the nitrogen remained boiling in the cooled mortar for a sufficient length of time. 4 ml of diethylether (Merck, *pro analysi*) were added to the nitrogen and the frozen ether was reduced to a powder by a precooled pestle. The leaf holder carrying the irradiated sample was taken from the Dewar where it had been stored under liquid nitrogen. The holder was placed above the mortar and the relevant tissue was carefully taken out of the holder in such a way that the fracture line coincided with the edge of the irradiated region. The tissue was reduced to a fine powder under liquid nitrogen using a precooled pestle (more liquid nitrogen was added from time to time). The powder was mixed with the powder of solid ether. After complete evaporation of the nitrogen (during which time the powder mixture was ground continuously in the mortar), the ether started melting (−120 °C). Grinding was then continued carefully until room temperature was reached. Finally, the contents of the mortar were poured into a centrifugal tube. The mortar was rinsed twice with ether drops which were subsequently added to the extract to make a final volume of 5 ml. The closed tube remained in darkness overnight at room temperature. The extract was subjected to centrifugation for 10 min at 2000 × g and the supernatant was collected for estimation of the pigment content.

Estimation of the pigment content of the extracts: The extracts were a very dilute solution of pigment mixtures. Their content was estimated fluorometrically as follows:

Several standard solutions containing different pigment mixtures were prepared by extracting several sets of primary, etiolated bean leaves into ether, each having received a certain amount of polychromatic radiation (different for each set; 1 to 5 g fresh leaf material per set extracted in 25 ml ether). After the centrifugation pigment concentrations of the standards were measured by absorption spectrophotometry in 10 cm cuvettes using the coefficients given by SMITH, BENITEZ and KOSKI in FRENCH (1960) at 624 nm and 662 nm for protochlorophyll(ide) and chlorophyll(ide) respectively. Then, the standards were diluted 10 times and recordings were made of their fluorescence excited at room temperature with 436 nm radiation (from a Hg lamp) using a *Zeiss ZFM 4* device placed in front of the monochromator of the spectrofluorimeter described in SIRONVAL *et al.* (1968) (the *Zeiss ZFM 4* device reproduces constant physical conditions for excitation and reception). The height (H_{664}) of the emission due to chlorophyllide at 664 nm was corrected for the emission of protochlorophyllide at the same wavelength (it was found that $H_{664,corrected} = H_{664,observed} - 0.035 H_{625,observed}$). Finally, the heights of the emissions of protochlorophyll(ide) at 625 nm (H_{625}) and chlorophyll(ide) at 664 nm (H_{664}) were plotted against concentration. Straight lines were obtained.

The fluorescence of the unknown extracts was measured by using the *ZMF 4* device in the same physical conditions as for the standard samples. The proportion of chlorophyll(ide) in the total pigment content [chlorophyll(ide) + protochlorophyll(ide)] was calculated from the calibration lines.

RESULTS

(*1*) Definition of $P_{688-676}$ as a 688 nm light emitting species

It was generally concluded from the results of SHIBATA (1957) that there was no intermediate step between $P_{657-647}$ and $C_{697-684}$. But these results did not preclude the existence of an intermediate step with a shorter life-time than the time required to record the effect of light in SHIBATA's experiments. By means of the apparatus in Fig. 1, SIRONVAL and MICHEL (1967) trapped a 688 nm emitting, 676 nm absorbing intermediate species ($P_{688-676}$) which has also been obtained by GASSMAN, GRANICK and MAUZERALL (1968) and others. When examining low temperature emission, isosbestic points have been demonstrated between $P_{688-676}$ and the initial $P_{657-647}$. This has been done as follows:

The fluorescence spectrum of an etiolated leaf was recorded at the temperature of liquid nitrogen (Fig. 3; curve *1*). The leaf was thereafter warmed to −15 °C and irradiated with a 1 ms flash which produced about a 25% transformation of the spectrum; it was then immediately frozen to −196 °C using the apparatus of Fig. 1 and its fluorescence recorded again (Fig. 3; curve *2*). Several leaves were handled successively in the same way, each receiving an increasing amount of radiation within a 1 ms flash (Fig. 3; curves *3*, *4*, *5* and *6*). The spectra of etiolated and

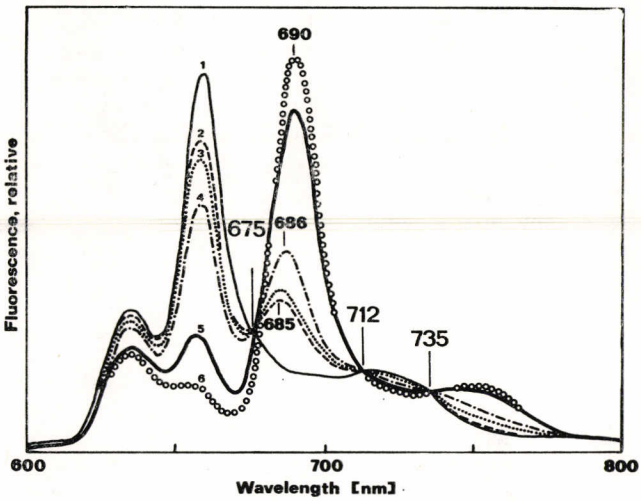

Fig. 3. Occurrence of isosbestic points during phototransformation of low temperature emission of etiolated bean leaves. The actinic light from the flash passed through a 660 nm filter. Irradiations at −15 °C. Curve *1*: etiolated, non irradiated leaf sample. Curves *2* to *6*: samples showing increasing phototransformation. The control etiolated spectra of samples *2* to *6* are not reproduced; after normalization, they were identical to curve *1*. All spectra normalized for the same total emission (same value of the integral below the curves) and recorded in liquid nitrogen.

Table 1

Constancy of the ratio (r) between the increase of low temperature emission at 688 nm and the corresponding decrease at 657 nm when irradiating etiolated leaves.

Each etiolated leaf sample was frozen in liquid nitrogen before irradiation and the initial low temperature emission was recorded ("*et*" emission). After this recording, the sample was warmed to −15 °C. It received a 1 ms polychromatic flash from a distance chosen so as to achieve either about 45, or 100% spectral transformation. The apparatus of Fig. 1 was used in order to freeze the sample in liquid nitrogen immediately after the flash. The emission of the irradiated sample ("*ir*" emission) was recorded. Identical physical conditions were used for recording both "*et*" and "*ir*" emissions (the position of the sample in its holder reproduced a maximum signal in both cases). The values of ($\Delta H_{657} = et_{657} - ir_{657}$) and ($\Delta H_{688} = ir_{688} - et_{688}$) were calculated from the spectra, appropriate corrections being made (see SIRONVAL *et al.* 1968, Fig. 8). The conditions of light reception and the properties of the photomultiplier were selected in order to make $r \cong 1.0$.

