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The Reduction of Protochlorophyllide into Chlorophyllide.

I. The Kinetics of the $P_{657-647} \rightarrow P_{688-676}$ Phototransformation*

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Abstract

The action of light on the protochlorophyllide-protein complex was studied in intact, etiolated leaves using low-temperature spectrofluorometry and short-time illuminations (less than 1 s to 1 ms). The first spectral changes observed were shown to correspond to the photoproduction within very short times of a particular pigment form, different from the chlorophyllide-lipoprotein complex, which was called $P_{688-676}$. The kinetics of these changes was found to be of the first order when 647 nm photons (or photons of longer wavelengths) were used; it was not of the first order when 630 nm photons (or photons of shorter wavelengths) were used. A plausible preliminary model involving two kinds of lipoprotein-protochlorophyllide complexes, one of which receives light energy from an accessory pigment, is proposed to account for the experimental facts.

SMITH and BENITEZ (1954) concluded that in etiolated barley the photoreduction of protochlorophyllide into chlorophyllide was not strictly photochemical and that it involved intermolecular interactions, the nature of which was unknown. Working with a protochlorophyllide-lipoprotein complex from etiolated bean leaves, BOARDMAN (1962) came to a somewhat different conclusion. He found that, although not purely photochemical, the process did not involve a collision between two independent molecules. Both authors agree that the kinetics of the photoreduction was not a first order one and that the rate of the conversion was dependent on temperature. BOARDMAN proposed three models in order to explain the kinetics data, but he was unable to decide between them.

We recently obtained evidence indicating that the reduction of protochlorophyllide into chlorophyllide was indeed a rather complicated process involving at least two steps. A first step occurs as the result of photon absorption by pigment(s); it is followed by another step which leads to the reduced product. We previously summarized the experimental evidence and the preliminary conclusions to be derived (SIRONVAL and MICHEL 1967; SIRONVAL *et al.* 1967). A somewhat more extensive and more exact treatment (although still preliminary) is given in SIRONVAL *et al.* (1968).

The present paper deals in detail with some properties of the first, light dependent, step. This step in some way modifies the protochlorophyllide-lipoprotein complex; it produces an optical shift, the characteristics of which are described. The effects of temperature on the light step, as well as other information on the overall process of protochlorophyllide reduction will be given in subsequent papers.

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The separation of the events into several steps was obtained by performing the light reaction within very short times (from 1 ms to less than 0·5 s) and by freezing the material in liquid nitrogen immediately after irradiation. Preliminary experiments showed that the rate of the optical shift depended essentially on the number of photons I reaching the sample per unit time and unit area (light intensity = $= I_{einstein\ s^{-1}\ cm^{-2}}$). Using very high values of I it was possible to complete the shift in times much shorter than 1 ms. For convenience, one ms "saturating" flashes are used in the present paper as a standard procedure for performing the complete phototransformation within very short time intervals. The use of 1 ms flashes for the phototransformation of protochlorophyllide was introduced by MADSEN (1963).

MATERIAL AND METHODS

Leaf material: Beans were sown in complete darkness at 23°C ± 2 in pots containing vermiculite moistened with tap water. Germination generally occurred 2 days after sowing. The two primary etiolated leaves were used between the 20th and the 30th day after germination. All manipulations

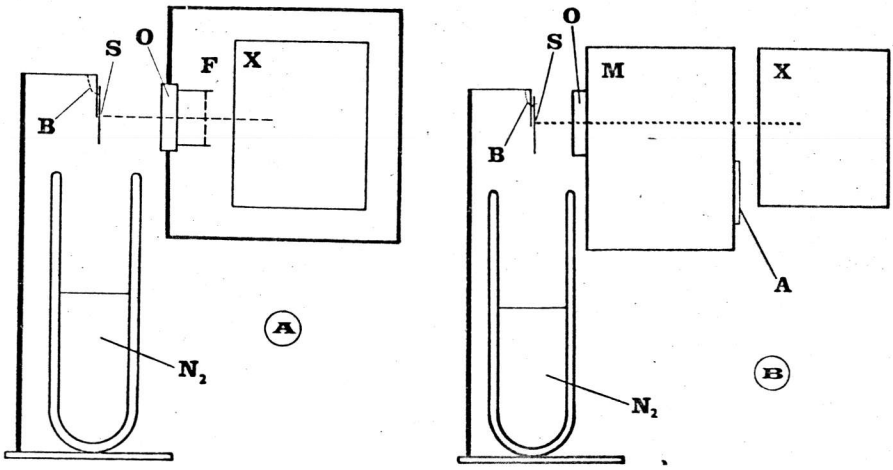

Fig. 1. The devices *A* and *B* used for sample illumination (explanations in the text). A glass infrared filter and a layer of water were placed between the filter *F* and the Xenon lamp *X* (not drawn on Fig. 1A) — air being continuously blown around the Xenon lamp and the filters. The lamp itself was stabilized by the standard *Zeiss* stabilizing device.

for growing the plants or preparing the samples for the illumination occurred in the presence of a green safe light of low intensity and consisting of an *Osram 4543* lamp surrounded by a neutral filter plus green cellophane (maximum transmission at 520 nm). It was verified that this light had no effect on the spectroscopic properties of the etiolated leaves. Only mature leaves were used in the experiments reported here. Such leaves are characterized by a low temperature emission at 657 nm 5 to 7 times more intense than the corresponding emission at 630 nm (see Figs. 4, 5, 7 and 8).

Sample illumination: The experimental devices for leaf illumination are shown in Fig. 1A and B. The light was provided by a Xenonlamp *X* (*Osram XBO 450 W/P*) especially adapted by *Zeiss Oberkochen*, Germany (*LX 501* device) for use with the monochromator *M* as in Fig. 1B. *M* was

a double prism monochromator (*Zeiss MM 12*) which gave a bandwidth of the order of 10 nm in the 650 nm region for a slitwidth of 0·9 mm, the intensity of the light being of the order of $I = 100$ to $150 \cdot 10^{-11}$ einstein s^{-1} cm^{-2} at 5 cm from the exit of the monochromator. The monochromator could be replaced by interference filters (*F* in Fig. 1A). In this case, 10 times higher light intensities were obtained.

A photographic shutter *O* was placed at the exit of the monochromator or in front of *F*. It could be opened during times ranging from 0·002 to 0·881 s. The times were calibrated with an oscilloscope. The sample (a leaf or a half-leaf) was placed at *S* on a plate of metal covered with black paper. *B* could be moved away by a magnet, liberating the sampleholder *S*. The fall of *S* in liquid nitrogen N_2 was regulated in such a way that it occurred at the very end of the irradiation.

When the monochromator *M* was used (Fig. 1B), *S* was placed about 5 cm away from *O*, and the illuminated area was a rectangle of 0·5 × 1·2 cm. It exceeded this surface when the monochromator was removed (Fig. 1A).

