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CIRCULAR DICHROISM AND FLUORESCENCE CHANGES ACCOMPANYING
THE PROTOCHLOROPHYLLIDE TO CHLOROPHYLLIDE
TRANSFORMATION IN GREENING LEAVES AND HOLOCHROME
PREPARATIONS

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SUMMARY

The photoreduction of protochlorophyllide to chlorophyllide in holochrome preparations or homogenates from etiolated bean seedlings (*Phaseolus vulgaris* L.) is followed by a series of dark steps involving changes in the absorption and circular dichroism spectra and in the polarization of the chlorophyllide fluorescence at 7 °C. Related changes can be seen in the pigment-protein, protochlorophyllide holochrome, isolated from bean etioplasts. The circular dichroism and fluorescence polarization spectra, which are particularly sensitive to close pigment association, suggest that the unilluminated holochrome contains protochlorophyllide *a* in an aggregated form, probably as dimers. Illumination produces a chlorophyllide *a* holochrome which initially exhibits evidence of chlorophyll aggregation, but which undergoes dissociation to perhaps a monomeric form within a few minutes at 0 °C. If etiolated leaves are illuminated for periods longer than 1 h, they then appear to accumulate chlorophyll *b* and chlorophyll *a* in aggregated forms that are distinct from one another. The relevance of these findings to the development of photosynthetically active plastid membranes is discussed.

INTRODUCTION

Leaves of angiosperms grown in the dark synthesize small amounts of protochlorophyllide *a* and of protochlorophyll *a*, but the final steps in the synthesis of chlorophyll *a* have a mandatory requirement for light¹. The photochemical reduction of protochlorophyllide *a* results in the stereospecific addition of two hydrogen atoms to ring IV of the porphyrin. This photoreduction exhibits many characteristics of an enzymatic reaction². It can be carried out in the intact leaf, in leaf homogenates, by isolated etioplasts or prolamellar bodies, and by a purified protochlorophyllide-protein complex known as holochrome³. Light absorbed by protochlorophyll(ide) is effective in initiating this conversion⁴.

Abbreviation: MCD, magnetic circular dichroism.

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The preparation of protochlorophyllide holochrome was first reported by Krasnovskii and Kosobutskaya⁵ and by Smith and Benitez⁶. Subsequently, preparations of higher purity were obtained by Smith⁷, by Boardman⁸ and by Schopfer and Siegelman⁹. The molecular weight of the major component is 550 000 (ref. 9) to 600 000 (ref. 8), but there is evidence of dissociation into subunits of about half this value⁹. The content of protochlorophyllide is the subject of some difference of opinion: values of 1–4 protochlorophyllides per protein of 550 000 daltons are variously reported^{8–10}.

Following the first illumination of etiolated leaves to form chlorophyllide *a*, there occurs a series of dark spectral shifts during intervals of a few seconds to a few hours¹¹. During this interval the chlorophyllide becomes esterified with phytol¹². The spectral shifts are believed to reflect a varying environment and/or state of aggregation of the chlorophyll pigments. Boardman¹³ and Bogorad *et al.*¹⁴ present evidence that the protochlorophyllide–protein complex undergoes dissociation following photoreduction. This step may result in the relocation of a smaller pigment–protein fragment by incorporation into the germinal chloroplast lamellae¹⁴.

In this study we have investigated the state of the pigment molecules at the different stages of greening through the use of circular dichroism and fluorescence spectrometry and fluorescence polarization. These methods are particularly well suited to the study of interacting or aggregated chromophoric molecules^{15,16}.

EXPERIMENTAL

Growth of etiolated bean seedlings

Red kidney beans (*Phaseolus vulgaris* L.) were germinated and grown in vermiculite at room temperature for 7–11 days in complete darkness. The seedlings were harvested under a dim green safelight, except for those experiments where a preillumination was given.

Barley (*Hordeum vulgaris*, strain Lyon) and the barley mutant lacking chlorophyll *b* (*H. vulgaris*, strain Chlorina)¹⁷ were grown in vermiculite in a growth chamber under fluorescent lights. Chloroplast fragments were prepared essentially by the method of Park and Pon¹⁸.

