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The Reduction of Protochlorophyllide into Chlorophyllide

III. The Phototransformability of the Forms of the Protochlorophyllide-Lipoprotein Complex Found in Darkness*

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Abstract

Three distinct forms of the protochlorophyllide lipoprotein complex are found in darkness within the etiolated leaf: a native form absorbing at 647—648 nm, a transformed, still active 639—640 nm form, and a transformed, denatured 627—628 nm form. Methods for producing the transformed forms are freezing and thawing, extraction, heat and acid treatments. The significance of these forms is discussed in relation to the phototransformability of the complex; it is suggested that pigment—pigment interaction is not required for phototransformation, while a given binding to the protein is required.

The native protochlorophyllide-lipoprotein is found in the plastids of the etiolated leaf. When light is given the pigment is reduced and chlorophyll *a* is formed. The process occurs in several steps (SIRONVAL, MICHEL-WOLWERTZ and MADSEN 1965; SIRONVAL *et al.* 1968a, b):

(*a*) Protochlorophyllide-lipoprotein $\xrightarrow{h\nu}$ "intermediary state" (stable in liquid N_2);

(*b*) "Intermediary state" $\xrightarrow{+2H}$ chlorophyllide-lipoprotein;

(*c*) Chlorophyllide + phytol $\xrightarrow{\text{enz.}}$ chlorophyll *a*.

The two last steps are dark steps. The first step needs light; it occurs only when the protochlorophyllide molecule is suitably linked to the lipoprotein.

Details of the red, low temperature absorption (−196°C) of an intact etiolated leaf shows: (*1*) a main peak at 647 nm, (*2*) a shoulder at about 638 nm, (*3*) a shoulder in the 630 nm region. If the leaf receives a flash of white or red light (being immediately frozen afterwards by falling in liquid nitrogen just after the flash) the location of the principal red band shifts (within less than 1 ms) from 647 nm to 676 nm while the band at 630 nm remains unchanged (SIRONVAL *et al.* 1968a). The 676 nm peak consists of a mixture of molecules in the "intermediary state" and of molecules of the reduced chlorophyllide-lipoprotein; the chlorophyllide proportion in the mixture depends on the extent of the phototransformation in step (*a*) (SIRONVAL *et al.* 1968b).

We intend to describe some possible alterations of the native protochlorophyllide-lipoprotein and to discuss these alterations in comparison with the native complex. Earlier papers related to the subject are those of KRASNOVSKIĬ and KOSOBUTSKAYA (1952), SMITH *et al.* (1957),

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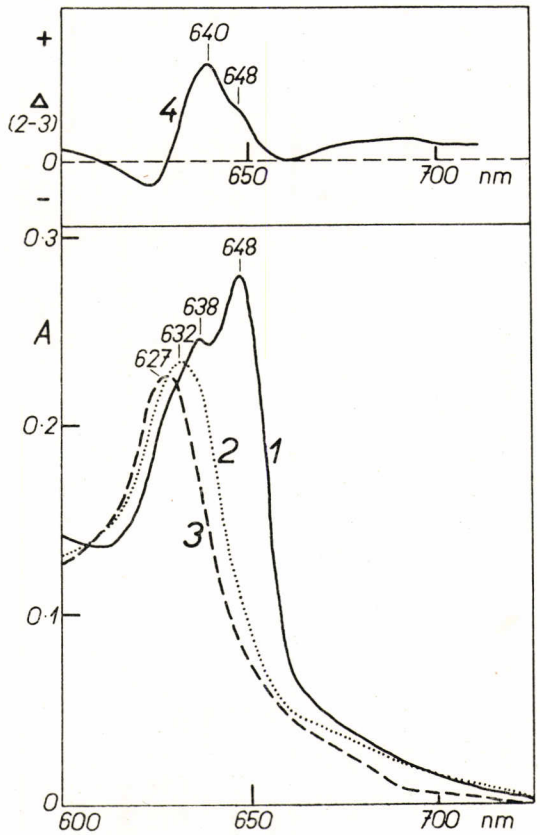

Fig. 1. Details of the red part of the absorption of an etiolated, primary bean leaf as measured at −196 °C. *1*: intact, etiolated primary leaf. *2*: The leaf has been frozen to liquid nitrogen temperature, warmed up to room temperature and examined later on at −196 °C. *3*: Five (freezing−thawing) treatments before examination of the spectrum at −196 °C. *4*: Difference (*2* − *3*). All manipulations made in darkness.