Electronic flash: For illumination with high light intensities during very short times, the Xenon lamp of Fig. 1A was replaced by an electronic flash (*Multiblitz 50* from *Gesellschaft für Multiblitzgeräte, Dr. Ing. Mannesmann*, Porz-Westhoven, Germany). The shutter *O* was removed and replaced by the filter *F*. The flash was placed as near the sample as possible, against the filter. Some experiments were performed without any filter; as a rule, this was the case when a complete phototransformation was required. The sample fell into liquid nitrogen at the very end of the flash.

Temperature during illumination: When not otherwise indicated, all illuminations were performed at a temperature of 23°C ± 2.

Characteristics of the filters: Two of the interference filters were constructed by *Barr and Stroud Ltd*, England. The transmission peaks of these filters were centred at 630 and 646·5 nm respectively, the bandwidth at half peak being of the order of 3·0—3·5 nm; the transmission at the peak maximum was 70% for both filters. The *Barr* and *Stroud* filters were not completely blocked in the infrared. The infrared light was removed, when necessary, by interference filters constructed by *Baird Atomic Europe*, Holland. The transmission peaks of the *Baird* filters were centred at 630 and 647 nm, the bandwidth at half peak being of 4·2 nm, and the transmission at the peak maxima being of the order of 70%; the transmission outside the passband was less than 0·1%. In addition, an ordinary infrared filter was placed in front of the Xenon lamp. In the flash experiments a *Baird Atomic* 660 nm filter was sometimes used (bandwidth at half peak 4·2 nm; transmission at the peak maximum about 70%).

Registration of the low-temperature fluorescence spectra: The excitation light was provided by a mercury lamp *E* equipped with a *Zeiss* filter (n° C) selecting the 434 to 436 nm photons (Fig. 2). The light was reflected by a mirror *G* on the sample holder *SH* in the *Dewar D*. The fluorescence was registered through the grating *Zeiss* monochromator *M 19* equipped with a filter for removing the light below 600 nm. The photomultiplier *PM* was either a *Dumont 6911* or a *Philips XP 1005* cooled to liquid nitrogen temperature.

The leaf sample was placed behind the hole *S* on a plate *P* which was adjustable in the sample holder *SH* as shown on the right part of Fig. 2. *SH* could be moved either horizontally or vertically inside the *Dewar* using the screw *Q*. In this way, the orientation of the sample in the *Dewar* could be adjusted to reproduce identical physical conditions of excitation and of reception of the emitted light in the monochromator. The fulfilment of this requirement was checked as follows: the sample *S* was surrounded by a circle *C* of a plastic material emitting a green fluorescence; when *S* was correctly placed, the intensity of the fluorescence of *C* reached a known minimum value (at the wavelength of its maximum emission).

The sample was kept under liquid nitrogen during the measurements; it was placed against the wall of the *Dewar* before a slit *0*, 2 cm wide. Different values of the entrance and exit slits

of the monochromator were used. It was found that these values could be varied within a rather large range without appreciably influencing the form of the measured spectra.

Measurements of the low temperature absorption spectra: The absorption of the leaves was measured at liquid nitrogen temperature using the device of Fig. 3. MM_{12} was a standard double prism monochromator (*Zeiss* MM_{12}). *A* was a photomultiplier *1P 28*, or a photoelectric cell *MC 100 2V*, as used in the standard *Zeiss* equipment. *X* was the standard *Zeiss* light source. The *Dewar D* was provided by 2 longitudinal slits, 2 cm wide, the place of which is indicated by *O* on Fig. 3. A special sample holder *H* was placed in the *Dewar*; it made it possible to put the frozen leaf or the blank alternately into the light beam. The blank was prepared with an acetone leaf powder frozen in water in suitable concentration. The sample holder was continuously frozen in liquid nitrogen. The holder is shown on the right side of Fig. 3.

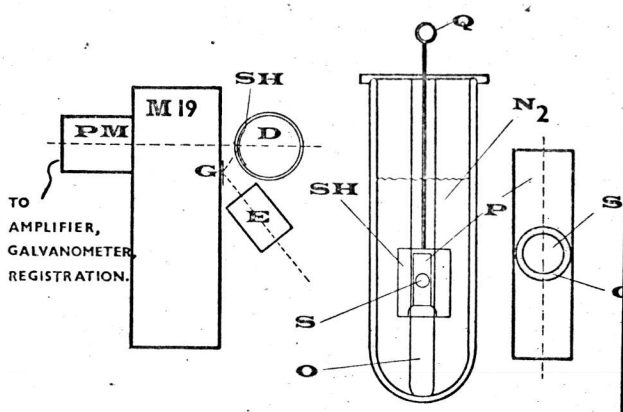

Fig. 2. The apparatus used for registration of the low temperature fluorescence of the leaf samples. It makes it possible to reproduce identical physical conditions of excitation and reception of fluorescence at liquid nitrogen temperature (explanations in the text).

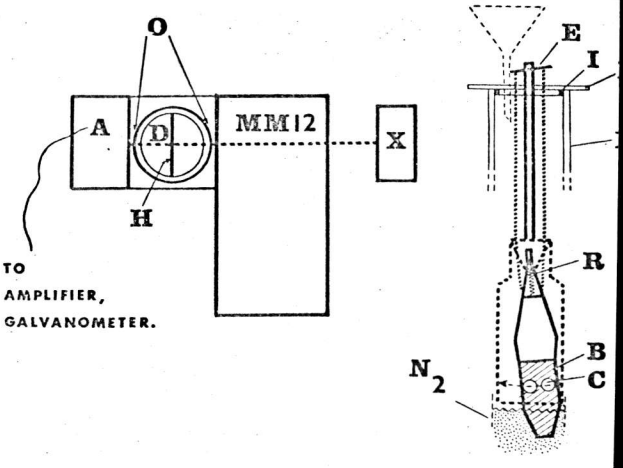

Fig. 3. The apparatus for measuring the absorption spectra at liquid nitrogen temperature. The sample holder *H* is seen in detail to the right of the figure. It consists of a plate of metal *B* with two apertures *C* which can be moved alternately in the light beam using the "balance" *E*; the positions of *B* are stabilized by the spring *R*. The lower part of *B* is immersed in liquid nitrogen. The holder is inserted into the dewar *D* by a cover *F*; *I* is a rubber ring (other explanations in the text).

The light intensity was estimated in a thermopile, type E_1 *special* (*Kipp, Delft*, Holland) using a microammeter (*Kipp, Microwa AL 4*). The thermopile was calibrated for the filters used with the aid of a *NBS* 1000 Watt quartz iodine lamp (standard of spectral irradiant) from the *Eppley Laboratory*, Newport, USA, serial number *EPI-1129*. However, the intensities given in the text are liable to a relatively wide margin of error ($\pm 10\%$ at least). All intensities are measured at the surface of the leaf sample.

RESULTS

1. Transformation of the fluorescence spectrum of etiolated leaves by short time illumination

The low temperature fluorescence (77°K) of any etiolated leaf specimen was characterized by a principal peak at 657 nm (protochlorophyllide) accompanied by 5 minor peaks or shoulders respectively found at 629, 674, 690, 713 and 728 nm (Fig. 4; compare with GOEDHEER 1967). These wavelengths form 3 pairs of values 55 to 60 nm apart from one another: 629—690 (d = 61 nm); 657—713 (d = 56 nm); 674—728 (d = 54 nm).