Number of the leaf sample	Changes observed between 0 to ~ 45% spectral transformation*		$r = \frac{\Delta H_{688}}{\Delta H_{657}}$	Changes observed between 0 to 100 % spectral transformation*		$r = \frac{\Delta H_{688}}{\Delta H_{657}}$
	ΔH_{657}	ΔH_{688}		ΔH_{657}	ΔH_{688}	
1	48	45 (39.9)**	0.94	118	124	1.05
2	44	51 (44.0)	1.16	110	116	1.05
3	39	43 (40.4)	1.10	127	129	1.02
4	69	68 (45.5)	0.99	130	135	1.04
5	61	59 (44.7)	0.97	113	115	1.02
6	46	50 (44.0)	1.09	119	124	1.04
7	76	71 (45.0)	0.93	142	148	1.04
8	55	56 (42.3)	1.02	122	127	1.04
9	64	63 (47.3)	0.98	109	110	1.01
10	50	52 (41.6)	1.04	114	120	1.05
11	53	56 (42.6)	1.06	140	144	1.03
12	46	48 (42.0)	1.04	119	122	1.03
13	55	48 (46.7)	0.87	100	106	1.06
14	31	38 (39.2)	1.23	121	122	1.01
15	48	50 (43.9)	1.04	132	131	0.99
16	47	50 (42.0)	1.06	143	147	1.03
17	40	46 (40.6)	1.15	138	135	0.98
18	40	38 (43.0)	0.95	97	96	0.99
19	41	40 (43.4)	0.98	117	121	1.03
20	55	57 (45.7)	1.04	135	138	1.02
21	47	44 (36.6)	0.94	122	116	0.95
22	63	66 (47.8)	1.05	137	139	1.01
23	66	64 (47.9)	0.97	104	109	1.05
24	83	89 (56.6)	1.07	113	116	1.03
25	75	77 (49.8)	1.03	117	120	1.03
	$\Sigma\,\Delta H_{657} = 1\,342$	$\Sigma\,\Delta H_{688} = 1\,369$		$\Sigma\,\Delta H_{657} = 3\,039$	$\Sigma\,\Delta H_{688} = 3\,110$	
	$\frac{\Sigma\,\Delta H_{688}}{\Sigma\,\Delta H_{657}} = 1.02$			$\frac{\Sigma\,\Delta H_{688}}{\Sigma\,\Delta H_{657}} = 1.02$		

* Arbitrary units.

** The numbers in brackets are values of the extent of spectral transformation (T %) computed as indicated in SIRONVAL *et al.* (1968).

260 irradiated leaves were normalized for the same total emission. Isosbestic points were found at 675, 712 and 735 nm. The isosbestic point at 675 nm was somewhat less evident than those at 712 and 735 nm. Fig. 3 shows that this fact was apparently due to a shift of the emission of $P_{688-676}$ from 685 nm at the start, to 690 nm at the end of the spectral transformation (688 nm is some "mean value" of the location of the $P_{688-676}$ main emission band; SIRONVAL *et al.* 1968; LITVIN and BELYAEVA 1971; see the "Discussion").

The occurrence of isosbestic points agrees with a fact which we previously used as a basis for estimating the extent of the change of the low temperature emission in the course of the $P_{657-647} \xrightarrow{h\upsilon} P_{688-676}$ transformation, namely that the ratio

$$r = \frac{\Delta H_{688}}{\Delta H_{657}}$$

of any increase of the 688 nm emission (ΔH_{688}) to the corresponding simultaneous decrease of the 657 nm emission (ΔH_{657}) remains constant throughout the transformation. The value of r varies according to working conditions, but once these conditions are fixed, r stays constant. In particular, by adjusting the experimental conditions appropriately, r may be made equal to 1 (Table 1).

The constancy of r and the occurrence of isosbestic points show that, as regards their low temperature emission, both $P_{688-676}$ and $P_{657-647}$ behave as indivisible entities one of which is produced from the other in the light. The same conclusion follows also from the kinetics of the changes of low temperature emission (SIRONVAL *et al.* 1968) and from certain other characteristic features (SIRONVAL and BROUERS 1970). $P_{688-676}$ may thus be defined empirically as "an intermediate pigment-lipoprotein complex which originates in a very short space of time from illuminated $P_{657-647}$ and behaves as a molecular unit as regards its principal low temperature emission around 688 nm", $P_{657-647}$ being "the original active pigment-lipoprotein complex found in etiolated leaves with a principal low temperature emission located around 657 nm". These definitions are sufficient as long as low temperature emission spectra of intact leaves are considered.

It is to be emphasized that:

(*a*) the definitions do not specify anything about the composition (or content) of the complexes: the latter, and especially $P_{688-676}$ may be formed by several protein subunits, each linked to several pigment molecules of different kinds, *etc.*;

(*b*) the definitions are essentially empirical since the action of light on the original $P_{657-647}$ is described as consisting in the transformation of low temperature emission. This transformation was called "phototransformation", or "spectral transformation of protochlorophyllide molecules by light" in previous papers of this series (see SIRONVAL and MICHEL 1967; SIRONVAL *et al.* 1967). Thus we have distinguished between "spectral transformation" and "reduction" of protochlorophyllide;

(*c*) the definitions deny any energy transfer from molecules belonging to $P_{657-647}$ to molecules belonging to $P_{688-676}$ during recording of low temperature emission (Appendix I).

On the basis of points (*b*) and (*c*) we are in a position to put forward two assumptions, namely: (A) that in a given volume of an etiolated leaf (whatever its dimensions) the number of spectrally transformable protochlorophyllide molecules, *i.e.* the number of molecules belonging to $P_{657-647}$ complexes (in this volume) whose low temperature emission is transformed by reaction [I] at infinite fluence* (in appropriate circumstances), is equal to the number of reducible protochlorophyllide molecules, *i.e.* to the number of molecules which are found reduced in this volume at infinite fluence (in the same circumstances). This may be written as: $C^t_{p=\infty} = C^r_{p=\infty}$ in which

* The fluence p (or "dose") is the number of photons reaching the leaf surface during a given irradiation: $p_{(photon\ cm^{-2})} = I_{(photon\ cm^{-2}\ s^{-1})} \times (t_e - t_0)_s$, t_0 being the moment when illumination begins and t_e the moment when it ends (see SIRONVAL *et al.* 1968, Appendix I).

$C^t_{p=\infty}$ and $C^r_{p=\infty}$ are the respective concentrations of spectrally transformable protochlorophyllide and reducible protochlorophyllide in the etiolated leaf. The magnitude of these concentrations is an inherent property of the etiolated leaves in the manner that we grow and collect them. In our experiments it does not depend on the conditions to which the leaves are submitted after etiolation;

(B) that after any given fluence p including zero the intensity of the 657 nm low temperature emission is directly proportional to the concentration of spectrally transformable protochlorophyllide (as defined here in Section (*1*)) which remains transformable in the leaf after fluence p.

Hereafter we refer to these assumptions as assumption (A) and assumption (B). Discussion of the experimental data will furnish arguments to prove their validity.

(*2*) Non-identity of intermediate $P_{688-676}$ with a chlorophyllide-lipoprotein complex

(a) Condition of identity

In order to decide whether the $P_{688-676}$ species as defined in Section (*1*) is identical to a chlorophyllide-lipoprotein or not, we have to compare the extent of $P_{657-647} \xrightarrow{h\upsilon} P_{688-676}$ spectral transformation with the extent of reduction.

Let x be the number of spectrally transformable protochlorophyllide molecules [in the sense of Section (*1*)] in a given volume v of an etiolated leaf; the concentration of spectrally transformable protochlorophyllide in the leaf is $x/v = C^t_{p=\infty}$. Let y_p be the number of molecules whose low temperature emission is transformed (by reaction [I]) in the same volume v after a fluence of p photons; the concentration of spectrally transformed protochlorophyllide after fluence p is $y_p/v = C^t_p$. The extent of the spectral transformation of the pigments after fluence p is defined as

$$i_p = \frac{y_p}{x} = \frac{C^t_p}{C^t_{p=\infty}}$$

Using assumption (B) from Section (*1*), i_p is found to be related to $T_p\,\%$, the empirical percentage of the spectral transformation as defined in SIRONVAL *et al.* (1968). In our working conditions $100 i_p = T_p\,\%$ (Appendix II).