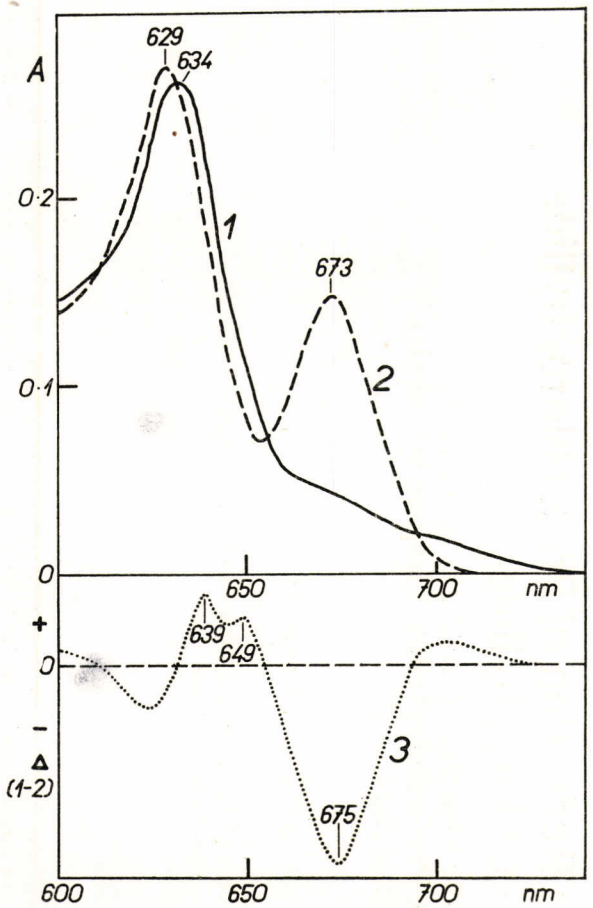

Fig. 2. *1*: An etiolated leaf has been frozen at liquid nitrogen temperature, warmed up to room temperature in the dark and examined after freezing again at −196 °C. *2*: Another identical leaf has been frozen at liquid nitrogen temperature, warmed up in the dark to room temperature, submitted to a flash of polychromatic light and immediately frozen again at −196 °C. *3*: Difference (*1* − *2*).

KRASNOVSKIĬ, BYSTROVA and SOROKINA (1961), BUTLER and BRIGGS (1966) and others (see the review by BOARDMAN 1966).

MATERIAL AND METHODS

Primary bean leaves of the "Commodore" variety were used. Methods for growing the leaves in complete darkness, for illuminating them, for measuring the low temperature absorption and for registering the low temperature emission spectra are described in SIRONVAL *et al.* (1968a). For the method of keeping the leaves at a temperature higher than 40 °C see SIRONVAL and BROUERS (1969).

Preparation of leaf extracts: Etiolated primary leaves (= 1 g) were carefully ground in a little mortar either in about 5 ml 0·1M phosphate buffer, pH 7·0, or in about 5 ml of a mixture of this buffer and glycerine, 50 or 30% glycerine in volume. The resulting liquid was centrifuged at 1000 × g for 20 min. The supernatant was used for the experiments.

Illumination of the extracts: A drop from the extracts was placed in the centre of a sample holder and was submitted, at room temperature, to a 1 ms polychromatic flash as described in SIRONVAL *et al.* (1968a).

Vacuum infiltration with 0·01N HCl: Etiolated primary leaves were dipped in 0·01N HCl. The vessel was placed under vacuum and the air was allowed to penetrate again after 1 to 2 min vacuum. This treatment was repeated until the leaves had fallen to the bottom of the vessel (usually 3 times).