When the three following conditions were fulfilled:

1) leaf frozen at 77°K at the very end of the illumination using one of the set-ups of Fig. 1;

2) any red monochromatic light;

3) sufficient light intensity at the leaf level (I higher than $100 \cdot 10^{-11}$ einstein s^{-1} cm^{-2}); we found that within some fractions of a second to about 1 s (depending on I and on the wavelength), the principal low temperature fluorescence peak of the leaf moved from 657 in the dark to about 688 nm in the light. The position of the newly formed peak varied from one experiment to another between 686 and 691 nm, depending on the speed and on the extent of the phototransformation. In particular, when the transformation was performed or nearly completed in a very short time (for instance by a flash of 1 ms), the peak was found at 688 nm (Fig. 4 and 7).

The 688 species showed a secondary peak at about 747 nm. Its fluorescence was characterized by the wavelength pair 688—747 nm (d = 59 nm).

That the 688 species was derived from the 657 species in the light was indicated by the fact that for a given number of photons of a given wavelength reaching the sample per unit time, the fluorescence intensity at 657 nm decreased, while the fluorescence intensity at 688 nm increased with increased duration of the illumination, whereas no appreciable change was found in the 629 nm fluorescence. Similarly for a fixed duration of the illumination the 657 fluorescence decreased and the 688 increased when the intensity of the light was increased. Fig. 5 reproduces stages of the disappearance of the 657 nm fluorescing material and the simultaneous appearance of the 688 species for increased duration of an illumination of a given intensity and wavelength. The absence of a well defined isosbestic point is due to a slight displacement from 686 to 689 nm of the band formed in the light.

2. Transformation of the absorption spectrum of etiolated leaves by short time illumination

The absorption spectra of an etiolated leaf and of the same leaf having received a saturating 1 ms flash (647 nm photons) are given in Fig. 6, as measured at liquid nitrogen temperature. A similar phototransformation can be obtained with any sufficiently intense red light.

The etiolated leaf shows essentially a double band formed by pigments absorbing at 647 and around 630—635 nm. The flash transforms the 647 nm material into a 676—678 nm material, while the 630 nm material remains unchanged. Compared with the low temperature fluorescence spectra of Figs. 4, 5 and 7, it becomes clear that the 647 - and 676 nm - absorbing materials emit the bands at 657 and 688 nm respectively, while the 630 material does not fluoresce (or fluoresces weakly?) when the intact leaf is frozen at liquid nitrogen temperature.

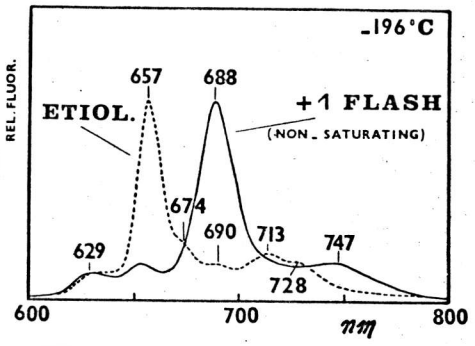

Fig. 4. Phototransformation of the low-temperature fluorescence of an etiolated bean leaf by a non saturating 1 ms flash (660 nm light). *Etiol* = spectrum of the etiolated leaf. + *1 Flash* = spectrum of a leaf frozen at liquid nitrogen temperature in the second following the flash (using the device of Fig. 1).

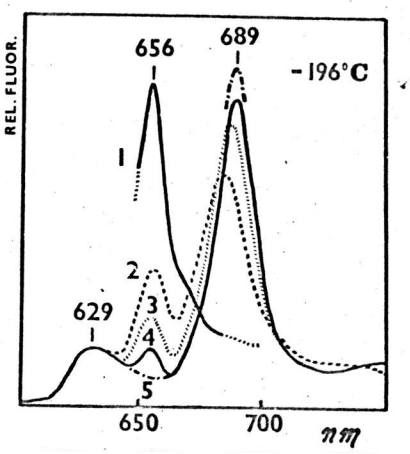

Fig. 5. Stages of the phototransformation of the low-temperature fluorescence of an etiolated bean leaf. Monochromatic actinic light, 647 nm. (*1*) fluorescence of the etiolated sample. (*2*), (*3*), (*4*) and (*5*) = = fluorescence of samples after 0·0667, 0·121, 0·241 and 1 s of illumination respectively. Intensity of the light of the order of $750 \cdot 10^{-11}$ einstein $cm^{-2}\,s^{-1}$.

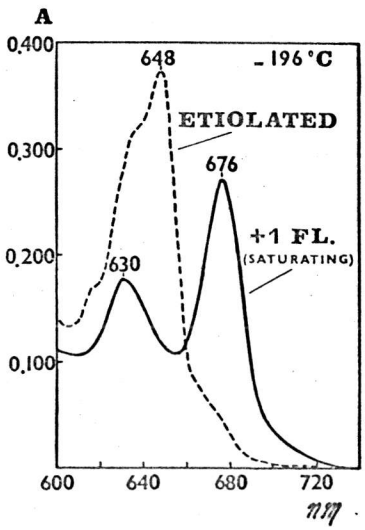

Fig. 6. Phototransformation of the low temperature absorption of an etiolated bean leaf by a saturating 1 ms flash (647 nm light). *Etiolated* = spectrum of the etiolated leaf. + *1 Fl* = spectrum of a leaf frozen at liquid nitrogen temperature in the second following the flash (using the device of Fig. 1).

This picture is similar to that described by some authors (for instance, LITVIN and KRASNOVSKIĬ 1957; KRASNOVSKIĬ, BYSTROVA and SOROKINA 1961) who have also found a phototransformable (647 nm absorbing) and a non-phototransformable (630—635 nm absorbing) pigment in the etiolated leaf. However, in the reports in the literature, the maximum absorption of the transformed material is located at 682 nm, while in our experiments it is found at 676 to 678 nm, provided very short-time irradiations of sufficient light intensity, followed by immediate freezing at liquid nitrogen temperature, are used.

We call the species present in the etiolated leaf P 657—647 (initial protochlorophyllide-lipoprotein complex, SIRONVAL, MICHEL-WOLWERTZ and MADSEN 1965), and the species formed by short time irradiation P 688—676 — the numbers pertaining to the respective positions of low temperature fluorescence and absorption maxima of the pigments.

3. Irreversibility of the P 657−647 → P 688−676 photoconversion

We never observed any dark reversion from P 688—676 to P 657—647 after a short-time illumination. Nor did we succeed in reversing the formation of P688—676 by light.

Etiolated samples were first irradiated with 647 nm light of high intensity to produce the total conversion of P 657—647 into P 688—676 in a time less than 0·1 s. This irradiation was followed by a second irradiation with 675 nm light. Different intensities of this light were used, as well as different time intervals (from 0·1 to about 1 s) between the first and the second irradiation. The photoconversion of P 657—647 into P 688—676 was never reversed.