Let x' be the number of reducible protochlorophyllide molecules in a given volume v of the etiolated leaf; the concentration of reducible protochlorophyllide is $x'/v = C^r_{p=\infty}$. Let y'_p be the number of molecules that are reduced in the same volume v after a fluence of p photons; the concentration of reduced protochlorophyllide after fluence p is $y'_p/v = C^r_p$. The extent of the reduction of the pigments after fluence p is defined as:

$$c_p = \frac{y'_p}{x'} = \frac{C^r_p}{C^r_{p=\infty}}.$$

c_p is the proportion of chlorophyllide in the pigment content (chlorophyllide + protochlorophyllide) of leaves exposed to fluence p, a correction being made for non-reducible protochlorophyllide. In the leaf this correction is less than 10%. It may be omitted in most cases since its exact value is not easily appreciated (magnitude of the error: $\pm 5\%$). We shall ignore this correction in the present paper (further discussion of this point will be given elsewhere).

Introducing assumption (A), the condition for identifying $P_{688-676}$ with a chlorophyllide-lipoprotein complex is given by:

$$\left(\frac{y_p}{y'_p}\right)_v = 1, \text{ or } \frac{i^n_p}{c^n_p} = 1 \qquad (1)$$

Condition (1) holds for any fluence p. The superscript n pertains to any given, definite set of experimental conditions for estimating both i_p and c_p. In particular we shall write below i_p^0 and c_p^0 for standard values of i_p^n and c_p^n obtained when the experimental conditions are: (*a*) brief irradiation of the leaf (less than 0·5 s); (*b*) immediate cooling of the leaf in liquid nitrogen and recording of its emission in liquid nitrogen; (*c*) extraction of the pigments from the irradiated volume as described in "Methods" after step (*b*) without any additional treatment of the leaf (see Section *3*). If any special treatment is included during or between steps (*a*) and (*b*), and/or (*b*) and (*c*), the corresponding i_p and c_p should accordingly be labelled i_p^1 and c_p^1; *etc.*

(b) Testing the condition of identity

We describe a first circumstance in which the i_p^n/c_p^n ratio appears essentially variable and different from unity.

Three etiolated leaves (group *A*) were irradiated for 15, 30 and 60 min in liquid nitrogen with an intense, polychromatic radiation from a Xenon lamp passing through a 6 cm water filter. This was done in a *Dewar* with a thin vertical unsilvered area. At the end of actinic irradiation the extent of spectral transformation (i_p^1) was estimated for each leaf from its low temperature emission. Immediately after recording this emission, an extract was made for each leaf separately as described in "Methods" and the extent of reduction (c_p^1) was estimated from measurements made on each extract.

A second group of three etiolated leaves (group *B*) was irradiated for 15, 30 and 60 min in the same conditions as the leaves in group *A* and the extent of spectral transformation (i_p^1) was estimated at the end of actinic irradiation from the low temperature emission of each leaf. Then the leaves were warmed in darkness and kept at −25° C in darkness for 20 min. After refreezing in liquid nitrogen the extent of spectral transformation was estimated a second time (i_p^2). Each leaf was finally extracted separately as described in "Methods" and the extent of the reduction (c_p^2) was estimated for each leaf as in group *A*.

Table 2

A situation in which the i_p^n/c_p^n ratios were essentially variable and differed from unity; explanations in the text.

Group *A*					Group *B*					
Leaf number	Duration of the irradiation* at −196 °C	$i_p^1 \times 100$	$c_p^1 \times 100$	$\frac{i_p^1}{c_p^1}$	Leaf number	Duration of the irradiation* at −196 °C	$i_p^1 \times 100$	$i_p^2 \times 100$	$c_p^2 \times 100$	$\frac{i_p^2}{c_p^2}$
1A	15 min	4.3	5.1	0.8	*1B*	15 min	3.3	29.6	4.0	7.4
2A	30 min	6.1	3.4	1.8	*2B*	30 min	6.9	30.9	5.6	5.5
3A	60 min	12.6	5.8	2.1	*3B*	60 min	8.7	35.8	5.6	6.4

* The fluence is proportional to the time, the irradiance being constant (1 840 W m^{-2} at the leaf surface; polychromatic radiation from a Xenon lamp *OSRAM XBO*, 450 W/P).

Table 2 shows that, for any fluence, the extent of spectral transformation increased in darkness after the irradiation: i_p^1 (group *A* or *B*) $< i_p^2$ (group *B*), whereas the extent of the reduction did not change markedly ($c_p^1 \cong c_p^2$). Consequently the values of the i_p^n/c_p^n ratios ranged from 1 to 8.

In general, when etiolated leaves were first irradiated from an intense Xenon source at temperatures below −100 °C and then warmed in darkness to temperatures higher than −70 to −80 °C, they behaved essentially like those in Table 2: the i_p^n/c_p^n ratio rose in darkness to assume final values different from unity. This being so, and the condition of identity being as defined in (1), we conclude that $P_{688-676}$ cannot be identical to a chlorophyllide-lipoprotein complex.

(3) Experimental relation between the extent of the $P_{657-647} \rightarrow P_{688-676}$ transformation (i_p^0) and, the corresponding extent of protochlorophyllide reduction (c_p^0)

(a) Establishing the relation

Several series of ten etiolated leaves were taken from a homogeneous set of leaves. In each series, the separate leaves received identical quantities of radiation per surface unit from a 1 ms flash through a 660 nm interference filter, at a selected temperature (*e.g.* at +23 °C; ± 1 °C). Each leaf fell in liquid nitrogen immediately after the flash using the apparatus in Fig. 1, and its low temperature emission was recorded. The extent of the $P_{657-647} \rightarrow P_{688-676}$ transformation was estimated from low temperature emission of each leaf, and the mean was calculated for each series ($= \overline{i_p^0}$). The fluence was varied from one series to another in order to produce different values of $\overline{i_p^0}$ (ranging between 0·20 and 0·95).

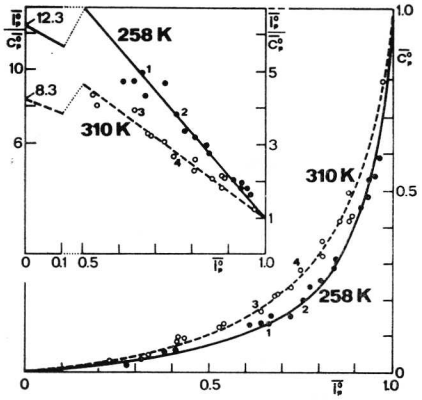

Fig. 4. Relation between the extent of phototransformation of low temperature emission (i_p^0) and the extent of reduction (c_p^0). In the insert, another plot of the results. Open circles: mean values at +37 °C; full circles: mean values at −15 °C. The lines were computed from formula (2), the values of A and K being taken from Table 3 (for the relevant temperatures; ——— = −15° C; — — — = +37 °C). All circles represent means of 10 measurements except circles *1* to *4* which are means of 25 measurements; at these points the standard deviations of i_p^0 and c_p^0 respectively were: $d \times 10^2$ = point *1*: ±0.3 and ±0.4: point *2*: ±0.4 and ±0.4; point *3*: ±0.5 and ±0.3; point *4*: ±0.3 and ±0.6.

The irradiated leaves were kept under liquid nitrogen in darkness until extraction. They were extracted separately in darkness as described in "Methods". The extract of each leaf was kept overnight in darkness at room temperature, submitted separately to centrifugation and its pigment content estimated separately. The average proportion of chlorophyllide in the pigment content of the leaves was calculated for each series ($= \overline{c_p^0}$).