Preparation of holochrome

Holochrome preparation followed the general procedure of Schopfer and Siegelman⁹, except that the DEAE-cellulose and agarose chromatographies were omitted*. Our method is described in full elsewhere¹⁹.

Protochlorophyllide holochrome in 0.02 M Tris–HCl was prepared as follows: The crude extract was chromatographed on hydroxylapatite, as described by Schopfer and Siegelman. Fractions containing protochlorophyllide holochrome were then centrifuged at $360\,000 \times g$ (Spinco L2-65B ultracentrifuge, Type 60 TI rotor) for 4 h at 1°C. The sediment was gently resuspended in 0.02 M Tris–HCl, and unsuspended material was removed from the solution by centrifugation at $20\,000 \times g$ for 30 min at 0°C. This material, designated protochlorophyllide holochrome, was used directly in the spectrometric studies.

* In all of our preparations, Tris buffer was substituted for the tricine used by Schopfer and Siegelman. The pH of the buffers was adjusted to 8.0 throughout.

In an alternative preparation in approx. 2 M sucrose, 0.25 M potassium phosphate and 0.02 M Tris-HCl, pH 8.0, the fractions eluted from the hydroxyl-apatite column were first clarified at $78000 \times g$ (30 min) and then concentrated by ultrafiltration against powdered sucrose. This preparation, designated protochlorophyllide holochrome (sucrose), was found to be considerably more stable than the preparations with sucrose absent. In some cases the normal protochlorophyllide holochrome was diluted with an equal volume of 4 M sucrose, 0.02 M Tris-HCl, in order to prepare the stabilized material.

Chlorophyllide holochrome was produced by illuminating the protochlorophyllide holochrome or protochlorophyllide holochrome (sucrose) solution in a 1-cm cuvette immersed in a beaker of ice-water using a 150-W floodlamp about 12 cm distant for 5–10 sec. Such preparations contain not only chlorophyllide *a*, but also protochlorophyllide *a* which did not photoreduce (inactive protochlorophyllide *a*).

Preparation of homogenates of post-etiolated leaves

Etiolated bean seedlings (7–11 days old) were illuminated for various periods from 50 min to 73 h under overhead room lights (GE Warm White fluorescent). The illumination measured at the specimens was 90 ± 10 ft candles. Following illumination, the two primary leaves were plucked from about 100 plants, the samples were weighed and then homogenized at 0–5 °C in a blender (Waring) with 4 ml 0.05 M potassium phosphate (pH 8.0) per g of leaves. The homogenate was strained through 8 layers of cheesecloth and sonicated (Biosonik) for 10 sec at full power. The sonicate was centrifuged at $14000 \times g$ (Type 40 rotor) for 10 min. The supernatant was then centrifuged at $100000 \times g$ for 30 min, and the sediment resuspended in 1 ml of 0.05 M potassium phosphate buffer. This was clarified by centrifugation at $10000 \times g$ for 10 min and the supernatant was used for the spectral measurements.

Absorption and CD spectra

Absorption spectra were measured using a Cary Model 14 spectrophotometer. CD and magnetic circular dichroism (MCD) spectra of the holochrome preparations were measured using an instrument built originally by Dr E. A. Dratz²⁰ and provided with a multi-channel analyzer for signal improvement through multiple scanning. The magnetic field strength was 11 kG. The CD spectra of the leaf homogenates were obtained using a Cary Model 60 spectropolarimeter with a Model 6001 CD accessory and a special red-sensitive photomultiplier (Hamamatsu TV Co., R136). The CD instruments were calibrated using a solution of (+)-camphorsulfonic acid (1 mg/ml)²¹.

All spectra were recorded at 0–5 °C. The presence of high concentrations of sucrose in some of the samples did not interfere with the CD measurements, as would have been the case for ORD measurements.