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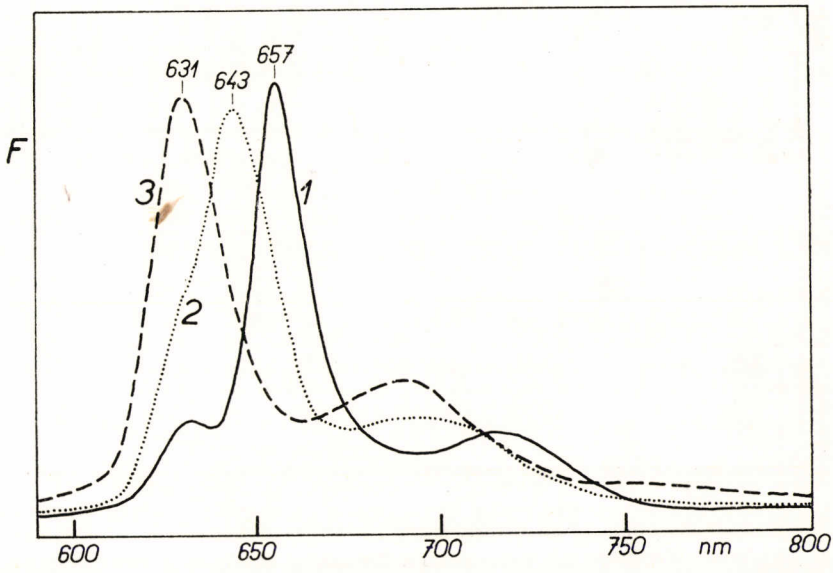

Fig. 3. *1*: Fluorescence spectrum of an intact, etiolated leaf at −196 °C. *2*: Fluorescence at −196 °C of an extract of a same leaf made in the mixture glycerine — 0·1M phosphate buffer, pH 7·0 (30/70, *v*/*v*). *3*: like curve *2*, but the extract was made in the 0·1M phosphate buffer, pH 7·0. The extractions and freezing at liquid nitrogen temperature occurred in darkness.