Values of $\overline{c_p^0}$ are plotted against $\overline{i_p^0}$ in Fig. 4. They refer to experiments performed at +37 and −15 °C. The values found at +23 °C were near those at +37 °C and have not been reproduced on the graph. Fig. 4 shows that the ratio of $\overline{i_p^0}$ to $\overline{c_p^0}$ varied more or less linearly with the degree of spectral transformation, and approached unity in the limiting case where $\overline{i_p^0}$ and $\overline{c_p^0}$

tended to 1, *i.e.* for a fluence tending to infinity. The following empirical formula seems to fit the experimental data well:

$$(A - \overline{i_p^0})\,\overline{c_p^0} = K\overline{i_p^0} \qquad (2)$$

in which A and K are constant. Eq. (2) may be written as:

$$\frac{\overline{i_p^0}}{\overline{c_p^0}} = \frac{A}{K} - \frac{1}{K}\overline{i_p^0}$$

A and K appeared to depend on temperature as shown in Table 3.

Table 3

Temperature dependence of A and K in formula (2).

Temperature* [°C]	A	K
−15	1.089	0.089
+23	1.134	0.133
+37	1.138	0.138

* ±1 °C.

Similar results were obtained when irradiating for short periods with polychromatic light (instead of red). It was also possible to obtain relation (2) roughly inside the leaf by freezing to −196 °C immediately after short-time irradiation, recording the low temperature emission for estimating i_p^0, thawing the leaf to room temperature, then refreezing to −196 °C and rethawing several times in darkness. Repeated freezing and thawing had the effect illustrated in Fig. 5. The

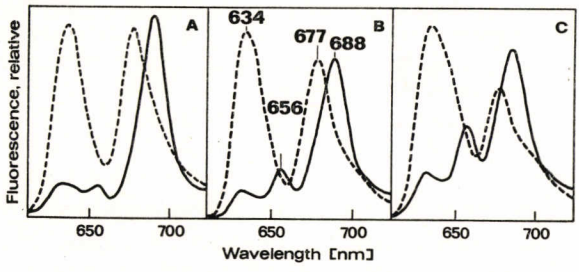

Fig. 5. Comparison of low temperature emission of three irradiated, intact leaves (continuous curves) with low temperature emission of the same leaves after 10 freezing and thawing treatments (dashed curves). All spectra recorded at −196 °C. The values of i_p^0 may be computed from the continuous curves as described in Section (*2*)(a) and Appendix II. The dashed curves give rough estimates of c_p^0. In leaf A for instance i_p^0 amounts to 0.9, whereas emission at 677 nm after freezing and thawing is half the total emission; this proportion is about equal to c_p^0 for $i_p^0 = 0.9$, as seen in Fig. 4. Similar rough estimates of c_p^0 may be made for leaves B and C.

extracted, free pigments emitted at 634 nm (protochlorophyllide) and 677 nm (chlorophyllide). Estimates of the relative chlorophyllide content from the intensities of the 634 nm and the 677 nm emissions in spectra like those in Fig. 5 agreed roughly, for the values of i_p^0 ,with the results of pigment extraction in ether.

(b) Preliminary discussion of the facts in Fig. 4

Fig. 4 shows that the $\overline{i_p^0}/\overline{c_p^0}$ ratio tended to unity for $\overline{i_p^0}$ and $\overline{c_p^0}$ tending to 1. Under assumptions (A) and (B) this means that the condition (1) of identity was satisfied at infinite fluence, when all transformable and reducible pigment molecules were spectrally transformed and reduced. On the other hand condition (1) was not satisfied elsewhere, since $\overline{i_p^0}/\overline{c_p^0}$ was found to be higher than unity at all finite fluences (for $1 > [\overline{c_p^0}$ or $\overline{i_p^0}] > 0$). Under the assumptions made, this means that the number of spectrally transformed molecules (y_p) generally exceeded the number of reduced molecules (y'_p) in a given leaf volume, or that protochlorophyllide molecules generally changed as regards low temperature emission without being reduced.

Fig. 4 also shows that $\overline{i_p^0}/\overline{c_p^0}$ ratios below unity were never found. A ratio lower than unity would indeed imply a number of spectrally transformed molecules lower than the number of reduced molecules; this situation is apparently impossible since any single protochlorophyllide must change its low temperature emission on reduction. It is therefore reasonable to admit that the inequality:

$$\frac{i_p^n}{c_p^n} \geqq 1 \tag{3}$$

must generally be verified.

(4) Separation of the light absorption act from spectral transformation and reduction; reductions of the first and of the second kind

On the basis of inequality [Eq. (3)] and assumptions (A) and (B) the following deductions will be made from experiments performed at low temperatures:

(1) pigment reduction and spectral transformation are separable from the light absorption act;

(2) there are two kinds of reduction.

(a) Reductions of the first kind

A set of four etiolated leaves was cooled to -125 °C. The leaves were irradiated separately at -125 °C (± 2 °C) with an intense, polychromatic light from a Xenon source through a 6 cm water filter. This was done in a Dewar with a thin vertical unsilvered area. After 1.5 to 3 min of irradiation, depending on the leaf, each leaf fell into liquid nitrogen. The low temperature emission of each leaf was recorded and i_p^1 values were calculated from the spectra. The leaves were then warmed in darkness from liquid nitrogen temperature to -125 °C; they remained in darkness at -125 °C for 15 min. After this time, they were cooled in liquid nitrogen a second time and i_p^2 values were estimated in each leaf from new recordings of low temperature emission. The pigments were finally extracted in ether as usual and estimates of c_p^2 values were made.

The i_p^2/c_I^2 ratios were found equal to, or somewhat higher than, unity whatever the fluence (Table 4). Keeping the leaves much longer than 15 min in darkness at -125 °C did not raise the ratios appreciably above unity.

Table 4

Evidence of the occurrence of a reduction of the first kind; explanation in the text.

Leaf number*	$i_p^1 \times 100$	$i_p^2 \times 100$	$c_p^2 \times 100$	$\frac{i_p^2}{c_p^2}$	$\frac{i_p^1}{c_p^2}$
1	11.5	17.9	13.4	1.3	0.86
2	13.5	21.2	21.5	1.0	0.63
3	15.0	29.0	19.0	1.5	0.79
4	26.9	31.7	32.5	1.0	0.85

* Increasing numbers correspond to increasing fluence. The irradiance was 1840 W m^{-2} at the leaf surface (polychromatic light from a *Xenon* lamp *OSRAM XBO*, 450 W/P). The duration of the irradiation was varied between 1.5 and 3 min in order to obtain (i_p^1)'s between 0.10 and 0.25.

However the i_p^1/c_p^2 ratios appeared lower than unity. These ratios compare, for the same fluence p, the extent of the spectral transformation seen when cooling the leaves in liquid nitrogen immediately after their irradiation (i_p^1), to the reduction found later after the irradiated leaves have been kept in darkness for a further 15 min at -125 °C (c_p^2). Since, according to Eq. (3), any i_p^1/c_p^1 ratio should always be equal to or greater than unity, it follows from the values of i_p^1/c_p^2 in Table 4 that c_p^2 exceeded c_p^1 in this experiment. We conclude that a reduction of protochlorophyllide into chlorophyllide occurred in darkness.

On the other hand since the i_p^2/c_p^2 ratios (or any other i_p^n/c_p^n) after more than 15 min in darkness at -125 °C approximate unity in Table 4, it is found, assuming the validity of both assumptions (A) and (B), that at -125 °C the reduction of a protochlorophyllide molecule corresponds to its spectral transformation. Between the statements:

α: the given protochlorophyllide molecule has been reduced;

β: the given protochlorophyllide has changed its low temperature emission;

we have at -125 °C the equivalence relation:

$$\alpha \Leftrightarrow \beta \tag{4}$$

The reduction will be termed "of the first kind" if and only if Eq. (4) is true.

As seen in Table 4 the amount of reduction of the first kind depends on the fluence. We are led to suppose: (*a*) that light absorption results primarily in the production of some quantity of an unknown, activated species; and (*b*) that lowering the temperature to -125 °C increases the time necessary for the conversion of the activated into the reduced (and spectrally transformed) product. Results like those in Table 4 were found for similar experiments performed at temperature below -125°C. Above -80°C reductions of the first kind were never separated from light absorption.