Fluorescence measurements

An Aminco-Bowman spectrophotofluorometer (American Instrument Co., Baltimore, Md.) was modified as follows: The emission monochromator grating was replaced with one blazed for optimal efficiency at 700 nm. Wavelength calibration was carried out using a low pressure mercury arc. The excitation beam was modulated using a vibrating slit operating at 200 Hz (American Time products, Woodside, N.Y., Type 40 Light Chopper) and phase detected (Princeton Applied Research Corp.,

Princeton, N.J.; Model 210 selective amplifier, Model 220 lock-in amplifier and Model 221 high-voltage power supply). A red-sensitive photomultiplier (RCA 7102, Type S-1 photocathode) was cooled by solid CO_2 . Excitation and emission spectra were recorded using an XY-recorder (Moseley/Hewlett Packard, Palo Alto, Calif., Model 2D-2A) and were not corrected for wavelength variation of the efficiency of the optical system. Monochromator bandwidths (at half maxima) noted in the figure captions were estimated from the widths of the peaks resulting from light scattering. Sample temperatures were controlled to about 7 °C. Cuvettes with four clear sides had square cross-sections of 10.0 mm internal diameter.

Excitation spectra were recorded using a supplementary sharp-cut glass filter (Corning C.S. 3-73) in the emission beam just before the photomultiplier. Fluorescence polarization spectra were recorded using similar filters (Corning C.S. 3-68 or 3-66). Glan-type crystal polarizers (American Instrument Co.) were used in both the excitation and emission beams. Polarization values were measured and corrected as described by Houssier and Sauer²².

RESULTS

CD spectra

Protochlorophyllide holochrome in 2 M sucrose. The absorption, CD and MCD spectra of protochlorophyllide holochrome (sucrose) are presented in Fig. 1. The

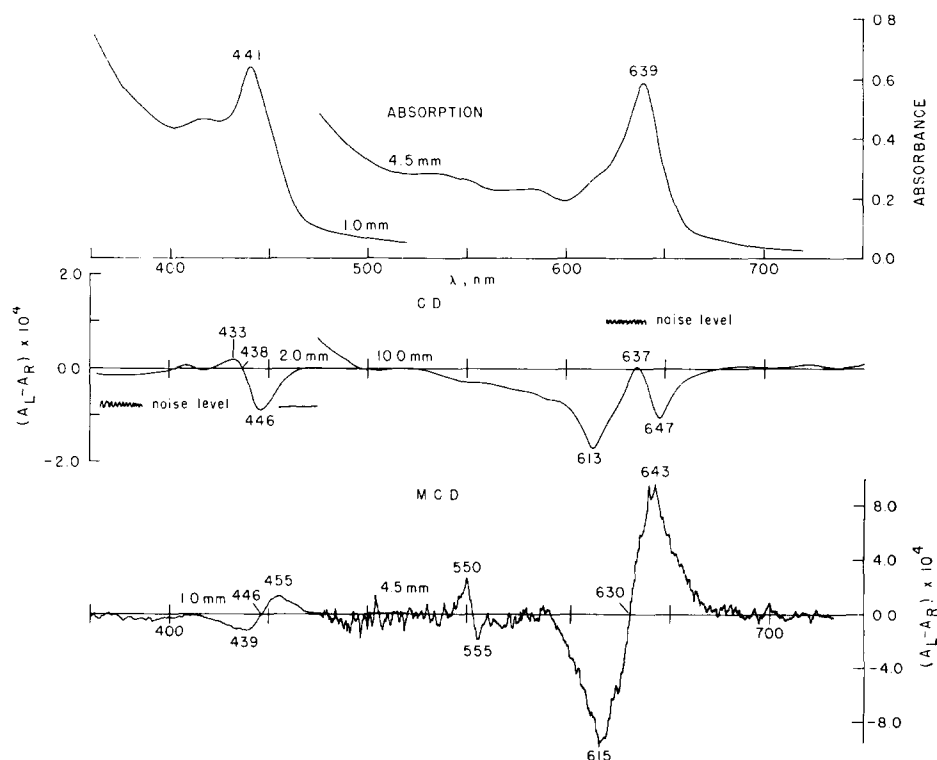


Fig. 1. Absorption, CD and MCD (at 11 kG) spectra of protochlorophyllide holochrome (sucrose). Optical path lengths and typical noise levels of the CD spectrum are indicated.