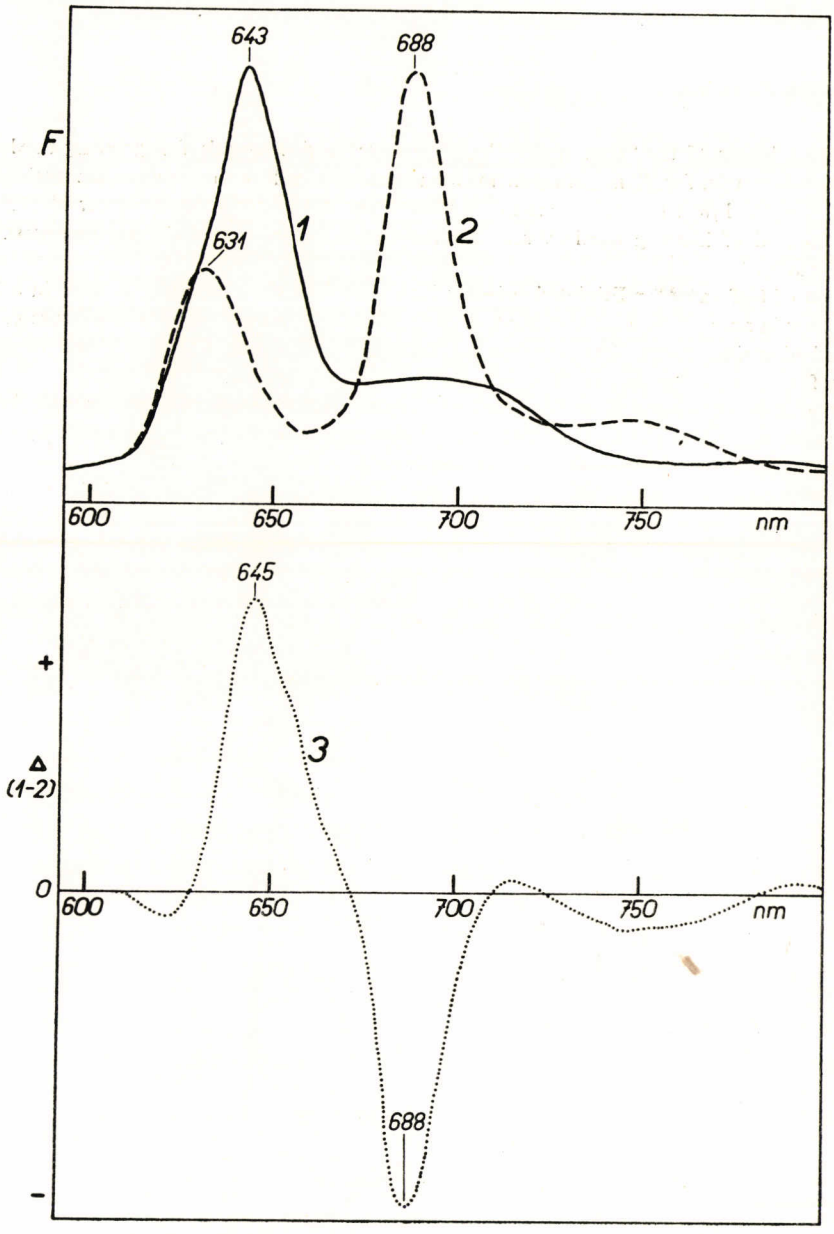

Fig. 4. Phototransformation of an extract made in the mixture glycerine — 0·1M phosphate buffer, pH 7·0 (30/70, *v*/*v*). *1*: Fluorescence of the extract frozen at −196°C; extraction and freezing at nitrogen temperature occurred in darkness. *2*: The same extract (made in darkness) received a polychromatic flash and was thereafter frozen at liquid nitrogen temperature. *3*: Difference (*1* — *2*).

RESULTS

1. Freezing and thawing

Fig. 1, curve *2*, reproduces the spectrum obtained in the red when an etiolated leaf is first frozen in liquid nitrogen, then quickly thawed to +37 °C and frozen again at −196 °C. The absorption maximum has been shifted from 648 nm to 632 nm. If the freezing-thawing-freezing treatment is repeated several times, the maximum shifts further to 627 nm (Fig. 1, curve *3*). When absorbing at 627−628 nm, the protochlorophyllide is no longer transformed by light.

However after the first freezing-thawing treatment a fraction of the pigment remains phototransformable. This fraction is characterized by red peaks at about 640 nm (principal peak) and at 647−648 nm (secondary peak) as shown by the difference (spectrum before − spectrum after a flash) (Fig. 2, curve *3*). The immediate product of the phototransformation of the 640 nm fraction absorbs at 675 nm. The 640 nm active fraction disappears by repeating the freezing-thawing treatment (Fig. 1, curve *4*).

2. Extraction

Fig. 3 gives in solid line (curve *1*) the fluorescence spectrum of an etiolated leaf, when cooled at liquid nitrogen temperature; the maximum is situated at 657 nm. The dashed line (curve *3*) gives the low temperature fluorescence of the pigment-lipoprotein extracted in a 0·1M phosphate buffer, pH 7·0; its maximum is at 631 nm. When a flash is given to this extract the phototransformation does not occur. If the extract is made in a solvent of higher viscosity (able to protect the protein configuration, for instance a glycerine-phosphate buffer mixture, — 0·1 M phosphate,

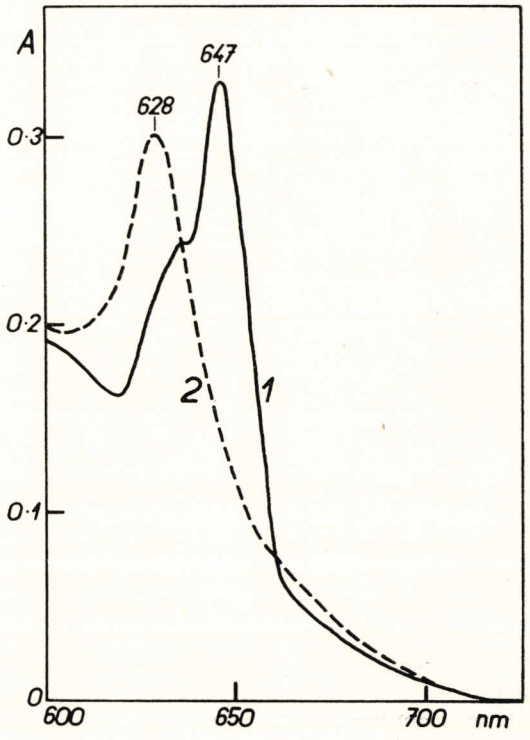

Fig. 5. Red part of the −196 °C absorption spectrum of an intact, etiolated leaf (*1*) and of a same leaf which had been incubated at 47 °C for 60 min in the dark (*2*).

pH 7·0—, 30 or 50% glycerine), the fluorescence maximum is found at about 643 nm (Fig. 3, curve *2*). In this case the pigment-lipoprotein is still phototransformable as can be seen in Fig. 4.