(b) Reductions of the second kind

Etiolated leaves from a homogeneous set were irradiated separately at low temperatures (Table 5). They were frozen as usual in liquid nitrogen at the end of irradiation. Their emission spectra were recorded in liquid nitrogen, i_p^1 being calculated for each leaf. Then each leaf was, either extracted as described in "Methods", c_p^1 being calculated for each leaf from measurements made on the

Table 5

Evidence of the occurrence of a reduction of the second kind; explanations in the text.

Group *A*						Group *B*							
(*1*) Irradiations at −77 °C													
Leaf number	i_p^1 ×100	c_p^1 ×100	$c_{p,c}^1$ ×100	$\frac{i_p^1}{c_p^1}$	$\frac{i_p^1}{c_{p,c}^1}$	Leaf number	i_p^1 ×100	$c_{p,c}^1$ ×100	i_p^2 ×100	c_p^2 ×100	$c_{p,c}^2$ ×100	$\frac{i_p^2}{c_p^2}$	$\frac{i_p^2}{c_{p,c}^2}$
1 A	6.1	0.9	0.5	6.8	12.2	*1 B**	5.5	0.5	23.1	3.2	2.4	7.2	9.6
2 A	28.2	3.8	3.1	7.4	9.0	*2 B*	22.1	2.3	50.5	7.4	7.7	6.8	6.5
3 A	56.8	9.1	9.8	6.2	5.8	*3 B*	35.0	4.2	64.5	19.0	12.9	3.4	5.0
4 A	97.8	64.0	75.0	1.5	1.3	*4 B*	49.6	7.4	77.9	26.0	22.2	3.0	3.5
(*2*) Irradiations at −95 °C													
*1 A**	17.4	8.5	1.7	2.0	10.2	*1 B**	20.8	2.2	57.1	10.4	10.0	5.5	5.7
2 A	47.4	34.0	6.9	1.4	6.8	*2 B*	39.5	5.1	82.6	26.0	27.5	3.2	3.0
3 A	63.1	45.0	12.2	1.4	5.1	*3 B*	55.0	9.0	85.1	30.0	31.5	2.8	2.7
4 A	70.0	42.0	15.9	1.6	4.4	*4 B*	71.1	17.3	87.1	35.0	35.0	2.5	2.5
5 A	81.4	57.5	26.2	1.4	2.1	*5 B*	91.1	45.5	97.8	67.0	78.0	1.5	1.2

* Increasing numbers mean increasing fluence. The duration of the irradiation was 30 s at −77 °C and 60 s at −95 °C. The different values of i_p^1 were obtained placing the source of radiation at different distances from the leaf. The irradiance was 1 840 W m^{-2} at 18.5 cm from the point of focus formed by the optical device surrounding the Xenon lamp (*OSRAM XBO*, 450 W/P).

extracts (group *A*), or warmed in darkness to −25 °C (group *B*). In group *B*, the leaves remained at −25 °C in darkness for 15 min before being recooled in liquid nitrogen for a further recording of low temperature emission, from which i_p^2 was calculated. Finally each leaf in group *B* was extracted in ether as usual and c_p^2 was calculated from measurements made on the extracts. The same experiment was made: (*1*) at −95 °C, and (*2*) at −77 °C. The results are shown in Table 5.

In order to make the evidence clearer we calculated $c_{p,c}^1$ and $c_{p,c}^2$ from the experimental i_p^1 and i_p^2, assuming the validity of expression (2). The $c_{p,c}^2$ were near to the corresponding experimental c_p^2, when irradiations occurred at −77 °C or at −95 °C. But $c_{p,c}^1$ agreed with the corresponding experimental c_p^1 at −77 °C only. In other words:

(*a*) when irradiating at −95 °C, the i_p^1/c_p^1 were found to be different from the $i_p^1/c_{p,c}^1$ ratios, whereas the i_p^2/c_p^2 were similar to the $i_p^2/c_{p,c}^2$; from this, we see that i_p^2/c_p^2, but not i_p^1/c_p^1, behaved according to expression (2);

(*b*) when irradiating at −77 °C, both experimental i_p^1/c_p^1 and i_p^2/c_p^2 ratios appeared to conform to expression (2).

This behaviour is interpreted as follows:

(*1*) All i_p^1/c_p^1 values at the end of irradiation at −95 °C in group *A* were near unity; this shows that, when irradiating at −95 °C, the reductions were essentially of the first kind. Something happened however in darkness after raising the temperature from −95 °C up to −25 °C in group *B:* some protochlorophyllide molecules changed spectra without being reduced (*cf.* Table 2).

(*2*) But at −77 °C, reductions of the first kind were not seen. At this temperature, since in group *A* c_p^1 and $c_{p,c}^1$ values were found nearly equal, the same must be true in group *B*. If we introduce the $c_{p,c}^1$ values into group *B* instead of the missing c_p^1, the difference $[c_p^2$ (or $c_{p,c}^2) - c_{p,c}^1]$ gives the amount of dark reduction which occurred after the temperature was raised from −77 °C up to −25 °C, while the difference $[i_p^2 - i_p^1]$ gives the amount of corresponding dark spectral transformation.

Assuming again the validity of assumptions (A) and (B), it may be seen that at −77 °C the reductions did not take place simultaneously with the spectral transformation but followed it, since i_p^n/c_p^n ratios higher than 1 were found. Between statements α and β in Section (*4*)(a), we now have the relation:

$$\alpha \rightarrow \beta \qquad (5)$$

instead of Eq. (4). The reduction will be termed "of the second kind" if and only if Eq. (5) is true.

DISCUSSION

I. In Section (*3*)(b) we concluded from our discussion of the data in Fig. 4 that, if assumptions (A) and (B) are true, the number of spectrally transformed molecules (y_p) exceeds the number of reduced molecules (y_p') in the leaf at any time of spectral transformation, except infinity. This conclusion applies to any leaf volume whatever its dimensions. Consequently, the pigments inside the intermediate $P_{688-676}$ molecular entity should consist of a mixture of chlorophyllide and protochlorophyllide. This deduction has been verified as follows:

If the pigments actually are a mixture in $P_{688-676}$, since $P_{688-676}$ behaves as a molecular unit as regards its low temperature emission as seen in Section (*1*), some kind of cooperation must be required between protochlorophyllide and chlorophyllide molecules for this emission. It might be imagined, *e.g.*, that inside one $P_{688-676}$ unit, chlorophyllide traps energy from neighbouring excited protochlorophyllide belonging to the same unit* and emits the 688 nm

* We already stressed in Section (*1*) that per definition transfer processes do not occur from $P_{657-647}$ to $P_{688-676}$, but transfers inside one $P_{688-676}$ unit are allowed (see Appendix I).

fluorescence. This was shown to be the case. Excitation spectra and some other experimental data (BROUERS, KUYPER and SIRONVAL 1972) proved: (*1*) that *in vivo* a 676—678 nm absorbing pigment emitted the fluorescence from the $P_{688-676}$ unit; (*2*) that the concentration of this pigment in the leaf varied with the concentration of chlorophyllide in the ether extracts; and (*3*) that at liquid nitrogen temperature pigments absorbing around 637 to 647 nm (protochlorophyllide) transferred energy inside the unit to the 676—678 nm absorbing pigment. Energy transfer from protochlorophyllide to chlorophyllide at −196 °C was also demonstrated by KAHN, BOARDMAN and THORNE (1970).