CD spectrum exhibits troughs at 446, 613, and 647 nm, a small maximum at 433 and a relative maximum at 637 nm. This spectrum differs strikingly from that of protochlorophyll *a* in ether solution²³, both in the signs and the apparent multiplicities of the CD components. By contrast, the MCD and, superficially, the absorption spectra of protochlorophyllide holochrome (sucrose) resemble those of protochlorophyll *a* in ether rather closely, apart from the wavelength shifts. The absorption and CD spectra of protochlorophyllide holochrome in the absence of sucrose were indistinguishable from those shown in Fig. 1.

Pigment aggregation is the origin of multiplicity (band splitting) in the spectra of the chlorophyll pigments^{23,24}. In dry non-polar solvents, such as carbon tetrachloride, these pigments are known to undergo dimerization and higher aggregation^{23,25}. The CD spectrum of protochlorophyll *a* dimers in carbon tetrachloride exhibits dramatic evidence of this band splitting, whereas the absorption and MCD spectra are superficially similar to those of monomeric protochlorophyll *a* in ether²³.

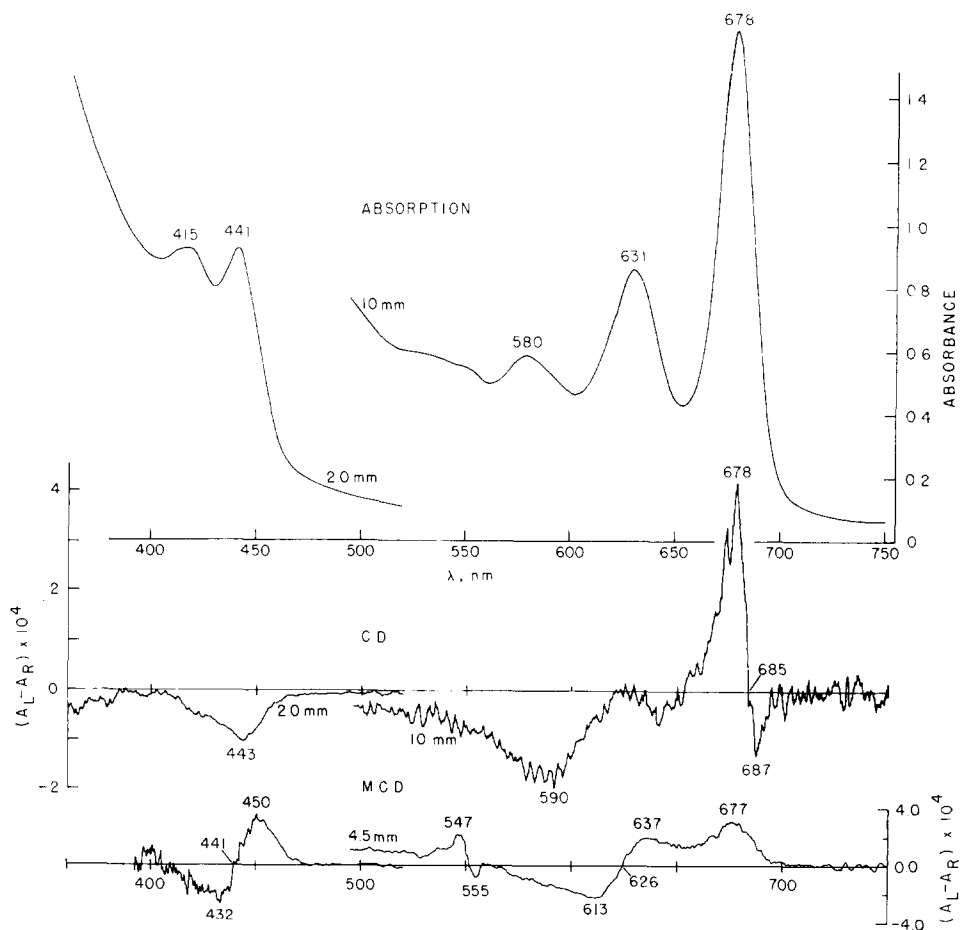


Fig. 2. Absorption, CD and MCD spectra of chlorophyllide holochrome (sucrose). Notations as in Fig. 1.