Absorption spectra of the extracts show that the inactive pigment absorbs at 627 nm, while the absorption of the glycerine-phosphate buffer extract is located at 639 nm.

3. Thermal denaturation

Fig. 5 compares the absorption of an intact, etiolated leaf (curve *1*) to the spectrum obtained after incubating the leaf at 47 °C for 60 min (curve *2*). The absorption is shifted from 647 nm to

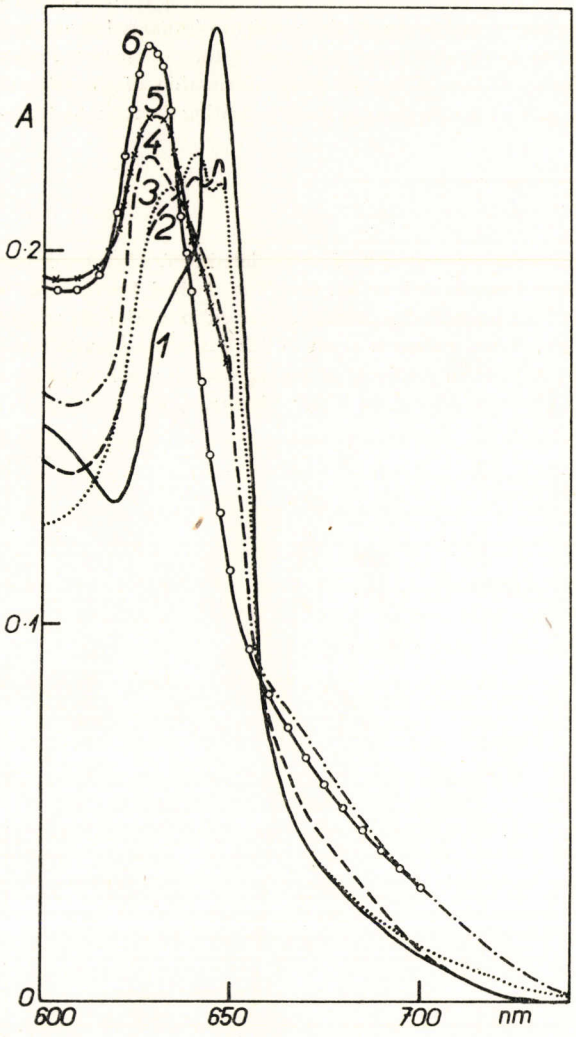

Fig. 6. Time course of the denaturation of the protochlorophyllide-lipoprotein complex in the leaf at 45 °C. *1*: —196 °C spectrum of an intact, etiolated leaf. *2* to *6*: —196 °C spectra respectively after 20, 40, 50, 60 and 150 min at 45 °C in the dark.

628 nm by the heat treatment. The warmed, 628 nm absorbing pigment-lipoprotein complex is completely photoinactive. Thermal denaturation of the complex is known to occur when the temperature of the leaf is kept higher than +40 °C (SMITH and BENITEZ 1954; see other details in SIRONVAL and BROUERS 1969).

The shift of the red absorption was followed in the course of time at 45 °C (Fig. 6). Already after 20 min the decrease at 647 nm was evident (curve *2*) while the absorption around 640 nm appeared. Within the same time interval, the 630 nm shoulder increased somewhat. After 40 min (curve *3*) the 640 nm band was clearly seen. After 50 min, the 647 nm and 640 nm peaks were strongly reduced, while the 630 nm peak had become by far the most important (curve *4*). Curve *6* (150 min) shows only the 627 nm, photoinactive pigment.

4. Denaturation in the presence of HCl

Fig. 7*A* gives the low temperature emission of an etiolated leaf after vacuum infiltration in the dark with 0·01N HCl. It appears that, from the first to the third hour which followed infiltration, the low-temperature fluorescence increased between 630 and 640 nm, while the 657 nm peak shifted to about 649 nm (curve *2*). At that stage, a saturating white flash completely transformed the 649 nm emitting species into a 688 nm emitting species, while an emission persisted in the 630—640 nm region (curve *3*; sometimes two distinct bands were seen at 630 and 645 nm, respectively).