We are thus led by the data: (*1*) to accept assumptions (A) and (B) [Section (*1*)] as true; and (*2*) to amplify the definition of the $P_{688-676}$ unit in Section (*1*) by adding that:

"The intermediate $P_{688-676}$ molecular unit contains (n) pigment molecules of which (m) molecules absorb in the red at 676—678 nm and ($n-m$) molecules absorb in the red at 647 and 637 nm, (m) being an integer such as $0 < m \ll n$. At low temperature the ($n - m$) 647 and 637 nm absorbing pigment molecules transfer energy to the (m) 676—678 nm absorbing pigment molecules which are the only radiation emitting molecules of the unit". By this definition, a single molecule of the $P_{688-676}$ complex appears essentially as a kind of "energy transfer unit".

II. The definition of $P_{688-676}$ implies the following situation: (*a*) on the one hand, as is easily deduced from assumption (B) and from the constancy of r [Section (*1*)], the transformation of the low temperature emission of a number L of pigment molecules at the start, or end of exposure to radiation corresponds to an identical increase in 688 nm emission; (*b*) on the other hand, given an identical 688 nm increase at the beginning or end of exposure, L should consist of a mixture of protochlorophyllide and chlorophyllide molecules whose composition varies according to expression (2), the proportion of chlorophyllide increasing considerably between the beginning and end of spectral transformation. Comparing (*a*) to (*b*), and since chlorophyllide is the light emitter, it is seen that, when exciting protochlorophyllide and chlorophyllide simultaneously (*e.g.*, in the blue) the quantity of photons emitted per chlorophyllide molecule at liquid nitrogen temperature should decrease when the extent of the spectral transformation increases.

We have been able to demonstrate this decrease in the leaf (BROUERS, KUYPER and SIRONVAL 1972). On the other hand, the results of KAHN, BOARDMAN and THORNE (1970) clearly established the decrease using preparations of pigment-lipoprotein complexes (measurements of emission at liquid nitrogen temperature). These results again confirm the validity of both assumptions (A) and (B). We shall therefore no longer refer to these assumptions.

A decrease in fluorescence emitted by chlorophyllide per unit absorbance was also seen by SCHULTZ (1970) when illuminating preparations of purified pigment-lipoprotein complex at +7 °C. SCHULTZ thinks that "the most reasonable explanation of the decrease would appear to be concentration quenching of Chlide [chlorophyllide] fluorescence, such as one finds for Chl [chlorophyll] solutions in solvents favouring its aggregation". He argues that, if it is assumed that at +7 °C protochlorophyllide uses almost all the absorbed energy for its own phototransformation, energy transfer is unlikely.

III. We have inferred in Section (*4*) from low temperature experiments that two kinds of reductions are distinguishable. This is also true at higher temperatures including room temperature as shown by the two following arguments:

(*a*) It is derived from expression (2) that at the start of spectral transformation each of the first $P_{688-676}$ units comprises 1 chlorophyllide and ($n - 1$) protochlorophyllide molecules (SIRONVAL 1972). Afterwards the ($n - 1$) protochlorophyllide molecules are reduced inside the units as spectral transformation proceeds. The initial reduction of 1 chlorophyllide per 1 unit is a reduction of "the first kind" since, when dealing with this particular chlorophyllide, relation (4) is obviously true. Later reductions inside the unit belong to "the second kind" since relation (5) replaces (4) in this case.

(*b*) We showed earlier that the principal low temperature emission of $P_{688-676}$ was around 685 nm at the start of the spectral transformation and that it shifted to 690 nm during the transformation (688 nm is a mean value; see Fig. 3 in SIRONVAL *et al.* 1968). LITVIN and BELYAEVA (1971) describe this as reflecting a succession of two photoreactions: in a first step photoreaction I produces "$Chl_{684-676}$" from $P_{657-647}$ (= $P_{655-650}$ in LITVIN and BELYAEVA); in a second step photoreaction II produces "$Chl_{690-680}$" from "$Chl_{684-676}$". "$Chl_{684-676}$" thus prevails temporarily at the start of irradiation while "$Chl_{690-680}$" is the product found at the end.

LITVIN and BELYAEVA (1971) give evidence that, contrary to "$Chl_{690-680}$", "$Chl_{684-676}$" transforms itself in darkness at room temperature within 1 to 3 min into "$Chl_{675-670}$", a chlorophyll(ide) form which is not otherwise found as a single product in the leaf. They suggest that:

(*1*) either "$Chl_{684-676}$" consists of a "half-reduced form or a stabilized radical" which is transformed (reduced?) in darkness in the cell, or during extraction, into "$Chl_{675-670}$"; (*2*) or "$Chl_{684-676}$" is the product of "complete reduction of a portion of precursor molecules [= = $P_{657-647}$] leading to the formation of a mixed aggregate (protochlorophyllide + chlorophyllide)" after photoreaction I; in this case "photoreaction II accomplishes the reduction of aggregate molecules", producing "$Chl_{690-680}$".

It is easily seen from the above discussion that suggestion (*2*) contains a good deal of truth: "photoreactions I and II" apparently correspond respectively to reductions of the first and second kind which prevail successively in the course of spectral transformation at room temperature. The same conclusion holds for the interpretation of the data of THORNE (1971).

IV. The experiment in Section (*2*) (b) (Table 2) and other similar experiments show that leaves in which reductions of the first kind have been produced at a temperature below −100 °C undergo a marked change of low temperature emission when warmed in darkness to a temperature above −80 °C. Comparing group *A* to group *B* in Table 2 it is seen that the spectral change is due to the appearance of energy transfer units. This was not understood by RUBIN *et al.* (1962) who already described this change.

The formation of transfer units depends on two conditions: (*1*) at least one chlorophyllide molecule must be available ; (*2*) some arrangement must be produced which enables energy transfer to this chlorophyllide. Condition (*2*) is met in darkness when the temperature is raised after intense low temperature irradiation satisfying condition (*1*) (Table 2, −196 °C irradiation, group *B*; and Table 5, −95 °C irradiation, group *B*). Obviously, the temperature rise permits the necessary arrangement; when the temperature is raised the pigments assume a geometrical pattern different from the one found initially in the etiolated leaf, and this pattern permits energy transfer to chlorophyll(ide). This implies some transconformation of the lipoprotein as soon as the temperature climbs sufficiently high after the absorption of light. Table 5, group *A*, shows that the temperature limit for this process lies between −80 and −90 °C (see GOEDHEER and VERHÜLSDONK 1970, for related observations).

To sum up, we may write reaction [I] as:

$$Pr_{657-647} \xrightarrow{h\nu} P_{688-676} \quad \text{(scheme, Fig. 6)}$$

in which $Pr_{657-647}$ is the initial protochlorophyllide-lipoprotein complex and $P_{688-676}$ refers to energy transfer units. Reaction [I] appears to comprise *in vivo* several steps, one of which plays a central role which we have been able to distinguish (step *2* in the scheme, Fig. 6). If *A* describes the initial state of the lipoprotein complex in the etiolated leaf (in $Pr_{657-647}$) this step consists in a change from *A* into another state, *B*:

$$A \rightarrow B$$

The state transformation is triggered off by absorption of radiation in some unknown way.

It enables energy to be transferred from protochlorophyllide to chlorophyllide as a result of the transformation of the protein moiety of the pigment-lipoprotein complex. Before the transconformation, reductions are of the first kind. Afterwards, reductions of the second kind occur. Two kinds of reductions thus follow in sequence as shown by LITVIN and BELYAEVA (1968, 1971) and by THORNE (1971).

How light absorption induces the reductions in reaction [I] is the key problem. As far as we can see: *a* — there exists some unknown step (X) between absorption of radiation and the reductions since the reductions appear to be separable from absorption of radiation; *b* — the situation after the protein transconformation essentially differs from the one existing before.