4. Estimation of the extent of the phototransformation

It was found that, — keeping constant all physical conditions of excitation, reception and registration of fluorescence and exciting with the 434—436 nm emission of a mercury lamp as indicated under "Methods" (set-up of Fig. 2) —, the height of the low temperature fluorescence peak of an etiolated leaf at 657 nm was equal to that of the flashed leaf at 688 nm provided this leaf had been frozen immediately after total transformation of the pigment using the set-up of Fig. 1 (Fig. 7). The percentage of the phototransformation ($T\%$) could therefore be estimated from the ratio:

$$r = \frac{\text{(intensity of fluorescence at 657 nm after phototransformation)}}{\text{(intensity of fluorescence at 688 nm after phototransformation)}} = \frac{\bar{H}\ 657}{\bar{H}\ 688}$$

applying the formula:

$$T\% = \frac{100}{1 + r}.$$

Fig. 8 reproduces an example of $T\%$ estimation. It shows how the intensities of the fluorescence at 657 and 688 nm were corrected for contributions from other pigments. The contribution of the 630 nm fluorescing pigment at 657 nm was estimated after the complete transformation of P 657—647 into P 688—676 as shown in Fig. 8a.

The ratio

$$R\,657 = \frac{H_L\,657}{H_L\,630}$$

was obtained from spectra of control leaves which had been illuminated by a polychromatic saturating electronic flash (light control samples *L*). The corrected value $\overline{H}$ 657 of the emission of a partially transformed sample at 657 nm was calculated from the registered spectra as follows:

$$\overline{H}\,657 = H\,657 - (H\,630\,.\,R\,657) \quad [1]$$

H 657 and *H* 630 being the heights of the peaks of the partially transformed sample at 657 (before correction) and 630 nm respectively (Fig. 8c).

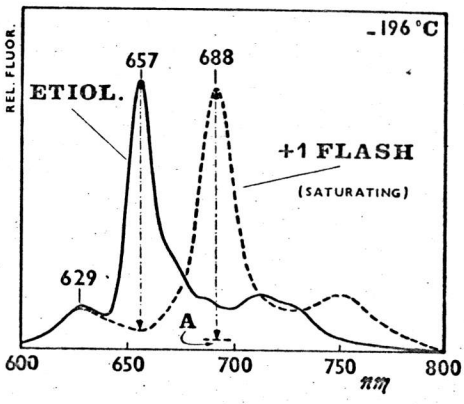

Fig. 7. Low temperature spectra of one half of an etiolated leaf (*Etiol.*) and of the other half of the same leaf having received a saturating polychromatic 1 ms flash (+ *1 flash*), — the sample being frozen at liquid nitrogen temperature immediately after the flash. (*A*) is the correction to be subtracted from the height of the fluorescence at 688 nm of the flashed sample calculated from ($\overline{H}$ 657 + *H* 688) *R* 688, in which $\overline{H}$ 657 is equal to zero (see Fig. 8). The registrations were made with the set-up of Fig. 2, which makes it possible to reproduce identical physical conditions of excitation and reception of fluorescence in a series of samples.

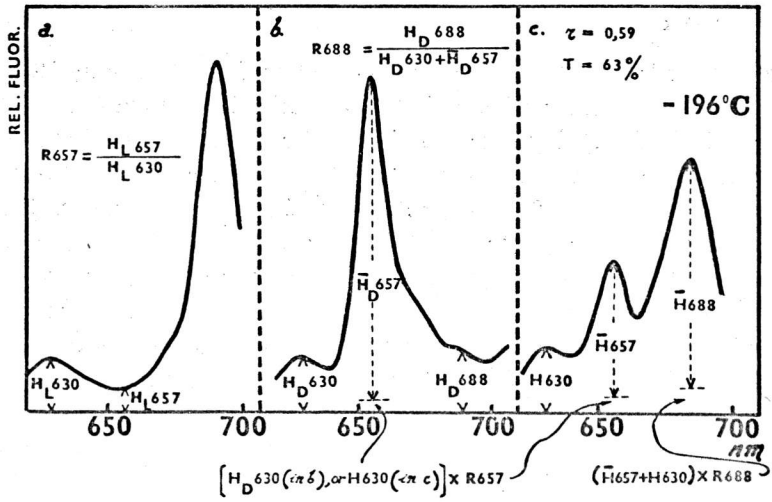

Fig. 8. An illustration of the method for calculating the percentage transformation of the pigment fluorescing at 657 nm (P 657—647) into the pigment fluorescing at 688 nm (P 688—676). The spectra were registered at liquid nitrogen temperature using the set-up of Fig. 2. (Other explanations in the text.) The line of zero fluorescence is drawn in admitting that the emission at 800 nm is zero (see Fig. 7).

The contributions of the 630 and 657 fluorescent pigments at 688 nm were estimated before any phototransformation, as shown in Fig. 8b. The ratio

$$R\,688 = \frac{H_D\,688}{H_D\,630 + \overline{H}_D\,657}$$

was measured on fluorescence spectra of control etiolated leaves (dark control samples D), — $\overline{H}_D\,657$ being the corrected values of the emission at 657 nm for control etiolated leaves calculated using formula [1] (Fig. 8b). The corrected values $\overline{H}\,688$ of the emission of a partially transformed sample at 688 nm was calculated from the registered spectra as follows:

$$\overline{H}\,688 = H\,688 - (\overline{H}\,657 + H\,630)\,R\,688 \qquad [2]$$

$H\,688$ and $H\,630$ being the heights of the peaks of the partially transformed sample at 688 (before correction) and 630 nm respectively, and $\overline{H}\,657$ being the height of the emission of the same sample at 657 nm corrected using formula [1] (Fig. 8c).

In practice, for the estimation of the extent of the phototransformation in any given illumination conditions, a series of 6 repetitions were registered (6 illuminations on separate samples), plus 3 dark controls (D) and 3 saturated light controls (L) from which the ratios $R\,657$ and $R\,688$ were calculated (for each series of 6 repetitions). Controls and illuminated samples came from the same culture of leaves. It was found that the values of $R\,688$ were fairly constant while $R\,657$ fluctuated more (Table 1). Values of $T\%$ for typical series of measurements at room temperature are given in Table 2.

5. Action spectrum

Action spectra for the P 657—647 → P 688—676 transformation were determined in measuring the percent transformation ($T\%$) as described in the previous section.

The etiolated leaf samples were illuminated for 0·881 s in the device shown in Fig. 1 B, with 630, 640, 650 and 660 nm photons; the relative values of the light intensity I at the different wavelengths were estimated using a bolometer. The percent transformation was thereafter calculated at every wavelength for a same value of I.

Typical results are shown in Fig. 9. It is clear that the maximum activity of the photons coincides with the maximum absorption of the etiolated leaf at 647 nm, and that the form of the action spectrum agrees with the form of the low temperature absorption of the etiolated leaf. It was concluded that the red absorbing pigments found in etiolated leaves were the photoreceptors for the transformation.

6. Kinetics of the phototransformation

The kinetics of the phototransformation was estimated for different light intensities I and for some wavelengths in the red. The illuminations were performed using the interference filters described in "Methods". The time was measured by the calibrated photographic shutter (Fig. 1), the leaf samples being frozen in liquid nitrogen at the

Table 1

A series of experimental values of R 657 and R 688.
(R 657 was calculated from completely transformed controls, and R 688, from etiolated controls, as shown in Fig. 8.)

