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# THE MECHANISM OF PHOTOREDUCTION OF PROTOCHLOROPHYLL(IDE)

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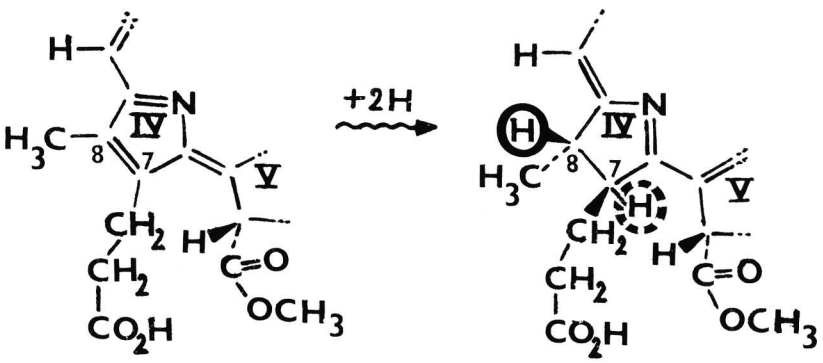
## INTRODUCTION

The protochlorophyll(ide)-protein complexes of higher plants are found in the membranes of dark grown plastids (etioplasts). Their chromophore (-protochlorophyll(ide)-) is a porphyrin. Light absorption by the chromophore results in its reduction to a chlorin (chlorophyll(ide)). This occurs in vivo in a time which does not exceed $10^{-4}$ s. Because, after light absorption, the apoprotein behaves as a catalyst, and because protochlorophyll(ide) behaves as a specific substrate for this catalyst, the reaction may be termed "photoenzymatic". The apoprotein controls the stereospecific addition of 2 hydrogen atoms, in the trans-configuration, at the double bound between carbons 7 and 8 in cycle IV of the tetrapyrrole ring (Fig. 1). The protochlorophyll(ide)-




Protochlorophyllide Chlorophyllide

Fig. 1

protein complex may be extracted in a suitable buffer from the etiolated leaf in a form which preserves its photoenzymatic activity.

Using ascorbic acid as a reductant, Suboch et al.[1,2] were able to photoreduce free protochlorophyll and other metalloporphyrins dissolved in pyridine/N-methyl-piperidine mixtures to chlorin. The hydrogen atoms are added to the 7-8 double bound of protochlorophyll in the cis-configuration; the yield is rather low (30% of the initial protochlorophyll is reduced). These results stress the catalytic role of the apoprotein in the enzymatic photoreduction of protochlorophyll(ide). The complexing of the porphyrin to the apoprotein increases the yield to 100% and insures that the hydrogenation occurs in the trans-configuration.

In 1966, Boardman[3] wrote an excellent review of the state of knowledge on the subject and the reader is referred to this for background material.

1) Structural features

Protochlorophyll(ide)-protein complexes have been extracted from several etiolated plants and they have been purified to variable extents using a variety of techniques. It appeared very difficult to reach a good state of purity whilst retaining a good photoactivity of the isolated complex, since , when extracted in an aqueous solvent, the complex denatures quickly, especially if no protective agent,for instance glycerol,is added to the medium.Even in the presence of such an agent, the active conformation of the complex is changed on extraction from the leaf, as indicated by the observed shifts of the absorption and fluorescence emission maxima (Fig. 2).

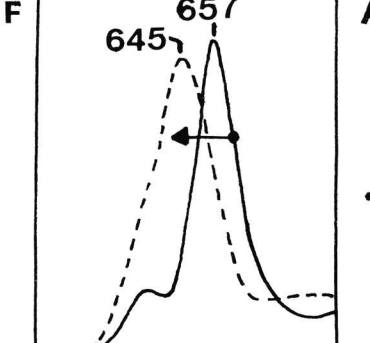



Fig.2. (—) : red absorption and emission spectrum of a fresh, etiolated leaf. Note the presence of a minor component of the absorption at 640 nm;(---) : the same after extraction in a buffer containing 25% glycerol.

