EARLY PHOTOACTIVITY IN ILLUMINATED, ETIOLATED BEAN LEAVES

Dedicated to Edgard Lederer at the occasion of his 70th birthday

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The Pathway to Chlorophyll-proteins

The red absorption band of many green plants (essentially algae and higher plants) has been resolved into several components. This has been generally made on the basis on an analysis of the derivative spectrum and of a reconstitution of the shape of the red absorption by addition of chosen Gaussian curves [2,6]. By changing the plant under study, by growing it in different conditions, by comparing mutant to wild type, intact chloroplasts to chloroplast fractions, etc...., the conclusion was drawn that a limited number of chlorophyll forms in suitable proportions (2 chlorophyll *b* forms and some 6 chlorophyll *a* forms) could account for the spectra.

The following principal components of the red band have often been quoted as pertaining to chlorophyll *a* forms:

Component:	Location (nm) of the red absorption between		
	x	and	y
1	667		670
2	672		675
3	683		685
4	around 695		

Some less abundant components absorbing at λ>700 nm are to be added to the list. Component (2) is generally thought to be the most abundant; sometimes two components are introduced instead of component 3 : 3', absorbing at 680-681, and 3", absorbing around 686 nm [6].

When they start greening etiolated plants exhibit a red absorption which appears simpler than that of fully green plants. In this case, one or two chlorophyll *a* components account for the shape of the band. Moreover the spectrum changes in time, reflecting the successive occurrence of distinct pigment-protein complexes. Because in this case the "components" appear isolated one from the other and successively, the study of greening did help a great deal for interpreting the spectrum of fully green plants.

30 s after an etiolated bean leaf has received a short flash of white light, which reduces the bulk of the original protochlorophyllide into chlorophyll *a*, the red absorption is made of the superposition of three components: a band at 628 nm, which belongs to the non-photoreducible protochlorophyll(ide)-protein complex $Pr_{630-628}$; a band appearing as a shoulder at 668 nm and a main band at 683 nm, which belong respectively to the chlorophyll *a*-protein complexes $C_{675-668}$ (minor complex) and $C_{696-683}$ (major complex) (the complexes are usually designated by letters, Pr for protochlorophyll(ide), C for chlorophyll(ide) and P for pigment, with subscripts recalling first the wavelength of the emission band and then the wavelength of the main red absorption band, in nm).

At room temperature the position of the absorption band found at 683 nm, 30 s after a short flash, shifts slowly to 672 nm. This shift - which was first seen by Shibata [9] - lasts more than 20 min. Its speed depends on leaf age and on temperature. It is due to a change of $C_{696-683}$ into $C_{685-672}$. Thus, in a relatively short time after its

illumination, the component bands 1, 2 and 3 of the fully green leaf are encountered in the spectrum of the etiolated leaf.

$C_{675-668}$ and $C_{685-672}$ are stable end products in the sense that, once they have been found, they are steadily found in the greening leaf and finally in the green leaf. On the contrary $C_{696-683}$ is a transient product, which disappears after a light flash as a consequence of the Shibata shift. It reappears, however, as soon as light is given again, as a product of the reduction of protochlorophyll(ide) molecules newly synthesized in the leaf. Thus, in the light, there is a flow from protochlorophyll(ide)-proteins to the chlorophyll *a* containing $C_{685-672}$ through $C_{696-683}$.

This pathway includes at least two intermediates between protochlorophyll(ide)-proteins and $C_{696-683}$:

One of these is the immediate precursor of $C_{696-683}$ which may be trapped in liquid nitrogen in times of the order of 30 ms after a 1 ms, intense flash. At that time and after such a flash a red absorption is found which culminates near 676-678 nm with an emission at 688-690 nm. This absorption shifts rapidly at room temperature and gives rise to the characteristic absorption of $C_{696-683}$ within less than 30 s [3,11]. The transient precursor of $C_{696-683}$ was called $P_{688-676}$ by Sironval and Michel [11].