	R 657	R 688
	0·286	0·129
	0·182	0·134
	0·200	0·165
	0·200	0·143
	0·333	0·129
	0·258	0·155
	0·373	0·145
	0·211	0·150
	0·111	0·128
	0·294	0·130
	0·100	0·132
	0·127	0·108
	0·130	0·122
$\bar{R}$	0·216	0·136
Standard error	0·024	0·004

Table 2

An example of $T\%$ estimation. (A 630 nm *Barr* and *Stroud* filter was used in the device of Fig. 1A; the temperature was $\pm 1°C$; the intensity of the light was of the order of $400 . 10^{-11}$ einstein . $cm^{-2}\ s^{-1}$.)

Repetition n°	Time (s)			
	0·881	0·461	0·241	0·121
1	59·8	37·7	24·6	18·3
2	55·6	40·0	32·4	17·1
3	59·7	39·1	30·2	15·1
4	58·9	46·3	29·2	18·2
5	63·8	42·9	25·3	18·7
6	60·5	51·7	24·6	20·2
7	57·8	49·0	30·2	16·0
8	63·7	47·6	27·9	15·9
9	58·9	46·7	25·9	14·4
10	—	41·2	30·7	16·9
11	—	49·2	—	—
$\bar{T}\%$	59·8	44·7	28·1	17·1
Standard error	0·86	2·41	0·90	0·58

278 very end of the illumination. The percent transformation was measured as described above (Fig. 8).

Fig. 10 shows that different kinetics were obtained depending on the wavelength of the photons. The phototransformation of P 657—647 followed, or tended towards a first order kinetics when 647 nm photons were used; it never followed a first order kinetics when 630 nm photons were used. This result did not depend on the intensity of the actinic light but it depended on the purity of the 647 nm photons as shown in Table 3.

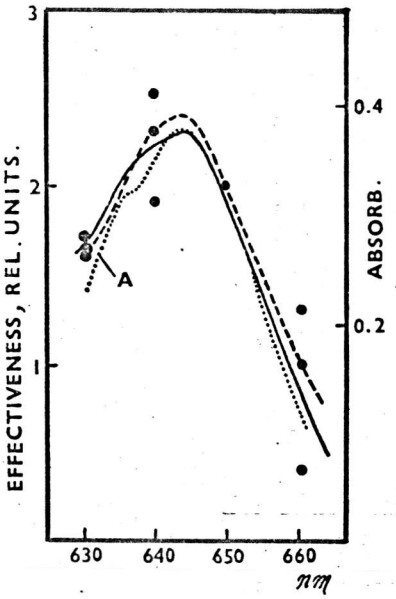

Fig. 9. Relative effectiveness of red photons of different wavelengths for the transformation of P 657—647 into P 688—676. Three series of measurements were made. Inside each series the percentage of phototransformation was calculated for a same light intensity and a same duration of illumination at the wavelengths experimented. The percentage transformation found at 650 nm was arbitrarily fixed at 2 relative units. Dashed curve, one series of measurement. Solid curve, average between the three series. Dotted curve (*A*), absorption spectrum of an etiolated leaf at liquid nitrogen temperature.

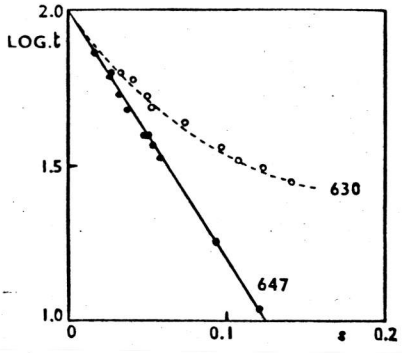

Fig. 10. Kinetics obtained for the transformation of P 657—647 into P 688—676 when illuminating during short times with 647 or 630 nm photons. $t = (100 - T\%)$. $T\%$ was estimated as indicated in Fig. 8; it was determined at a series of different light intensities for three time intervals: 0·461, 0·241 and 0·121 s. The light was filtered by *Barr* and *Stroud* filters in the device of Fig. 1A. The time scale of the graph was adjusted for a unique light intensity, *i. e.* a given, well defined e. m. f. at the exit of the bolometer (about $1{,}000 \times 10^{-11}$ einstein cm^{-2} s^{-1} at both wavelengths). This method, which makes use of formula [A 3], avoids the difficulties involved in the exact mechanical measurement of a great series of short times.

Since, when illuminating with (pure) 647 nm photons, the kinetics of the phototransformation was of the first order, it appeared necessary to assume that, in this case, it consisted of a photoreaction, the rate constant k of which depended on the intensity of the light I and on the absorption coefficient ε of the untransformed pigment (P 657—647), as follows:

$$k = n\varepsilon I\,, \qquad [3]$$

Table 3

Influence of the purity of the actinic photons on the order of the kinetics of the phototransformation. (In column *1*, the light was filtered by a *Baird* and a *Barr* and *Stroud* 647 nm filter; in column *2*, by a *Baird* 647 nm filter alone; in column *3*, by a *Barr* and *Stroud* 647 nm filter which had been damaged and was somewhat transparent for photons of other wavelengths. k is the first order constant per unit time).

Duration of the illumination	*1* (647 nm photons; half band width: 3·0 nm)		*2* (647 nm photons; half-band width: 4·2 nm)		*3* (contaminated 647 nm photons)	
	$T\%$	k	$T\%$	k	$T\%$	k
0·0677	25·6	4·4	36·0	6·6	38·7	7·2
0·121	39·7	4·2	59·6	7·5	52·3	6·1
0·241	62·9	4·1	82·2	7·1	69·4	4·9
$K_{max} - K_{min}$	—	0·3	—	0·9	—	2·3

$n \neq 0$ being the quantum yield of the phototransformation (Appendix I; in the prolamellar body, the magnitude of n seems to depend essentially on the age of the leaf and on the temperature). Formula [3] shows that, at a known light intensity, k measures ε multiplied by n; (k/n) also measures the frequency of the absorption acts which take part in the observed phototransformation. It follows that the kinetics can be written

$$T\% = 100\,(1 - e^{-kt}) \qquad [4]$$

in which $T\%$ is the percentage of the pigment molecules initially present which are transformed by light during time t.

If it is accepted that, at any wavelength, the phototransformation results from photoreactions like those found when illuminating with 647 nm photons, the kinetics found with 630 nm photons should result from a particular combination of photoreactions running at different rate. Indeed, the slope of $T\%$ versus time at 630 nm can be interpreted by such a combination.

The model of Fig. 11 represents a plausible, preliminary interpretation. It includes two lipoproteins bearing pigments: the first lipoprotein contains a 647 nm absorbing pigment (complex *a*). The second lipoprotein contains the same 647 nm absorbing pigment, but it is associated with a non fluorescent, accessory pigment responsible for the 630 nm absorption seen on Fig. 6 (complex *b*).