A = absorption

F = fluorescence emission spectra registered at -196°C

Schopfer and Siegelman[4] prepared a protochlorophyll(ide)-protein complex from etiolated bean leaves, while Henningsen and Kahn[5] used both barley and bean for their preparations. Molecular weight estimations by Schopfer and Siegelman revealed the presence of aggregates of approximately 550 and 300 KD in their purified preparation, with at least 2 protochlorophyll(ide)s per 550 KD aggregate. Henningsen and Kahn, using the detergent saponin obtained a photoactive protochlorophyll(ide)-protein complex from barley having a molecular weight of 63 KD or lower. Their measurements on energy transfer, circular dichroism (with Houssier[17]) and kinetics of photoreduction gave no indication of more than one protochlorophyll(ide) molecule per 63 KD subunit. They also prepared a photoactive complex from bean with a molecular weight of 100 KD, apparently a subunit of the aggregates of Schopfer and Siegelman. More recently Canaani and Sauer[6] showed that the 600 KD (550 KD) bean aggregate was composed of 12 to 14 polypeptides of 45 KD, each aggregate containing at least 4 protochlorophyll(ide) molecules.

Protochlorophyll(ide) aggregation has been thought to account for the spectral properties of the pigment *in vivo* and in extracted complexes.

1.- The photoactive complexes show a red absorption band at about 640 nm when extracted (Fig.**2** ---) and bands at about 650 nm (major component) and 640 nm (minor component) in the leaf (Fig.**2** ——). *In vitro* experiments proved that protochlorophyllide [7,8,9], protochlorophyll[10] and vinylprotochlorophyll[11] aggregate in non-polar solvents and molecular films.

Experiments of Brouers[14], extending those of Seliskar and Ke[7], led to the interpretation of the *in vivo* 650 nm absorption as due to rather large protochlorophyll(ide) aggregates of dimers ("dimères associés"), while the 640 nm absorption has been ascribed to isolated dimers. The "large" aggregates contain water and in dry benzene they have an absorption band in the blue at 469 nm. It is worth noting that a similar blue band characterises the complex which absorbs *in vivo* in the red at 650 nm[19].

2.- On the other hand, the splitting of the 640 nm band in the circular dichroïsm spectra of protochlorophyll(ide)-protein preparations (from bean) has been interpreted by Schultz and Sauer[15] as suggesting an aggregated form of protochlorophyll(ide), probably a

dimer. Similar observations led Mathis and Sauer[16] to state that "before illumination, protochlorophyllide occurs in a dimeric form"

However, circular dichroïsm spectra did not give any evidence for pigment aggregation in saponin-containing preparations of the protochlorophyll(ide)-proteins with an absorption peak at 640 nm prepared from barley and bean by Henningsen et al.[17]

Protochlorophyll(ide) is easily removed as a monomer from the apoprotein by leaf infiltration with acetone or ether. The forces existing in the pigment-protein interactions are thus rather weak. No more can be said on the pigment-protein interactions in the absence of any significant information on the apoprotein structure.

As far as we know, a 650 nm absorption band was observed in two cases *in vitro*, after extraction from the leaf :

a) In preparations of etioplasts, etioplast fragments and prolamellar bodies, to which NADPH had been added[18].

In this case, carotenoïds, cytochromes, other proteins, etc ... are obviously present in the preparation.

b) In protochlorophyll(ide)-protein preparations made after Schopfer and Siegelman[4] (up to the stage of precipitation by polyethylene-glycol) allowed to stand for days and to dry slowly under calcium chloride[19].

A 650 nm absorption band reappears consistently, but at a reduced intensity, during drying.

The shift of the red absorption maximum from 650 to 640 nm, seen after extraction of the protochlorophyll(ide)-protein complex from prolamellar body membranes, suggests that the *in vivo* structure of the complex is not fully retained after solubilisation, despite being carried out at a low temperature and in a viscous medium. This may reflect the fact that *in vitro*, in water containing media, the environmental conditions do not allow the complex to assume the "aggregation state", or it cannot associate with other vital factors existing *in vivo* (in the membranes). Thus although the photoenzymatic activity is more or less fully retained *in vitro*, one ought to be carefull when extrapolating from the *in vitro* to the *in vivo* situation.