In fact two pigment proteins, a major and a minor one, with two distinct fates are trapped 30 ms after a flash, if sufficiently strong. Following Litvin *et al.*[7], the absorption of a first photon by a protochlorophyll(ide) molecule produces a pigment-protein complex with an absorption at 676 and a fluorescence emission at 684 ($C_{684-676}$), and the absorption of another, second photon by $C_{684-676}$ transforms this species into the true, immediate precursor of $C_{696-683}$ ($C_{688-680}$). We do not intend to enter here into the discussion of the data which support this two-photon mechanism. It is, however, established

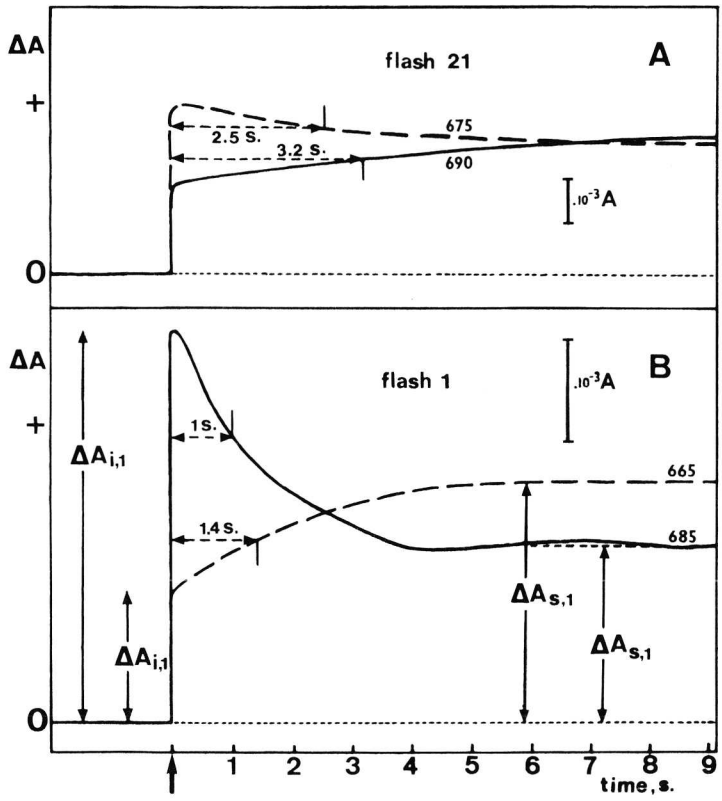

Fig. 1. Kinetics of the absorbance changes after successive, low intensity flashes. The flash is given at (o) time. Absorbances after flash 1 (B) and 21 (A) are observed respectively at 665 and 685 nm, and at 675 and 690 nm. The shift is towards 670 nm after the first flash; it is towards 690 nm after flash 21 (reproduced from Michel and Sironval, [8]; see this paper for details).

that, as shown by Litvin and Belyaeva [5] when an etiolated leaf has received a short, low intensity flash - thus at the very start of the photoreduction of protochlorophyll(ide) - the absorption band with a low absorbance near 676-678 nm, trapped immediately after the flash, does not shift to 683 nm - *i.e.* to a longer wavelength - but to a shorter wavelength - down to 667-670 nm.

Using repetitive (about 0.1 ms) low intensity flashes, we were not able to distinguish spectroscopically, 30 ms

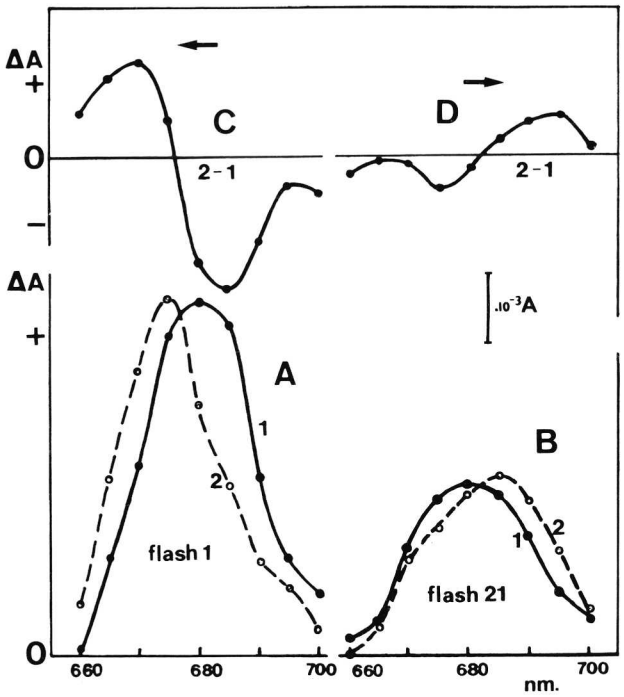

Fig. 2. Difference spectra: [absorbance 30 ms after the flash minus absorbance before the flash] (curves 1) and [absorbance 10 s after the flash minus absorbance before the flash] (curves 2) for flash 1 (A) and 21 (B) - Curves C and D are the differences: [absorbance 10 s after the flash minus absorbance 30 ms after the flash] for flash 1 and 21 respectively. In these experiments the change of the shift direction occurred around flash 4 (reproduced from Michel and Sironval [8]; see this paper for details).