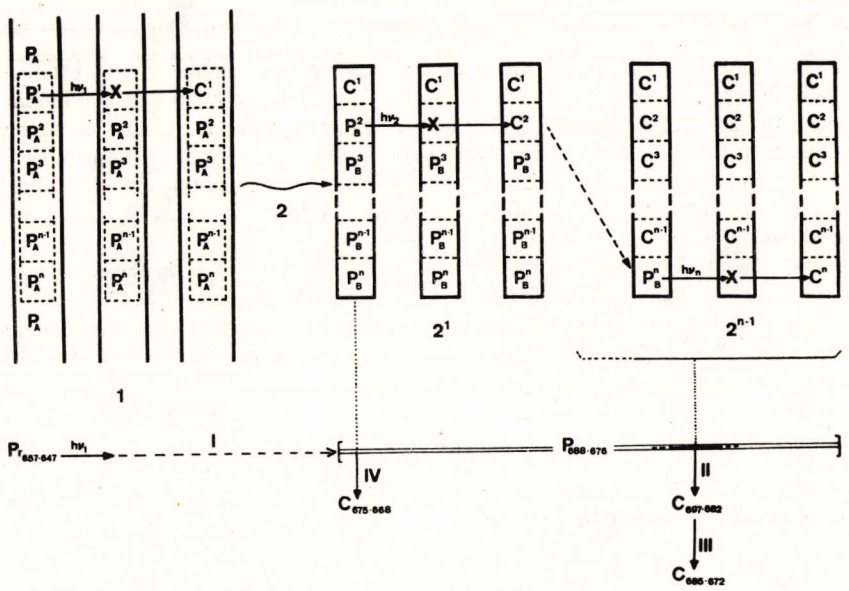

Fig. 6. Position of $P_{688-676}$ in the pathways leading from protochlorophyll(ide) to chlorophyll(ide) *a* forms *in vivo*. Tentative scheme. Above left a portion of a prolamellar body is symbolized by heavy vertical lines. Some protochlorophyllide-lipoprotein complexes in state $A(P_A)$ are seen. The absorption of a photon first creates in one complex some unknown state x. A reduction of the first kind ($P_A^1 \rightarrow C^1$; step 1) follows. In addition, or by virtue of this reduction a transconformation of the lipoprotein occurs (step 2); some neighbour protein complexes go from state $A(P_A)$ to state $B(P_B)$. This leads to the production of an energy transfer unit. Reductions of the second kind occur inside this unit ($P_B^2 \rightarrow C^2$, etc; steps $2^1, \ldots, 2^{n-1}$). The sequential relationships between the steps involved in reaction [I] and the subsequent dark steps (reactions [II], [III] and [IV]) are shown at the bottom of the scheme. Note that the scheme does not say anything about the changes of the protein state during reactions [II], [III] and [IV].

On the other hand, each kind of reduction is followed by dark step(s) leading to the accumulation in the leaf at room temperature of a specific stable product: the first kind of reduction results in the accumulation of $C_{675-668}$ through reaction IV (scheme, Fig. 6); the second kind in the accumulation of $C_{685-672}$ through reaction II, giving $C_{697-682}$, and reaction III (scheme, Fig. 6). It may be presumed that these chlorophyll(ide)s are (*a*) forms normally found in green leaves with red absorptions respectively at 668, 672 and 682—684 nm (FRENCH *et al.* 1968).

 In this respect, the scheme, Fig. 6 illustrates the pathways of the biosynthesis of chlorophyll (*a*) forms *in vivo*, the common intermediate precursor of which is $P_{688-676}$.

It is clear that the state of the protein moiety of the pigment-lipoprotein complex plays a prominent role both in the reduction acts and in the subsequent events. Generally speaking, we are faced with a situation which would be expected if the complex behaved as a photoenzyme. This comes as no surprise since certain other properties of the complex point to precisely the same conclusion (we have discussed this general idea elsewhere; SIRONVAL 1971; see also SÜZER and SAUER 1971).

APPENDIX

I. At different moments of a reaction ($A \rightarrow B$) in the course of which one species is transformed irreversibly into another, we excite at a temperature of -196 °C the fluorescence of the mixtures of the species involved. An isosbestic point appears by superposing the spectra of fluorescence corresponding to the moments chosen. This circumstance, together with certain others, leads us to define A and B by the properties of their emission at low temperature. We shall show that any transfer of electronic excitation energy from species A to species B, as defined in this way, is excluded per definition.

In general, the energy emitted by species A at the isosbestic point λ_i, in the $\Delta\lambda_i$ layer of the spectra, is a defined fraction Φ^A of the excitation energy at the disposal of A. If A transfers part of this energy to B, the energy at its disposal for emission is reduced by the same amount, and the energy emitted by A in the layer $\Delta\lambda_i$ is, per cm^3 and per s, at time t of the reaction ($A \rightarrow B$):

$$E^A_{e,t} = \left[E^A_a - e^{A\rightarrow B}\right] \quad \Phi^A \quad C^A_t \tag{1}$$

C^A_t being the concentration of A [mol cm^{-3}] at time t, E^A_a the energy absorbed by A (per mol of A and per s), and $e^{A\rightarrow B}$ the energy transmitted from A to B (per mol of A and per s). Similarly, the energy emitted by species B in the layer $\Delta\lambda_i$ per cm^3 and per s at time t is:

$$E^B_{e,t} = \left[E^B_a + e'^{A\rightarrow B}\right] \quad \Phi^B \quad C^B_t \tag{2}$$

where C^B_t is the concentration of B [mol cm^{-3}] at time t, E^B_a the energy absorbed by B (per mol of B and per s), $e^{A\rightarrow B}$ the energy received by B as a result of the transfer (per mol of B and per s), and Φ^B the fraction of energy at B's disposal emitted in the layer $\Delta\lambda_i$. Since A is transformed into B,

$$C^A_t + C^B_t = c \tag{3}$$

whatever the value of t. At time $t = 0$, $C^B_t = 0$ and $C^A_t = c$. The transfer is then impossible and the energy emitted by A in the layer $\Delta\lambda_i$ has the value:

$$E^A_{e,t=0} = E^A_a \Phi^A c = E_e \tag{4}$$

Similarly at time $t = \infty$, $C^A_{t=\infty} = 0$ and $C^B_{t=\infty} = c$. The transfer is again impossible and the energy emitted by B in the layer $\triangle\lambda_i$ is equal to:

$$E^B_{e,t=\infty} = E^B_a \Phi^B c = E_e \tag{5}$$

This energy is the same as that emitted by A at time t_0 since the layer $\Delta\lambda_i$ is situated at the isosbestic point λ_i. It is thus found that:

$$E^A_a \Phi^A = E^B_a \Phi^B \tag{6}$$

On the other hand, the energy emitted in layer $\Delta\lambda_i$ is, at any moment in time:

$$E_{e,t} = E^A_{e,t} + E^B_{e,t},$$

either by introducing Eqs. (1) and (2):

$$E_{e,t} = \left[E^A_a - e^{A\to B}\right] \Phi^A C^A_t + \left[E^B_a + e'^{A\to B}\right] \Phi^B C^B_t$$

which becomes (taking Eqs. (3) to (6) into account):

$$E_{e,t} = E_e - e^{A\to B}\Phi^A C^A_t + e'^{A\to B}\Phi^B C^B_t \tag{7}$$

The isosbesticity at wavelength λ_i requires that $E_{e,t}$ be constant, and, according to Eqs. (4) and (5), equal to E_e; this means that the equation:

$$e^{A\to B}\Phi^A C^A_t = e'^{A\to B}\Phi^B C^B_t \tag{8}$$

is the condition of isosbesticity of emission at wavelength λ_i. The left-hand side of equation (8) is the energy which, because of the transfer from A to B, is not emitted per s in layer $\Delta\lambda_i$ from the total number of A molecules contained in a cm^3. The right-hand side of the equation is the energy emitted in layer $\Delta\lambda_i$ by the total number of B molecules (per cm^3 and per s) as a result of the transfer. According to Eq. (8), if there is a transfer of electronic excitation energy from A to B, the existence of an isosbestic point λ_i requires that the energy not emitted by A in layer $\Delta\lambda_i$ be re-emitted by B in its entirety in the same layer. We shall show that, since species A and B are defined by the properties of their emission, this amounts to excluding any transfer from A to B and to admitting that a definite number of A molecules have been transformed into B molecules and may be described as such.