During the fourth hour infiltration, the 629 nm peak was predominant, while the 649 nm peak disappeared (Fig. 7*B*; curve *1*). Later on, a peak reappeared at 656 nm while the emission around 710 nm increased. This last process was shown to result from the pheophytinisation of protochlorophyllide (curve *2*).

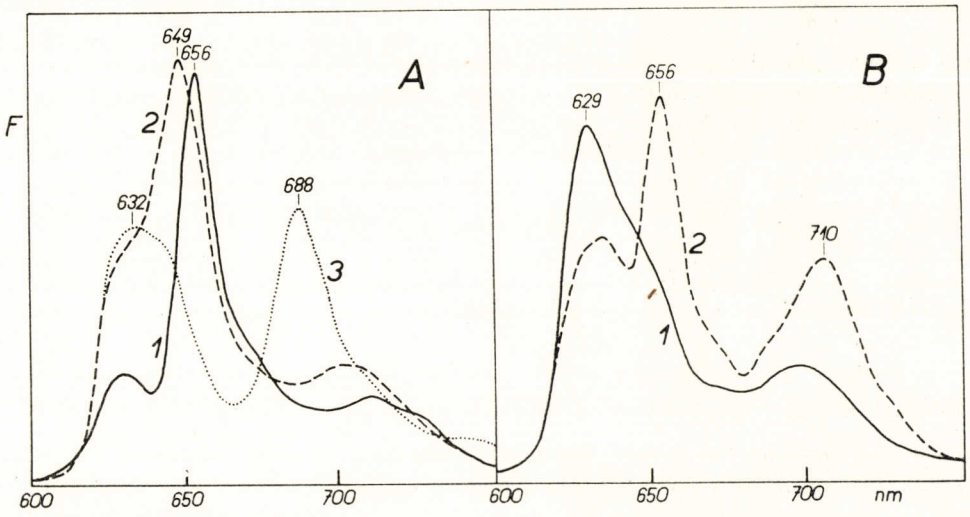

Fig. 7. Denaturation of the protochlorophyllide-lipoprotein complex after vacuum — infiltration of the leaves with HCl 0·01N. *A1*: —196 °C fluorescence of an intact, etiolated leaf. *A2*: —196 °C fluorescence 3 h after infiltration by 0·01N HCl in the dark. *A3*: a flash of polychromatic light was given to the leaf 3 h after infiltration; the leaf was immediately frozen to liquid nitrogen temperature after the flash and its —196 °C fluorescence examined. *B1* and *B2*: —196 °C fluorescence respectively 4 and 5 h after infiltration (in the dark).

Infiltration of the leaf with 0·01N HCl after thermal denaturation (1 h at 47 °C) did not change the location of the 629 nm emission peak characterizing the heat-denaturated pigment — lipoprotein. The pheophytinisation of protochlorophyllide had started 1 h after infiltration (Fig. 8*A*, curve *2*).

136 Fig. 8*B* (curve *2*) shows that the 629 nm emission was produced within a very short time when the pH of a leaf extract in a glycerine-phosphate buffer was lowered to 3·4 (with HCl). Also in this case, pheophytinisation followed as a consequence of lowering of the pH (curve *3*). We intend to describe the pheophytinisation process elsewhere.