after a flash, between the absorption of the product of the first flash and the absorption of the product of later flashes. But the half life time of the 30 ms product of the first flash was about 1 s, and the shift was towards 670 nm, yielding an absorption at 668 and an emission at 675 nm, the spectral characteristics of $C_{668-675}$. At later flashes, another 30 ms product appeared with a half life-time of about 3 s, whose absorption shifted towards 685 nm, yielding $C_{696-683}$ (Figs 1 and 2; see [8]). It has been computed that the proportion of the protochlorophyll(ide)-proteins of the etiolated leaf which transforms into

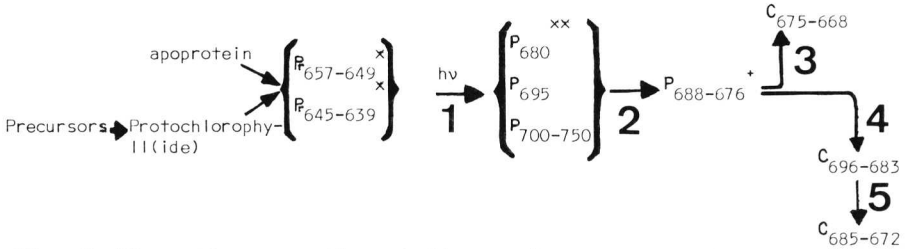

Fig. 3. The pathway to chlorophyll-proteins. Pr=protochlorophyll(ide); P=pigment; C=chlorophyll(ide). The first subscript refers to the wavelength of the main emission band of the pigment-protein complex; the second subscript refers to the wavelength of its main red absorption band; when a single subscript is given, it stays for the wavelength of the main red absorption band. (XX) P680 probably represents $P_{688-676}$ molecules with an efficient energy transfer to neighbour, long-wavelength absorbing molecules (Dujardin and Sironval [1]). (+) Litvin *et al.* [6] distinguish here between $C_{688-676}$ and $C_{688-680}$; they include a photoreaction leading from $C_{684-676}$ to $C_{688-680}$. The symbol $P_{688-676}$ covers both $C_{684-676}$ and $C_{688-680}$; it stays for the "energy transfer unit" (see Sironval and Kuiper [10]). The first $P_{688-676}$ molecules which appear are transformed into $C_{675-668}$ through pathway 3; some percent only of the total pigment content follows this pathway; the bulk of $P_{688-676}$ is transformed into $P_{685-672}$ through the pathway 4, 5.

$C_{668-675}$ through its 30 ms precursor at the very start of the illumination amounts to some percent only; the bulk of $P_{688-676}$ is made of the immediate precursor of $C_{696-683}$.

Intermediates which precede $P_{688-676}$ have been recently trapped in liquid nitrogen by illuminating etiolated bean leaves at temperatures from -95° to -120°C [1]. They include precursors whose life-time at room temperature is unknown, but lies below some ms. The absorption of these precursors shows a main band around 695 nm with a tail extending up to 750 nm - *i.e.* their absorption appears similar to that of component 4 and to that of the long wave-length components of the green spectrum. The paper of Dujardin at this symposium deals with these particular precursors. By warming up in darkness to temperatures higher than 90°C the long-wavelength pigment-proteins disappear while $P_{688-676}$ appears. It is remarkable that the transient, long-wavelength absorbing chlorophylls do not

seem to emit any fluorescence at -196°C.

The scheme of Fig. 3 depicts the pathway to stable chlorophyll-proteins (in the sense already mentioned) as we know it today. Since each chlorophyll *a*-protein found along this pathway has spectral characteristics of a component of the "fully green" spectrum and since this pathway is seen as soon as an etiolated leaf is illuminated, we conjectured that this leaf might exhibit early photobiochemical activities similar to some extent to those of the green leaf. This has already been shown to be the case when etiolated leaves are submitted to series of 1 ms flashes which trigger the mentioned pathway; although the total illumination does not exceed 1 s, photobiochemical activities are recorded in these leaves [13].