The model assumes that:

1) at wavelengths above 645 nm, the absorption of the 630 nm pigment is negligible, the only absorbing pigment being the 647 nm species in both complexes *a* and *b*,

280 while at wavelengths below 640 nm, the absorption of the 630 nm pigment becomes appreciable;

2) the molar absorption coefficient of the 647 nm pigment is the same for any wavelength in both complexes *a* and *b*; this assumption implies in particular that the orientation of this pigment and its interactions with the lipoprotein are the same in both complexes *a* and *b*;

3) the value of *n* associated in formula [3] with the 647 nm pigment (*n* 647) is the same, at any given wavelength, in both complexes *a* and *b*; there is also an *n* associated with the absorption of light by the 630 nm pigment (*n* 630);

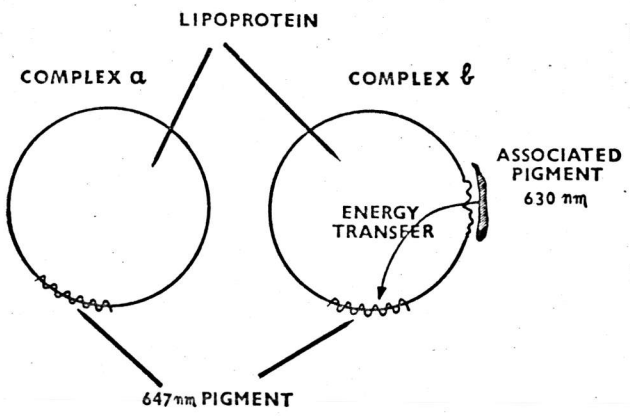

Fig. 11. Schematic representation of the model proposed for interpreting the kinetics data. The spherical form given to the lipoproteins is obviously arbitrary. The graphical representation of the links between protein and pigment only suggests that these links are not the same for the 647 nm pigment as for the associated (accessory) 630 nm pigment (see explanations in the text).

4) the light energy absorbed by the 630 nm pigment is used for the photoconversion of the 647 nm species in complex *b* only;

5) the rate constant for the photoconversion of the 647 nm pigment in complex *a* does not depend upon the photoconversion in complex *b*, and conversely.

Under these assumptions, the phototransformation follows a first order kinetics when the actinic light consists of 647 nm photons (one single absorbing pigment, the 647 nm species; one single absorption coefficient; one k for a given value of I in formula [3]). But it does not follow first order kinetics when the actinic light consists of 630 nm photons (two different absorption coefficients at 630 nm, originating from the 647 and from the 630 nm species respectively; two k's, $k_1 \neq k_2$, for a given value of I).

The model leads us to express the kinetics of the (P 657—647 → P 688—676) phototransformation as resulting from the sum of two first order kinetics as follows:

$$T\% = 100 - A e^{-k_1 t} - (100 - A)\, e^{-k_2 t} \qquad [5]$$

(see Appendix II), A being the proportion, in %, of the complex (*a* or *b*) which is transformed at rate k_1.

The analysis of the experimental kinetics for the transformation of P 657—647 by 630 nm photons using equation [5] showed that k_1 and k_2 tended to values which

remained constant in time for $A = 50$ (Fig. 12). This held t[illegible] any light intensity, *i.e.* for any value of k_1 and k_2 (we investigated values of k_1 and k_2 at 630 nm in range of 30 times). This feature seems to be related to the slope of the curve of $T\%$ in function of time. It implies that, in the etiolated leaf, the molecular ratio of complex *a* to complex *b* should be equal to 1. On the other hand, in the presence of (pure) 647 nm photons given alone (or of photons of longer wavelengths), A in [5] is either equal to zero or to 100, *i.e.* $k_1 = k_2$ (the kinetics are first order).

Values of k_1 and k_2 calculated for the kinetics produced by 630 nm photons in Fig. 10 are used in Table 4 to calculate the values of $T\%$. It is seen that the calculated and experimental values agree quite well, except in the region where $T\%$ exceeds 85%.

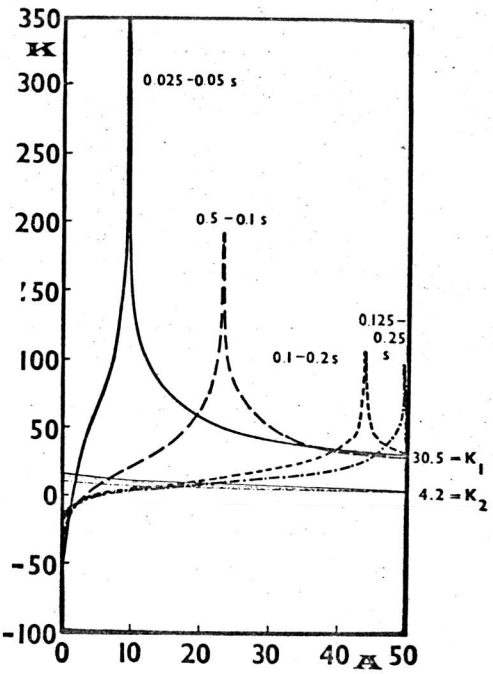

Fig. 12. Values for k_1 and k_2 found in applying equation [5] to the kinetics of the transformation of P 657—647 by 630 nm photons for different values of (A) and for different pairs of the duration of the illumination (0·025 and 0·05 s; *etc* ...). The calculations were made by means of an *IBM* computer. (A) intervals were 0·01. It is seen that both k_1 and k_2 tend to constant values for $A = 50$.

Table 4

Comparison of the experimental values of $T\%$ with the values calculated using the constants k_1 and k_2 derived by means of equation [5] for $A = 50$. (The experimental data are those of Fig. 10 for 630 nm photons; $k_{1(s^{-1})} = 30{\cdot}5$; $k_{2(s^{-1})} = 4{\cdot}2$.)

Time in s	Values of $T\%$	
	Calculated	Experimental
0·025	31·6	31·5
0·05	48·5	48·2
0·10	64·8	65·2
0·15	73·3	73·5
0·20	78·3	78·5
0·25	82·5	82·2
0·30	85·8	85·0
0·35	88·5	86·8
0·40	90·7	88·4
0·45	92·4	89·1
0·50	93·9	90·0

7. Estimating the *in vivo* absorption spectrum of P 657–647 from kinetics data

According to equation [3] and to the model of Fig. 11, one of the two values k_1 or k_2, as calculated from the kinetics produced by 630 nm photons (equation [5]), is related to the absorption of 630 nm photons by the 647 nm pigment in complex *a* (k_{630}^{647}), while the value of k for the kinetics produced by 647 nm photons (equation [4]) is related to the absorption of the 647 nm photons by the 647 nm pigment in both complexes *a* and *b* (k_{647}^{647}).

If k_1, k_2 and k are measured for the same intensity of the actinic light I (at a given, constant temperature), and if we assume that n 647 does not vary with wavelength, we find according to formula [3]:

$$\frac{k}{k_1 \text{ or } k_2} = \frac{I.n647.\varepsilon 647}{I.n647.\varepsilon 630} = \frac{\varepsilon 647}{\varepsilon 630} = C \qquad [6]$$

where $\varepsilon 647$ and $\varepsilon 630$ are the absorption coefficients of the 647 nm pigment for the 647 and for the 630 nm photons respectively.