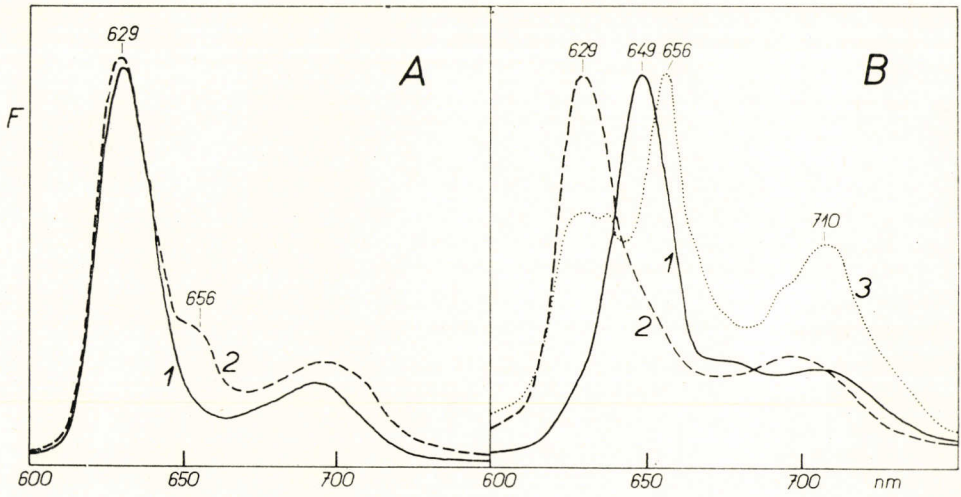

Fig. 8. *A*: *1*: —196 °C fluorescence of an etiolated leaf after 1 h at 47 °C in darkness. *2*: An etiolated leaf remained 1 h at 47 °C; it was afterwards infiltrated under vacuum with 0·01N HCl. 1 h later, the leaf was frozen at liquid nitrogen temperature and its fluorescence registered. *B*: *1*: —196 °C fluorescence of a glycerine — 0·1M phosphate buffer extract (50/50, *v*/*v*; pH 7·0) of an etiolated leaf. *2*: —196 °C fluorescence immediately after lowering the pH of the extract to 3·4 by addition of HCl. *3*: the same as curve *2*, 1 h later. All manipulations made in darkness.

DISCUSSION AND CONCLUSIONS

Two kinds of blue shifts of the 647—648 nm absorption are observed when an etiolated bean leaf is submitted, in the dark, to various treatments:

(*a*) — an 8 nm shift, from 647—648 to 639—640 nm;
(*b*) — a 20 nm shift, from 647—648 to 627—628 nm.

After having undergone the 8 nm shift, the pigment remains phototransformable; it is found inactivated when the 20 nm shift has occurred.

The shifts could reflect either changes in the links between the pigment and the protein, or changes concerned with the pigment-pigment interactions, or else chemical changes of the pigment. These changes do not exclude one another; they could possibly be involved at the same time. A stable, chemical modification of the pigment seems improbable since extraction always yields protochlorophyllide.

The 20 nm blue shift is produced by heat, by acidification, or by extracting the lipoprotein-pigment complex out of the leaf in a medium of low viscosity (phosphate buffer). It obviously appears to result from the denaturation of the lipoprotein.

SELISKAR and KE (1968) have found a red shift of some 20 nm when standing purified protochlorophyllide in a solvent of low dielectric constant (dry benzene for 5 h). In their experiments the main red absorption shifted from 634 to 651 nm; at the same time the intensity of the fluorescence decreased while the scattering of the 440 nm light (used for excitation) increased. SELISKAR and KE think that this behaviour is attributable to pigment aggregation. In their opinion, the *in vitro* aggregates are probably polymers rather than dimers owing to the "relatively long time necessary for the establishment of the equilibrium". They also suggest "a contribution by aggregation to the *in vivo* spectral properties" of protochlorophyllide.

On the other hand, BUTLER and BRIGGS (1966) freeze a leaf to −196 °C and find a clear phototransformation after subsequent thawing. Freezing, thawing and refreezing (once) in the dark showed a shift of the main absorption to 635 nm. A repetition of BUTLER and BRIGGS' experiment gave the spectra shown in Fig. 2 in good agreement with the results of the authors. The difference {[freezing — thawing — darkness — freezing] — [freezing — thawing — light — freezing]} showed that the phototransformed species absorbs at 640 nm, including the region around 650 nm. It is further seen in Fig. 1 that the 640 nm species disappears from the first to the 5th repetition of the (freezing — thawing) treatment, along with the inactivation of the photochlorophyllide-lipoprotein complex.

The 640 nm absorbing pigment-protein is isolated when extracting the leaf in a medium containing 30 to 50% glycerol (Figs. 3 and 4). The principal red absorption band of the active complex, when purified in the presence of glycerol and Triton X-100, has also its principal red band located at 639 nm (SCHOPFER and SIEGELMAN 1968). The activity appears to be lost and the red band shifts to 627 nm when the viscosity of the medium is lowered (Fig. 3). The loss of the activity was studied by several investigators (see the review of BOARDMAN 1966, pp. 456 and 457).

We may discuss the nature of the 627—628, 639—640 and 647—648 nm absorption bands as follows:

(*1*) The 627—628 nm absorption is very near or identical to the absorption of protochlorophyllide in solution. It corresponds to the situation of free pigment molecules (possibly with feeble links to the protein) behaving independently of another one. The pigment would be more or less liberated from the protein when the latter is denatured.