2) Reaction order; energy transfers

In 1954, Smith and Benitez[20] found that the photoreduction of

protochlorophyll(ide) was apparently a second-order reaction. However the reaction rate was found to be proportional to light intensity. On the other hand, using partially purified preparations of the protochlorophyll(ide)-protein complex, Boardman[21] showed that the reduction rate was not influenced by medium viscosity. This suggested that the second order does not result from a collision between two individual, separated molecules.

In fact, the photoreduction kinetics was first-order in the case of the protochlorophyll-protein subunits prepared by Nielsen and Kahn[22] from dark-grown barley. In contrast, it obeyed neither first- nor second- order laws in etiolated barley leaves. Experimenting on bean leaves and extracts, Vaughan and Sauer[23] found that the kinetics was neither first- nor second- order with respect to protochlorophyll(ide).

The first **attemps at** explaining the kinetic features have been made along essentially two lines. It has been proposed :
1) that protochlorophyll(ide) reduction results from a series of complicated, fast reactions inside the pigment-protein complex;
2) that the photons trigger reductions following different parallel routes, depending on the "type" of protochlorophyll(ide)-protein, or protochlorophyll(ide) molecules, by which they are absorbed.

Boardman[21] has studied various models along the second of these lines, but it has not been possible to make a selection from these models on the basis of kinetic data. Sironval et al.[24] followed the photoreduction at room temperature in etiolated bean leaves by measuring the increase of the fluorescence emission at 688 nm due to the formation of chlorophyll(ide). They found that the emission kinetics involved one or two exponential components depending on the actinic wavelength. In order to explain this result, they suggested the existence of two types of pigment-protein complex**es,one** of them involving energy transfer from a non-reducible protochlorophyll(ide) to a reducible protochlorophyll(ide) and the other one lacking the ability for such transfer.

High efficiency energy transfer from one protochlorophyll(ide) molecule to another protochlorophyll(ide) was demonstrated by Kahn et al.[25] who also proved that, after reduction of part of the protochlorophyll(ide) molecules, energy was transferred from protochlorophyll(ide) molecules to newly-formed chlorophyll(ide)s. These

results imply that a number of pigments are organized into units whose structure facilitates energy transfer. Sironval[26] has calculated the mean size of one transfer unit to be 18 pigment molecules at room temperature in the bean leaf. A similar figure has been obtained by Thorne[27] using another method of reasoning.

Sironval's calculation is based on a comparison of the kinetics of the photoreduction, with the kinetics of the phototransformation of the leaf emission spectrum, excited with 436 nm light at - 196°C. If the kinetics of the photoreduction are expressed as the change in time of the percentage (c) of chlorophyll(ide) (among all pigments : photoreduced and photoreducible), and if the kinetics of the phototransformation of the emission spectrum (i) are expressed as the change in time of the fraction of light emitted at -196°C by chlorophyll(ide) when excited with blue light, the following relationship holds :

$$(A - i)\ c = Ki \qquad (1)$$

in which A and K are constants[28]. Amongst other things, this relationship expresses the fact that, as a result of energy transfers, the changes in chlorophyll(ide) content during photoreduction do not coïncide with the changes of the low temperature fluorescence of chlorophyll(ide) excited using photons which are absorbed both by protochlorophyll(ide) and chlorophyll(ide). Comparison of the kinetics of the absorbance and fluorescence changes as registered at room temperature, led Vaughan and Sauer[23] to state that the complexity of the reduction kinetics was due to the transfer of electronic excitation from protochlorophyll(ide) to chlorophyll(ide) during photoreduction.

Indeed, as shown by Nielsen and Kahn[22], the photoreduction kinetics are accounted for by a dynamic model in which the deexcitation of excited protochlorophyll(ide) $P^*$ includes a term expressing energy transfer to chlorophyll(ide) C on the assumption that the transfer probability is linearly dependent upon chlorophyll(ide) concentration; thus :

$$-\frac{dP^*}{dt} = k_t\ P^*C + k\ P^* \qquad (2)$$

in which $k_t$ is the rate constant for deactivation of $P^*$ by transfer, and k accounts for all other first-order deactivation constants.

Under assumption (2), the data of Vaughan and Sauer are in full agreement with those of Nielsen and Kahn, and with those of Sironval and Kuiper (see Brouers and Sironval[29]).