Let us suppose per definition that, at time t of the reaction ($A \to B$), there exists a number of B molecules in concentration $+x^B_t$ [mol cm^{-3}] which absorb from the surroundings and emit, per s in layer $\Delta\lambda_i$, the equivalent of the energy not emitted per s in this layer at the same time t, by A as a result of the transfer. Thus:

$$E^B_a \Phi^B x^B_t - e^{A\to B}\Phi^A C^A_t = 0$$

Similarly let us now suppose per definition that at time t a number of A molecules in concentration $-x^A_t$ [mol cm^{-3}] are lacking, and do not therefore emit, per s in layer $\Delta\lambda_i$, the equivalent of the energy emitted in this layer by B as a result of the transfer (per s and at the same time t). Thus:

$$e'^{A\to B}\Phi^B C^B_t - E^A_a \Phi^A x^A_t = 0$$

In this case, the concentrations $(-x^A_t)$ and $(+x^B_t)$ of the molecules which we suppose are missing in A and appearing in B are necessarily equal because if they were not so, we would have the following inequality:

$$\frac{e^{A\to B}\Phi^A C^A_t}{E^B_a \Phi^B} \neq \frac{e'^{A\to B}\Phi^B C^B_t}{E^A_a \Phi^A}$$

which, bearing in mind Eq. (6), contradicts the condition (8) of isosbesticity of the emission at the wavelength λ_i. Thus, if the spectra of emission of the mixtures of A and B obtained at various moments of the transformation ($A \to B$) have an isosbestic point λ_i, the decision to define these species as emitting entities precludes *per se* any transfer of energy between them.

274 **II.** The percentage of spectral transformation ($T\%$) of the spectrally transformable pigment in a leaf sample was defined empirically as:

$$T_p\,\% = \frac{\bar{H}_{p,688}/r}{\bar{H}_{p,657} + \bar{H}_{p,688}/r} \times 100 \qquad (1)$$

in which r is the ratio $\Delta\bar{H}_{688}/\Delta\bar{H}_{657}$ between the increase of the 688 nm emission and the decrease of the 657 nm emission, $\bar{H}_{p,657}$ and $\bar{H}_{p,688}$ being the corrected intensities of the low temperature emission of the leaf sample at 657 and 688 nm respectively (measured in a given, constant set of physical conditions for excitation and reception) after exposure to fluence p. In our working conditions r is very near unity and may be omitted in the calculation of $T_p(\%)$ (SIRONVAL *et al.* 1968).

Assumption (B) [Section (*1*)] says that the corrected low temperature emission at 657 nm after the fluence p is:

$$\bar{H}_{p,657} = \alpha(x - y_p) \qquad (2)$$

α being a proportionality factor, x the number of spectrally transformable protochlorophyllide molecules in the leaf sample and y_p the number of spectrally transformed molecules. Since r is constant, the emission at 688 nm after fluence $p(\bar{H}_{p,688})$ is proportional to the number of spectrally transformed protochlorophyllide molecules:

$$\bar{H}_{p,688} = \beta(y_p) \qquad (3)$$

in which $\beta = r\alpha$.

By incorporating Eqs. (2) and (3) in Eq. (1) we see that

$$T_p(\%) = 100\, i_p$$

since $i_p = y_p/x$ [Section (*2*) (*a*)].

REFERENCES

BONNER, B. A.: A short-lived intermediate form in the *in vivo* conversion of protochlorophyllide 650 to chlorophyllide 684. — Plant Physiol. **44**: 739—747, 1969.

BROUERS, M., KUYPER, Y., SIRONVAL, C.: The reduction of protochlorophyllide into chlorophyllide. V. Demonstration of energy transfer inside the $P_{688-676}$ molecular units. — Photosynthetica **6**: 169—176, 1972.

DUJARDIN, E., SIRONVAL, C.: The reduction of protochlorophyllide into chlorophyllide. III. The phototransformability of the forms of the protochlorophyllide-lipoprotein complex found in darkness. — Photosynthetica **4**: 129—138, 1970.

FRENCH, C. S.: The chlorophylls *in vivo* and *in vitro*. — In: RUHLAND, W. (ed.): Handbuch der Pflanzenphysiologie. Vol. V/1. Pp. 252—297. Springer - Verlag, Berlin—Göttingen—Heidelberg 1960.

FRENCH, C. S., MICHEL-WOLWERTZ, M. R., MICHEL, J. M., BROWN, J. S., PRAGER, L. K.: Naturally occurring chlorophyll types and their function in photosynthesis. — In: GOODWIN, T. W. (ed.): Porphyrins and Related Compounds. Pp. 147—162. Academic Press, London—New York 1968.

GASSMAN, M., GRANICK, S., MAUZERALL, D.: A rapid spectral change in etiolated red kidney bean leaves following phototransformation of protochlorophyllide. — Biochem. biophys. Res. Commun. **32**: 295—300, 1968.

GOEDHEER, J. C., VERHÜLSDONK, C. A. H.: Fluorescence and phototransformation of protochlorophyll with etiolated bean leaves from −196 to +20 °C. — Biochem. biophys. Res. Commun. **39**: 260—266, 1970.

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.

LITVIN, F. F., BELYAEVA, O. B.: Issledovanie fotokhimicheskikh reaktsiĭ biosinteza khlorofilla. [Investigation of photochemical reactions of chlorophyll biosynthesis.] — Biokhimiya **33**: 928—936, 1968.

LITVIN, F. F., BELYAEVA, O. B.: Sequence of photochemical and dark reactions in the terminal stage of chlorophyll biosynthesis. — Photosynthetica **5**: 200—209, 1971.

RUBIN, A. B., MINCHENKOVA, L. E., KRASNOVSKIĬ, A. A., TUMERMAN, L. A.: Izuchenie sredneĭ dlitel'nosti fluorestsentsii protokhlorofilla v protsesse zeleneniya etiolirovannykh list'ev. [Study on the fluorescence life-time of protochlorophyll during the process of greening of etiolated leaves.] — Biofizika **7**: 571—577, 1962.

SCHULTZ, A. J.: The development and organization of photosynthetic pigment systems. — Thesis, Lawrence Radiation Laboratory, University of California, Berkeley 1970.

SHIBATA, K.: Spectroscopic studies on chlorophyll formation in intact leaves. — J. Biochem. (Tokyo) **44**: 147—173, 1957.

SIRONVAL, C.: The evolution of chlorophyll containing photoactive structures. — In: SCHOFFENIELS, E. (ed.): Chemical Evolution and the Origin of Life. Pp. 236—258. North-Holland Publ. Co., Amsterdam 1971.

SIRONVAL, C.: The reduction of protochlorophyllide into chlorophyllide. VI. Calculation of the size of the transfer unit and the initial quantum yield of the reduction *in vivo*. — Photosynthetica **6** (4): in press, 1972.

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., 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, Yugoslavia 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. **6**: 43—50, 1967.

SÜZER, S., SAUER, K.: The sites of photoconversion of protochlorophyllide to chlorophyllide in barley seedlings. — Plant Physiol. **48**: 60—63, 1971.

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