Fluorescence Kinetics in Etiolated Leaves

A very early photobiochemical activity, distinguishable from protochlorophyll(ide) reduction, was suggested by the kinetics of the fluorescence emitted by an etiolated leaf when illuminated for the first time. The experiment was essentially as follows [4]:

A continuous light from a He-Ne laser tube (632.8 nm) was sent on an etiolated leaf. The transmission of that light and the intensity of the excited fluorescence were simultaneously monitored at room temperature using a device similar to that previously used by Strasser [12]. The measured fluorescence emission included either all wavelengths above 665 nm, or (by means of a proper interference filter) one of the following selected wavelengths: 680, 690, 702 and 740 nm.

It was found that, after an initial, rapid rise which took about 2 s, the fluorescence emission of the etiolated leaf decreased to some 50% of the maximum in a time of the order of 60 s, and finally reached a steady state somewhat below the 50% level. The same kinetics was found when receiving the red light above 665 nm in the photomultiplier,

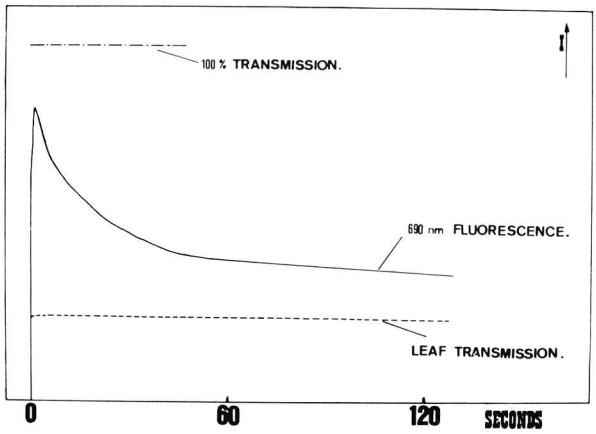

Fig. 4. An etiolated leaf was illuminated at (o) time by a red light from a He-Ne laser tube. The kinetics of the fluorescence emitted at 690 nm and the transmission of the 632.8 nm light through the leaf were recorded simultaneously. After an initial rise, the fluorescence emission decreases markedly while the leaf transmission stays constant. Illumination of the leaf at room temperature.

or any one of the selected wavelengths, showing that the decay could not be due to any spectral shift as such. On the other hand, the decay occurred while the leaf transmission remained constant at 632.8 nm. This forced us to admit that it was in essence a yield decay (Fig. 4).

The decay begins while light is still used for the reduction of protochlorophyll(ide) into chlorophyll(ide). This reduction is ended before the steady state level of the fluorescence emission is reached. Thus, the decay does not parallel protochlorophyll(ide) photoreduction.

On the other hand, it was shown by pouring liquid nitrogen around the leaf during the decay - without changing the physical conditions for excitation and reception of the fluorescence - that the -196°C emission at 690 nm increased along with the progression of protochlorophyll (ide) reduction, while the room temperature emission decreased (Fig. 5).

All these facts point to the establishment of some hitherto unnoticed photoactivity of the chlorophyll *a*

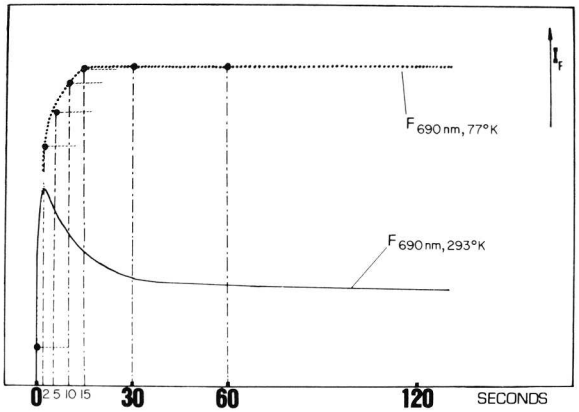

Fig. 5. Comparison of the fluorescence kinetics seen when etiolated leaves are illuminated for the first time at room temperature (full line), with the fluorescence emitted at 77 K when these leaves are frozen at increasing times during their room temperature illumination. For obtaining the points of the 77 K fluorescence (along the dotted line) liquid nitrogen was poured around the leaves during the recording of the room temperature kinetics; this occurred for successive leaves: 0, 2, 5, 10, 15, 30 and 60 s after the start of the first illumination. The room temperature and 77 K fluorescences have been measured under exactly the same physical conditions, the normalization of the leaf response being made using the room temperature spectrum. Illumination with light at 632.8 nm from a He-Ne laser. Recording of the intensity of the fluorescence emitted at 690 nm.

containing complexes which appear in the etiolated leaves when illuminated for the first time (especially of $C_{696-684}$).