Nielsen and Kahn believe that the energy transferred from protochlorophyll(ide) to chlorophyll(ide) is lost for photoreduction. What then is the fate of this energy ? It could be dissipated, or serve for some biochemical work. If it does not serve for protochlorophyll(ide) reduction, then an increase of the transfer probability would imply decreased reduction rates (all experimental conditions being kept equal). Dujardin[30] found that the probability of energy transfer to chlorophyll(ide) was much greater in lyophilised, etiolated bean leaves than in fresh leaves (Fig. 3). Despi-

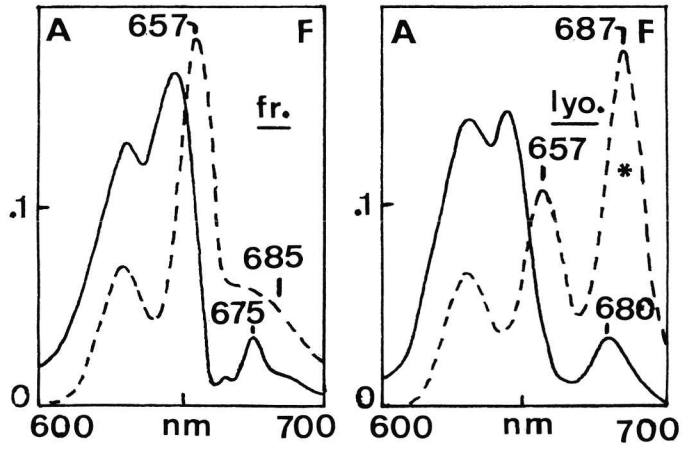



Fig. 3. Comparison of the emission spectrum of a fresh leaf with the emission spectrum of a lyophilised leaf (at -196°C). Both leaves have received the same amount of light energy. The lyophilised leaf has formed somewhat more chlorophyll(ide) (with red absorption at 680 nm; lyo) than the fresh leaf (red absorption at 675 nm; fr). In the lyophilised leaf, the 687 nm emission of chlorophyll(ide) (----; ✱) is greatly enhanced, while the 657 nm emission of protochlorophyll(ide) is partially quenched.

te this, the initial rate of photoreduction was found to be equal or even greater, in lyophilized than in fresh leaves, a result in contradiction to the expectation. This result is worth checking carefully using improved analytical methods.

A dark protochlorophyll(ide) reduction has been shown to occur in bean leaves by Sironval and Kuiper[28]. It is moreover certain that 695 nm photons are used for protochlorophyll(ide) photoreduc-

tion[31] especially when chlorophyll(ide) is present. (See also Fig. 4 and reference 33).

3) Intermediary, short-life states

The reduction of protochlorophyll(ide) (a porphyrin) into chlorophyll(ide) (a chlorin) consists of the addition of 2 electrons and 2 protons on the double bound between carbons 7 and 8 of ring IV of the porphyrin (Fig. 1). In the free pigment, in solution, the reaction is certainly biphotonic. It is not a concerted reaction and it should be possible to detect one or several intermediaries.

Chemists have established that porphyrin hydrogenation proceeds through the addition of hydrogen atoms on the methene bridges and on the pyrrole nitrogens. Products resulting from the reduction of the methene carbons have been called "phlorins". As a result of the rupture of bound resonances in the macrocycle, the phlorins exhibit absorption spectra with rather broad bands between 700 and 800 nm and a low intensity Soret band. They are unstable in the presence of oxygen and water. They isomerize into chlorins or reoxidise into the original porphyrins, depending on the conditions. Conditions may be found in vitro which demonstrate the equilibrium[32] :

$$\text{porphyrin} \underset{-\ 2H}{\overset{+\ 2H}{\rightleftharpoons}} \text{phlorin} \rightleftharpoons \text{chlorin}$$

Dujardin and Sironval[33] succeeded in isolating *in vivo* intermediaries on the route to protochlorophyll(ide) reduction; in the red, the spectral characteristics of these intermediaries resemble those of phlorins. (See fig.4).

When a lyophilised, etiolated bean leaf has been illuminated with sufficiently intense light at -100°C, the characteristic fluorescence of protochlorophyll(ide) at 657 nm is found to have disappeared from the emission spectrum (excited at 436 nm and registered in liquid nitrogen). But, the emission which should appear at 688 nm as a result of the reduction of protochlorophyll(ide) into chlorophyll(ide) is lacking.