We calculated k_1 and k_2 from kinetics data for a given illumination with 630 nm photons at 23°C ± 2. Thereafter using the same set of leaves and the same light intensity, we measured the value of k from the first order kinetics for 647 nm photons at the same temperature. k_1 was found equal to 0·158, k_2 to 1·058 and k to 0·608. C was therefore equal either to

$$C_1 = \frac{k}{k_1} = \frac{0{\cdot}608}{0{\cdot}158} = 3{\cdot}85\,,$$

or to

$$C_2 = \frac{k}{k_2} = \frac{0{\cdot}608}{1{\cdot}058} = 0{\cdot}58\,.$$

The second value was excluded, since the absorption of the 647 nm pigment cannot be higher at 630 nm than at 647 nm. k_1 was therefore identical to k_{630}^{647}.

Similar measurements and calculations may be made at different wavelength pairs, each of them including 647 nm and another wavelength. Theoretically, they should lead to the establishment of a spectrum for P 657—647 relatively to its 647 nm *in vivo* maximum, whose form should correspond to that of the *in vivo* absorption of P 657—647 if n 647 remains constant with wavelength.

We are not able to present this *in vivo* spectrum in detail, but the above value of C_1 (and some others) shows that its form is probably very near to that found for protochlorophyllide in solution. Indeed, the ratio between the *in vitro* absorption coefficients of protochlorophyllide at its absorption maximum (623 nm) and at a distance 17 nm apart in the direction of lower wavelengths (606 nm) is equal to

$$\frac{\varepsilon 623}{\varepsilon 606} = 3{\cdot}9$$

(in ethyl-ether), a value practically identical with C_1. It follows that the quantum yield 283
n 647 in formulae [3] and [6] probably does not vary with wavelength. Further information concerning n will be given elsewhere.

DISCUSSION

The model of Fig. 11 is reminiscent of the first of three hypotheses presented by BOARDMAN (1962) to account for the kinetics of the conversion of protochlorophyllide into chlorophyllide in red light. This hypothesis was formulated as follows: "A solution of the protochlorophyll-protein complex may be a mixture of two types of protochlorophyll-protein molecules..., but the protochlorophyll molecules of one complex are converted to chlorophyll *a* at a different rate from the protochlorophyll molecules in the other complex". On the basis of the experimental results available, BOARDMAN was unable to decide between the above hypothesis and two others; they appeared at that time as equally reasonable. He derived equation [5], but did not interpret explicitly the mechanism of the reaction-rate differentiation between the postulated complexes, although equation [5] implicitly included the interpretation (see Appendix I and II). BOARDMAN found that equation [5] fitted in well with the experimental data for A equal to 50.

BOARDMAN experimented with purified protochlorophyllide-protein complexes in solution. He was led to explain the experimental kinetics as the sum of two first order reactions because he found that any interaction between protochlorophyllide-protein molecules was "extremely unlikely". This conclusion was based on the fact that the reaction rate "is independent of the initial concentration of the protochlorophyll — protein molecules and is not influenced by the viscosity of the medium".

One may wonder about the coincidence between our model and BOARDMAN's hypothesis, since BOARDMAN measured chlorophyllide and probably chlorophyll formation, while our measurements are only concerned with the (P 657—647 → P 688—676) phototransformation. At first sight, this coincidence seems explainable since, in BOARDMAN's experiments, the rate limiting factor was the intensity of the light. He gave 8·5 $\mu W\ cm^{-2}$ to less than 1 $\mu W\ cm^{-2}$; in some experiments the phototransformation was followed for times as long as 150 min. We could suppose that, under these conditions, the kinetics was determined by the rate limiting light phase.

Our observation that the phototransformation follows a first order kinetics when illuminating with photons of 647 nm (or longer wavelengths) not only leads one to choose BOARDMAN's first hypothesis as the most suitable model for the P 657—647 → P 688—676 phototransformation. It also provides a simple, physical explanation of the differentiation of the reaction rates between the two protochlorophyllide—lipoprotein complexes.

The model designed in Fig. 11 is naturally not to be considered strictly. It provides a general idea rather than an exact picture. We do not know very much about the real links between pigments and lipoproteins. We do not know how the molecules are arranged inside the prolamellar body. It is, for instance, very likely that the relations between the 647 nm pigment and the protein differ from those between the 630 nm pigment and the protein (different protein subunits may be involved?).

It seems that facts related to those presented here were observed in 1962 by workers in KRASNOVSKII's group. RUBIN *et al.* (1962) found that both the 690 nm fluorescing pigment, — which first appears as a result of the phototransformation of the 657 nm species —, and this last species itself, exhibit the same value of the fluorescence lifetime τ at normal temperature as well as the same value of the absorption integral ($=\int \varepsilon_\nu\, d\nu$; ε_ν being the molar absorption at the frequency ν). On the other hand, the Russian authors describe experiments indicating that, when etiolated bean leaves are illuminated at —120°C, some pigment species is formed which is transformed into chlorophyllide in the dark. They however did not explicitly describe P 688—676 as a species distinct from the chlorophyllide—lipoprotein complex.

Appendix

I. During the phototransformation of P_0 molecules initially present at $t = 0$ (before illumination), the photons play the role of a reactant, the concentration of which remains constant, if it is assumed that there is no darkening of one pigment molecule by another, either before or after the phototransformation (*i.e.* the molecules are considered as arranged in a same plane, or their concentration per unit volume is low). We can write:

$$-\frac{dP}{dt} = kP \qquad [A1]$$

which gives after integration:

$$P = P_0 e^{-kt} \qquad [A2]$$

This equation describes the kinetics of any photoreaction in the case of one absorbing pigment. k has the dimension of s^{-1}.

If the photon fluence (BROWN and JOHNS 1967) or energy flux L, — defined as the number of photons having reached the P molecules at a given time t:

$$L_{\text{photons cm}^{-2}} = I_{\text{photons s}^{-1}\text{cm}^{-2}} \cdot t_s \qquad [A3]$$

I being the intensity of the incident beam—, is substituted for the time (I constant), we have, similarly to [A1] and [A2]:

$$-\frac{dP}{dL} = K'P \qquad [A4]$$

which gives after integration:

$$P = P_0 e^{-K'L} \qquad [A5]$$

K' has the dimensions of a cross-section, cm^2 molecule^{-1}. It is the virtual area to be ascribed to a phototransformable molecule for picking up the quantity of photons of a given wavelength necessary to account for the observed phototransformation (in our experiments, the photons are sent normally to the surface of the leaf). The cross-section K' is the photochemical equivalent of the molar absorption coefficient ε defined by the Lambert-Beer law:

$$\ln \frac{I_0}{I} = \varepsilon c d$$

(where I_0 is the intensity of the incident beam, I the intensity of the transmitted beam, c the concentration of the absorbing molecules and d the thickness of the solution), divided by the Avogadro number N:

$$K'_{\text{molecule}^{-1}\text{ cm}^2} = \frac{n\,10^3}{N}\,\varepsilon_{\text{molecule}^{-1}\text{ cm}^2} \qquad [A6]$$

n being some constant different from 0.