The establishment of this photoactivity requires the presence in the leaf of intact protochlorophyll(ide)-protein complexes. Heating the leaf to 50°C stops the fluorescence decay; the same is true for hydroxylammonium chloride infiltration. Infiltration of ethanol-water (5% in ethanol) also suppresses the decay, but in contrast to heating or infiltration of hydroxylammonium chloride, it does not increase the fluorescence yield when the steady state level of fluorescence emission has been reached. Addition of DCMU to ethanol-water does not change this result.

Early Cytochrome Photoreduction

In order to make more explicit the suspected photoactivity of the first chlorophylls, which appear in the etiolated leaf, we investigated absorption changes in the blue region of the spectrum under a red polychromatic illumination light from 600 to 750 nm (Schott RG 630 filter). Two kinds of changes have been recorded in this region: some pertain to protochlorophyll(ide) photoreduction, others pertain to the photoactivity of chlorophyll-protein complexes resulting from this reduction. We restrict our description to the photoreduction of a cytochrome in red light.

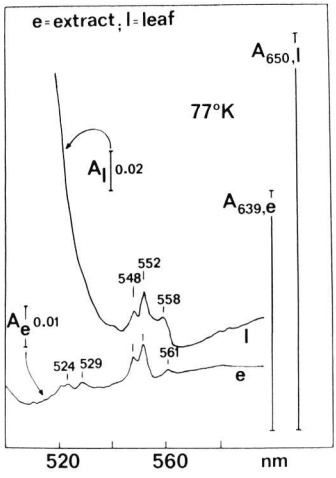

Fig. 6. Absorption spectra of an etiolated leaf and of a leaf extract showing the absorption bands of the cytochromes f and b_6. On the right, $A_{639,e}$ is the absorbance of the extract at its absorption maximum at 639 and $A_{650,l}$ is the absorbance of the leaf at its absorption maximum at 650 nm. Other explanations in the text.

Fig. 6 shows the low temperature absorption of an etiolated bean leaf and of a rather crude extract of the same leaf in the region of the α and β cytochrome absorption bands. The α_1 and α_2 bands of cytochrome f are respectively seen at 552 and 548 nm in the leaf and in the extract. The β_1 and β_2 bands of cytochrome f, respectively at 529 and 524 nm, are seen in the extract; they are hidden by the

carotenoid absorption in the leaf spectrum. The α band of cytochrome b_6 is seen at 557 nm in the leaf spectrum, but it seems to be absent in the extract.

When a red actinic light is sent on an etiolated leaf, the main absorption changes between 400 and 500 nm are due to the photoreduction of protochlorophyll(ide). However at 410-420 nm other changes are superimposed to those due to photoreduction. On the contrary at 430-440 nm, the kinetics are typical of the reduction. In this spectral region the absorbance decreases steadily when the actinic light is turned on - 80 to 90% of the maximum decrease being reached about 2 s after the light is set on in our illumination conditions. A characteristic feature of the spectral changes due (in the blue region) to protochlorophyll (ide) photoreduction is their irreversibility. An actinic flash of 0.1 s given to the etiolated leaf produces a difference: (absorption before - absorption after the flash) which essentially matches the blue absorption of the protochlorophyll(ide)-protein complexes around 440 nm; isosbestic points are found at 415 and 500 nm,

Between 525 and 565 nm the kinetics are more complex. At 550 nm, the wavelength of the α absorption band of cytochrome f, the leaf absorbance decreases during about 2 s (in our conditions!) after the onset of the actinic light. This 2 s decrease does not reverse when the actinic light is turned off. If the absorbance changes are measured from 500 to 570 nm for a 2 s actinic illumination, the difference spectrum: (absorption before - absorption after the illumination) fits the difference: (absorption of the protochlorophyll(ide)-proteins - absorption of the chlorophyll(ide)-proteins). It may be controlled that the 2 s changes pertain to the exclusive reduction of protochlorophyll(ide), in subtracting from the difference spectrum measured using etiolated leaves, the spectrum measured (with the same 2 s illumination) using leaves in which protochlorophyll(ide) has been already reduced by a

strong flash of light; this substraction does not alter the difference.