At the same time, the absorption shows a broad band which extends up to 750 nm and which peaks around 682-683 nm. This state is stable in liquid nitrogen.

When the leaf is then heated above -90°C in darkness, the long

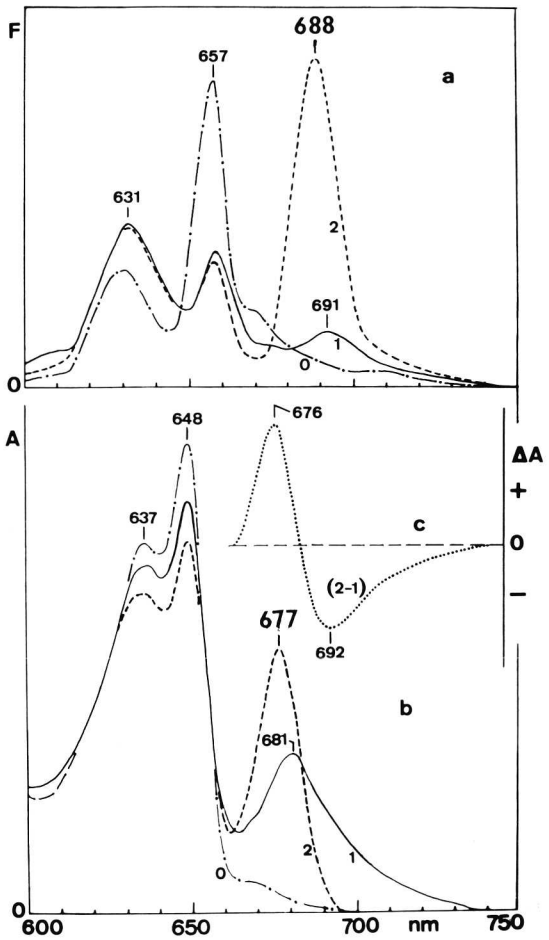



Fig. 4. The figure deals with the experiment which is described in the text, but performed with a fresh leaf. [F, (a)] are fluorescence spectra; [A, (b)] are absorption spectra, all registered at -196°C. Curves 0 (-.-.-.) are the spectra of an etiolated, fresh leaf. Curves 1 (——) are the spectra after having illuminated at -95°C; the fluorescence is quenched, while an absorption tail extends to nearly 750 nm (absorption of the intermediary pigment state (s)). Curves 2 (---) are the spectra after heating for 1 min. at -23°C. The chlorophyll(ide)-protein which absorbs at 677 nm and emits strongly at 688 nm has appeared. [A, (c)] is the absorption difference (after - before heating). Note the absorbance decrease around 640-650 nm after heating in darkness (see Dujardin and Sironval[33]).

wavelength absorption band disappears. The absorption spectrum registered at -196°C shows that an intense band centered at 678 nm has appeared : this is the characteristic red band of the chloro-

phyll(ide)-protein discovered some years ago by Gassman et al.[34], and by Sironval and Michel[24,35]. At the same time, the intense 688 nm fluorescence emission which originates from this chlorophyll(ide)-protein is observed. The difference spectrum (absorption after - absorption before heating) shows that the main absorption of the intermediary (ies) is at 695 nm.

The sequence has been demonstrated to occur not only in the lyophilised leaf, but also in the fresh leaf by illuminating at a low temperature and then warming in the dark. In the fresh leaf, the formation of the chlorophyll(ide)-protein in the dark is accompanied by a decrease of the absorbance at 650 nm (Fig.4).

Even when present in a very low amount the intermediaries are able to quench the fluorescence emitted at -196°C by protochlorophyll(ide) - and chlorophyll(ide)-proteins in the leaf. This may be shown by photoproducing intermediaries after having kept greening leaves in darkness for some hours. Recently, we have been able to follow at room temperature changes of chlorophyll(ide) fluorescence emission due to the transitory formation of the non-fluorescent, intermediary quenchers; this has been done by monitoring rapid kinetics in fresh and lyophilised, etiolated leaves and in fresh, greening leaves. The half life-time of the intermediaries is roughly 10 msec. in the lyophilised leaf and less than 10μsec. in the fresh leaf at room temperature (Franck, Dujardin and Sironval[36]).