(*2*) The 639—640 nm absorption does not necessarily require the assumption of any pigment-pigment interaction, since a 10 nm red shift from the location found in solution can result from pigment-protein interactions as discussed by BOARDMAN (1966). Indeed, the 639—640 nm absorption characterises the 300.000 MW phototransformable complex isolated by SCHOPFER and SIEGELMAN (1968) which apparently contains one single protochlorophyllide per protein molecule. Moreover this absorption band is not found *in vitro* upon pigment aggregation in the absence of protein (SELISKAR and KE 1968).

(*3*) Finally the 647—648 nm absorption of the etiolated leaf should result from pigment-pigment interaction (aggregation). Firstly, as stated above, it corresponds more or less to the absorption found upon aggregation of protochlorophyllide in dry benzene. Secondly, it is easily destroyed by mechanical treatments such as extraction, or freezing and thawing which are able to disrupt aggregates of subunits inside a protein.

138 This picture implies that pigment-pigment interaction is not required for phototransformation, while a given binding to the protein is required. We would like to point out that the expected limited number of suitable protein sites for this binding explains the behaviour of etiolated leaves when fed with δ-aminolevulinic acid. The excess of non-phototransformable protochlorophyllide found in this case is known to absorb around 630 nm. The wavelength of this absorption, as well as the non-transformability, reflect the same feature: the pigment in excess is (at least) unsuitably linked for phototransformation, — a consequence of the limitation of the number of suitable, active sites in the lipoprotein, and of lipoprotein molecules.

REFERENCES

BOARDMAN, N. K.: Protochlorophyll. — In: VERNON, L. P., SEELY, G. R. (ed.): The Chlorophylls. Pp. 437—479. Academic Press, New York 1966.

BUTLER, W. L., BRIGGS, W. R.: The relation between structure and pigments during the first stages of proplastid greening. — Biochim. biophys. Acta **112**: 45—53, 1966.

KRASNOVSKIĬ, A. A., KOSOBUTSKAYA, L. M.: Spektral'noe issledovanie sostoyaniya khlorofilla pri ego obrazovanii v rastenii i v kolloidnykh rastvorakh veshchestva etiolirovannykh list'ev. [Spectral investigation of chlorophyll state during its formation in plants and in colloid solutions from etiolated leaves.] — Dokl. Akad. Nauk SSSR **85**: 177—180, 1952.

KRASNOVSKIĬ, A. A., BYSTROVA, M. I., SOROKINA, A. D.: Fraktsionirovanie razlichnykh pigmentnykh form v gomogenatakh etiolirovannykh i osveshchennykh list'ev. [Fractionation of different pigment forms in homogenates of etiolated and illuminated leaves.] — Dokl. Akad. Nauk SSSR **136**: 1227—1230, 1961.

SCHOPFER, P., SIEGELMAN, H. W.: Purification of protochlorophyllide holochrome. — Plant Physiol. **43**: 990—996, 1968.

SELISKAR, C. J., KE, B.: Protochlorophyllide aggregation in solution and associated spectral changes. — Biochim. biophys. Acta **153**: 685—691, 1968.

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

SIRONVAL, C., MICHEL-WOLWERTZ, M.-R., MADSEN, A.: On the nature and possible functions of the 673- and 684 mμ forms *in vivo* of chlorophyll. — Biochim. biophys. Acta **94**: 344—354, 1965.

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

SIRONVAL, C., BRONCHART, R., MICHEL, J.-M., BROUERS, M., KUIPER, Y.: Structure macromoléculaire et activités photochimiques des lamelles plastidiales (essais). — Bull. Soc. franç. Physiol. vég. **14**: 2, 195—225, 1968 b.

SMITH, J. H. C., BENITEZ, A.: The effect of temperature on the conversion of protochlorophyll to chlorophyll *a* in etiolated barley leaves. — Plant Physiol. **29**: 135—143, 1954.

SMITH, J. H. C., KUPKE, D. W., LOEFFLER, J. E., BENITEZ, A., AHRNE, I., GIESE, A. T.: The natural state of protochlorophyll. — In: GAFFRON, H. (ed): Research in Photosynthesis. Pp. 467 to 474. J. Wiley (Interscience), New York 1957.