When the actinic illumination of the etiolated leaf is continued for times longer than 2 s, the 550 nm absorbance is seen to increase (Fig. 7). The increase starts between second 2 and second 3, apparently when the bulk of the protochlorophyll(ide) has been reduced. It is at once

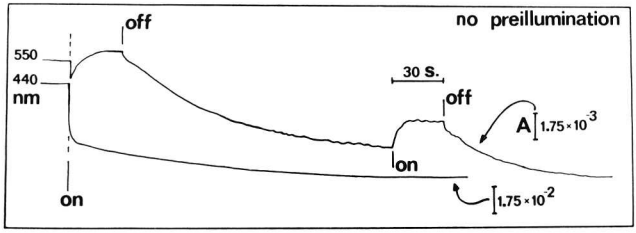

Fig. 7. Absorbance changes seen at 550 and 440 nm when an etiolated leaf is illuminated with red light for the first time. The same red actinic light was used for both traces. The changes at 440 nm are concerned with protochlorophyll(ide) photoreduction only. At 550 nm, this process dominates the kinetics during the first two seconds; after that time the changes are essentially due to the reduction of cytochrome f; the cytochrome is reduced in the light and reoxidised in darkness. At a 1 s illumination of the leaf (on the right) the trace records cytochrome changes only.

rather rapid. Within 2 to 5 s the absorbance rises over its value before leaf illumination. Later on it goes up more slowly, reaching a steady value 10 to 15 s after the start of the illumination. On turning the light off, a rapid decrease is observed. This phase lasts some seconds; it is followed by a slower dark absorbance decrease. In general a final minimum level is reached in darkness after some minutes. This minimum lies below the level reached 2 s after the start of the illumination, showing that the absorbance increase seen in the light after protochlorophyll(ide) photoreduction is fully reversible in darkness.

The 550 nm reversible changes can be repeated several times. After a first 30 s illumination of an etiolated leaf followed by a 60 s dark period, a new illumination provokes a new absorbance rise with a kinetics similar to that of the rise at the first illumination (Fig. 7). In

this case the initial decrease linked to protochlorophyll (ide) reduction does not occur, however. On turning the light off, the absorbance decreases immediately as it does after the first illumination. In our working conditions, a 2 s red illumination, or else a strong 1 ms white flash suffices to suppress the initial decrease of the absorbance at 550 nm. Then light causes the absorbance rise immediately.

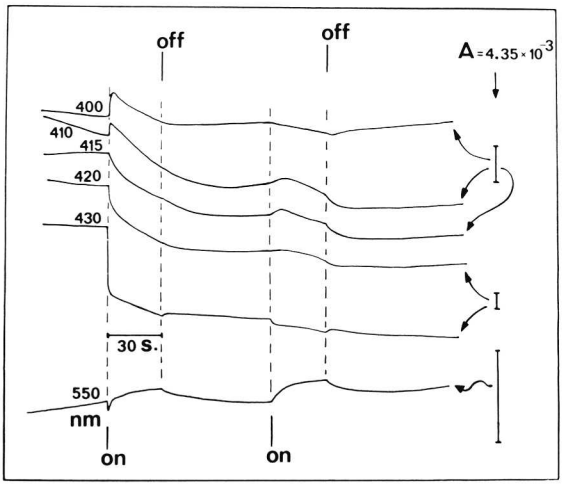

Fig. 8. Absorbance changes seen between 400 and 430 nm when an etiolated leaf is illuminated for the first time with red light. After completion of photochlorophyll(ide) reduction during the first 30 s illumination, the traces record cytochrome changes at 410-420 and in the region of 530-550 nm during a second illumination. The same actinic light was used in all experiments.

Events similar to those recorded at 550 nm are seen between 420 and 410 nm (Fig. 8). In this spectral region the absorbance changes due to protochlorophyll(ide) reduction are large at a first actinic illumination of the leaf. Opposite changes are, however, superposed to them after some seconds of illumination. After completion of protochlorophyll(ide) reduction, in particular at a 1 s illumination of the etiolated leaf, the kinetics are the same at 420-415 nm and at 550 nm. The similarity is restricted to these wavelengths, a circumstance which identifies a

cytochrome. A preliminary examination of the changes seen at several wavelengths between 520 and 560 nm indicates that cytochrome f absorption changes are involved.

Because the observed changes are recorded under a red illumination of the leaf, and because they follow the formation in the leaf of the first chlorophyll(ide)-protein complexes, there is little doubt left that these complexes, or some of them (possibly $C_{696-684}$), exhibit photochemical activity as soon as they appear. The data presently available let us propose that the light they absorb serves very early for reducing cytochrome f.

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Addendum

Since the presentation of this paper, additional measurements of the authors have shown that the absorption changes denoting an early photochemical activity of chlorophyllide in the illuminated, etiolated leaves, are not, or are not essentially, due to cytochrome f reduction. The data pertaining to the possible chemical nature of the products involved will be presented elsewhere (added 19.11.1977).

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