It is presently submitted as a working hypothesis that the pigments seen as *in vivo* intermediaries are of the phlorin type. Suboch et al.[1,2] found transitory products in a photoreduction of protochlorophyll in pyridine/N-$CH_3$-piperidine (2:1) using ascorbic acid as a reductant. They give arguments following which these products consist of a mixture of molecules with hydrogenated methene bridges[2]. By admitting air, a cis-chlorin is formed.

*In vivo*, the role of the apoprotein would be in particular to orient the hydrogenations in the trans-configuration. But its role is not necessarily restricted to those reactions which lead to chlorophyll(ide); the complex could as well take part in reactions involving newly-formed chlorophyll(ide) (excited directly or through energy transfer), and this could include for instance photocatalysed oxido-reduction cycles[37].

4) Enzymatic nature of the protochlorophyll(ide)-protein complex

Smith and Benitez[20] have shown that the rate of protochlorophyll(ide) photoreduction depends on temperature. This suggests that the reaction is not purely photochemical. In fact, the only agents known to inhibit reduction are protein-denaturating agents [3,38]. Dujardin[39] reversibly inhibited the photoactivity by heating leaves at 47°C, and then returning them to room temperature, exactly as may be done with some enzymes. Also, the *in vivo* reduction, -as well as the reduction in the isolated pigment -protein -, is highly stereospecific, - in trans -, a configuration which is not favoured by the steric conditions offered by the isolated pigment molecule, and which suggests the participation of a catalytic active centre[40]. Indeed, the enzymic activity requires the protochlorophyll(ide) molecule to be attached to the apoprotein in a characteristic way since the red absorption maximum of the active complex always peaks at 640 nm (in the isolated complex and in the leaf) or at 650 nm (in the leaf).

The substrate-apoprotein complex is remarkably stable in darkness; it persists for days in the leaf at room temperature (after lyophilisation, for years[30]). Light destabilizes the system, the substrate being the sensibilisator. Protochlorophyll(ide) is, however, exchangeable at the binding site as demonstrated by Manetas and Akoyounoglou[41] who incubated isolated protochlorophyll(ide)-proteins in darkness in the presence of radioactive protochlorophyll (ide) and found radioactive chlorophyll(ide) after illumination.

Sundqvist[42,43] and Sundqvist and Klockare[44] demonstrated that the product of the reaction, -chlorophyll(ide)-, leaves the apoprotein, and that it is replaced in darkness at the binding site by a new molecule of protochlorophyll(ide). Fig. 5 reproduces an experiment by Brouers and Sironval[45] which shows that the fluorescence of the protochlorophyll(ide)-proteins which are reconstituted in darkness after a first flash is completely quenched by the chlorophyll(ide) which has been produced by the flash. This indicates that the chlorophyll(ide) molecule does not move far away the protochlorophyll(ide) binding site. The cycle may be depicted as follows :

Protochlorophyll(ide) + apoprotein → [protochlorophyll(ide) ~ protein]

[Protochlorophyll(ide) ~ protein] $\xrightarrow{h\nu}$ [chlorophyll(ide) ~ protein]