It obviously follows from [A2], [A3] and [A5] that:

$$\frac{k}{K'} = \frac{L}{t} = \frac{It}{t} = I \qquad [A7]$$

Substituting for K' in [A7] its value in [A6], we obtain:

$$k_{s^{-1}} = \frac{n\,10^3}{N}\,\varepsilon_{\text{molecule}^{-1}\,\text{cm}^2}\,I_{\text{photons s}^{-1}\,\text{cm}^{-2}} \qquad \text{[A8]}$$

[A8] says that k represents the frequency of the absorption acts taking part in the observed phototransformation: it is the number of effective photons which are absorbed per molecule and per s during the phototransformation. This number of active photons is proportional to the intensity of the incident beam, to the molar absorption coefficient (or the cross-section) of the absorbing molecule, and to n.

It appears from [A8] that n is equal to 1 whenever every absorption act effectively takes part in 1 phototransformation: y molecules (P) which absorbs $yhv \rightarrow yP^* \rightarrow yp$, ($p$) being a molecule of the product. In this case, $n = y/y = 1$. n is lower than 1 when some of the absorption acts do not take part in the phototransformation: y molecules (P) which absorb $yhv \rightarrow yP^* \rightarrow \rightarrow (y-a)\,P + ap$; $n = a/y < 1$. Some of the P^*'s are de-excited before transformation into p. n is higher than 1 when each absorption act transforms on an average more than 1 molecule: y molecule (P) which absorb $yhv \rightarrow yP^* \rightarrow$ (some unknown mechanism) $\rightarrow (ay)\,p$, with $n = a > 1$. In this case, the excitation resulting from the absorption in one point is spread over a certain number of pigment molecules (there is a kind of chain reaction).

It is obvious that n represents the number of molecules (P) transformed into (p) per absorbed photon. It is the quantum yield of the photoreaction, the dimensions of which are molecules. photon^{-1} (or moles einstein^{-1}).

If α is substituted for ε in [A8], $-\alpha$ being defined by:

$$\log \frac{I_0}{I} = \alpha c d -,$$

[A8] is written:

$$k = \frac{n\,2{\cdot}3\,.\,10^3}{N}\,\alpha\,I \cong n\,4 \times 10^{-21}\alpha\,I \qquad \text{[A9]}$$

(see RABINOWITCH 1951).

II. The proposed model assumes that the photoconversion rate of the 647 nm pigment in one complex does not depend on the photoconversion rate in the other complex. This condition implies also that trapping a photon at a given pigment molecule does not modify the intensity of the light beam for another molecule (there is no chance of some molecules being darkened by other molecules, either before, or after the photoconversion). The complexes constitute molecule families independently transformed into the same product under the action of light. The transformation is not reversible.

Under these assumptions (see also section 6), BOARDMAN's (1962) derivation may be adapted to the model of Fig. 11 as follows:

If A_0 is the number of molecules of complex a which are initially exposed to the photon beam at $t = 0$, L being the photon fluence and K_1' the cross-section of the 647 nm pigment, we find, by applying [A5], that the kinetics of the photoreaction is given by:

$$A = A_0 e^{-K'_1 L}$$

Similarly, if B_0 is the number of molecules of complex b exposed to the same beam at $t = 0$ and K_2' is a certain function of the cross-sections of both 647 and 630 nm pigments, we have:

$$B = B_0 e^{-K'_2 L}$$

Since every molecule of complex a and of complex b bears 1 molecule of the 647 nm pigment, the sum $A_0 + B_0$ is equal to the number of 647 nm pigment molecules to be transformed (initial number of molecules of the protochlorophyllide—lipoprotein complex).

On the other hand, if (a) is the proportion of the molecules of complex a in $(A_0 + B_0)$, $(1 - a)$ is the proportion of molecules of complex b. The partial percentage transformation of the 647 nm pigment in complex a is:

$$T_a \text{ partial } (\%) = \frac{A_0 - A}{A_0} 100a = 100a(1 - e^{-K'_1 L}).$$

Similarly, in complex b, this percentage is:

$$T_b \text{ partial } (\%) = \frac{B_0 - B}{B_0} 100(1 - a) = 100(1 - a)\,(1 - e^{-K'_2 L}).$$

The total percentage transformation T of the 647 nm pigment for both complexes a and b is the sum of ($T_{a\ \text{partial}}$) and ($T_{b\ \text{partial}}$):

$$T(\%) = 100a(1 - e^{-K'_1 L}) + 100(1 - a)\,(1 - e^{-K'_2 L}) =$$
$$100\,(1 - a\,e^{-K'_2 L}) - (1 - a)\,e^{-K'_2 L}) \qquad \text{[A 10]}$$

[A 10] obviously reduces to: $T\% = 100(1 - ae^{KL})$ for $K'_1 = K'_2$, — a condition which is reached in the model when the 647 nm pigment absorbs (pure) monochromatic photons of a wavelength equal or higher to 645 nm (in this case, a single cross-section is involved).

Replacing K'_1 and K'_2 in [A 10] respectively by the frequency of absorption acts k_1 and k_2 and the photon fluence L by the time t (see Appendix I), we get:

$$T(\%) = 100(1 - ae^{-k_1 t} - (1 - a)\,e^{-k_2 t}) \qquad \text{[A11]}$$

The previous argument may be extended to any number of molecule families like complexes a and b, provided the assumptions made above are preserved. We can write that in general, for n families with n distinct cross-sections (K'_1 to K'_n):

$$T(\%) = 100[1 - a_1 e^{-K'_1 L} - a_2 e^{-K'_2 L} - \ldots - a_{n-1}\,e^{-K'_n - 1 L} -$$
$$- (1 - a_1 - a_2 - \ldots - a_{n-1})\,e^{-K'_n L}] \qquad \text{[A 12]}$$

in which a_1 to a_{n-1} represent the partial proportions (in %) of $n - 1$ families.

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Ц. Сиронвал, М. Броуерс, Й. М. Мишел и Й. Куипер (Лаборатория физиологии растений, Горсемская научно-исследовательская станция, Горсем Ст-Труиден, Бельгия): **Превращение протохлорофиллида в хлорофиллид. I. Кинетика трансформации $P_{657-647} \rightarrow P_{688-676}$.** — Photosynthetica **2**: 268—287, 1968.

Изучалось действие света на протохлорофиллид-протеиновый комплекс у интактных этиолированных листьев. Были применены низкотемпературная спектрофлуорометрия и импульсное освещение (менее чем 1 сек по 1 мсек). Обнаружено, что первые наблюдаемые спектральные изменения отвечают фотопродукции особой формы пигмента в течение очень короткого интервала времени. Эта форма отличается от хлорофиллид-липопротеинового комплекса и была названа $P_{688-676}$. При использовании 630 nm (или фотонов более короткой длины волны) было обнаружено, что кинетика этих изменений представляет собой изменения первого порядка. Для объяснения экспериментальных данных предложена предварительная модель, включающая два сорта липопротеин-протохлорофиллидового комплекса, один из которых получает световую энергию от добавочного пигмента.