[Chlorophyll(ide) ~ protein] → chlorophyll(ide) + apoprotein

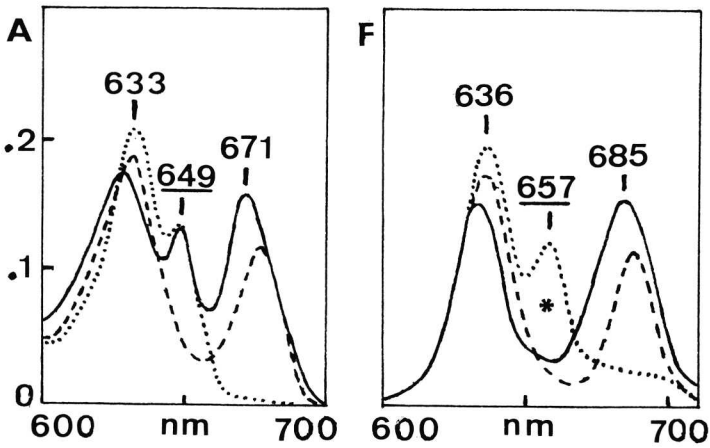



Fig. 5. The spectra (absorption = A; emission = F) of an etiolated leaf fed with Δ - aminolevulinic acid (.....) show the presence of a protochlorophyll(ide) which absorbs at 633 nm and emits at 636 nm and of the photoreducible protochlorophyll(ide) which absorbs at 649(650)nm and emits at 657 nm (underlined). On illumination (----) the photoreducible pigment is transformed into chlorophyll (ide) with absorption band at 676 nm and emission band at 688 nm. After some 30 min. in darkness (——) photoreducible pigments with absorption at 649 (650) nm have been reconstituted. But these pigments do not emit any appreciable fluorescence at 657 nm (*) because the energy is transferred to chlorophyll(ide). Spectra registered at -196°C (see Brouers and Sironval[45]).

All these facts indicate that the apoprotein behaves as an enzyme whose substrate is the protochlorophyll(ide) molecule (photoenzyme). It may be termed a protochlorophyll(ide) photoreductase.

In 1956 Röbbelen[46] produced evidence to suggest that a reduced pyridine nucleotide was the hydrogen donor for the photoreduction of protochlorophyll(ide). This has been demonstrated to be true by Rubin et al.[47] and Griffiths[18]. Griffiths et al.[48,49] have shown that most of the facts related to the above cycle also apply to isolated, barley etioplasts. A typical experiment demonstrates that the cycle is able to produce chlorophyll(ide) at a constant rate in flashing light, provided exogenous protochlorophyll(ide) and NADPH are supplied.

All authors stress the specificity of NADPH in the reduction process. NADPH appears to play a role in the formation and stabilisation of the photoactive complex with red absorption at 650 nm [18,50]. Apparently NADPH (or NADP) remains part of the complex in the dark to form a ternary complex; it does not seem that NADP

leaves the complex before illumination.
The data of Rubin et al.[47] indicate that in etiolated maize leaves reduced pyridine nucleotides are oxidised during the first minutes at the start of protochlorophyll(ide) photoreduction.

It is well known that in conifers, chlorophyll is formed in darkness, while protochlorophyll(ide) does usually not accumulate. Thus, the pathway to chlorophyll appears to be enzymatic in this case. However, in Pinus jeffreyi, Michel-Wolwertz[51] found that it was possible to inhibit dark chlorophyll accumulation, and to introduce a photobiochemical protochlorophyll(ide) to chlorophyll (ide) step by cultivating seedlings in darkness at 10°C. Two protochlorophyll(ide) forms accumulate in this case, one of which emits a fluorescence at 657 nm, the characteristic emission wavelength of the main photoactive protochlorophyll(ide)-protein in higher plants. A 1 msec. flash of light reduces this protochlorophyll(ide) to chlorophyll(ide); but the reduction is never seen in darkness, even after returning the plants to room temperature. A switch from an enzymatic to a photoenzymatic system seems to be here at hand. In this connection, it should be noted that in plastids isolated from dark grown spruce, Oku et al.[52] have obtained chlorophyll(ide) synthesis by flash illuminating in the presence of protochlorophyll(ide) and NADPH.

The following properties of the photoenzymatic system which reduces protochlorophyll(ide) should be stressed :

1) The pigments involved are highy organized; their complexes with proteins aggregate into large units inside the prolamellar body membranes; energy flows easily inside these units towards energy traps, or centres;

2) These centres, - in particular the transient pigments absorbing at 695 nm -, appear to be very efficient quenchers with an exceptional capacity for collecting energy from other pigments;

3) Basically, the system makes use of light energy for performing biochemical work; the fate of the energy collected by the centres may well be to participate to such a work;

4) The process involves a series of events from the singlet excited state of protochlorophyll(ide) to the final, reduced product, - chlorophyll(ide);

5) The reductant which acts in the system is NADPH.

**These properties support the idea that the etiolated system might be related ontogenetically and functionally to the active centres of photosynthesis. The system may be used as a model for the study of energy conversion in green plants, in particular for examining how high quantum yields are achieved in this case.**

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