Chlorophyllide holochrome in 2 M sucrose. Chlorophyllide holochrome (sucrose) was produced (see Experimental) by illumination of the protochlorophyllide holochrome (sucrose) solution used in recording the spectra shown in Fig. 1. The CD spectrum of chlorophyllide holochrome (sucrose) exhibits red band multiplicity that is not apparent in the absorption or MCD spectra (Fig. 2). The CD spectrum of chlorophyllide holochrome (sucrose) resembles neither that of chlorophyll *a* monomers in ether solution nor that of chlorophyll *a* dimers in carbon tetrachloride, where the signs and relative magnitudes of the two long wavelength components are reversed^{23,24}.

Inactive (unconverted) protochlorophyllide appears in both the absorption and MCD spectra in the 630-nm region in Fig. 2. It also undoubtedly contributes to the CD spectrum in this region; however, no distinctive features are apparent.

The presence of sucrose (2 M) in the holochrome preparations has the effect of stabilizing the holochrome against denaturation, dark bleaching and other dark spectral changes. In addition, sucrose appears to block the spectral shifts following illumination, first observed by Shibata¹¹. The absorption maximum appears initially at 678 nm in chlorophyllide holochrome (sucrose) and remains there for at least 1 h at 5 °C. The CD spectrum is also stable during this interval.

The sharp, double MCD band with a crossing at 552 nm, seen in Figs 1 and 2, is not present in MCD spectra of solutions of protochlorophyll *a* or chlorophyll *a*. It may represent the inclusion of the reduced form of cytochrome *b₆*, which Boardman has shown to be present in etioplasts but not in purified holochrome²⁶.

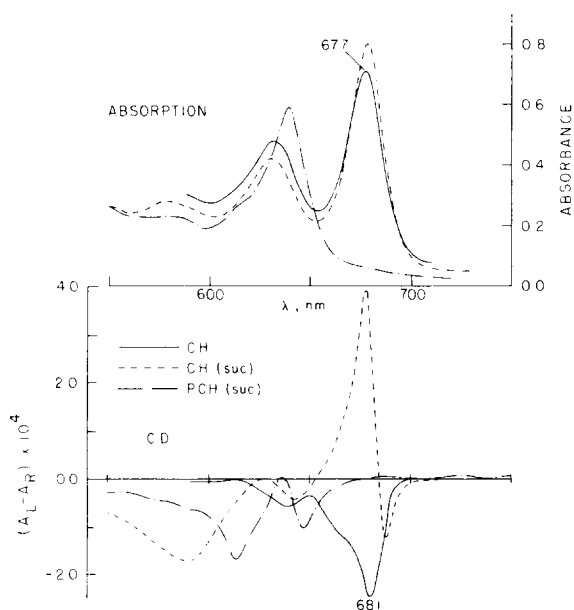


Fig. 3. Absorption and CD spectra of chlorophyllide holochrome (—), chlorophyllide holochrome (sucrose) (---) and protochlorophyllide holochrome (sucrose) (- · - ·). Optical path lengths: chlorophyllide holochrome (sucrose) and protochlorophyllide holochrome (sucrose) absorption, 4.5 mm; chlorophyllide holochrome absorption and all CD spectra, 10.0 mm. Chlorophyllide holochrome spectra were recorded within 30 min of the first illumination.

Chlorophyllide holochrome (sucrose absent). A sample of protochlorophyllide holochrome was transformed to chlorophyllide holochrome at 0°C and its absorption and CD spectra measured as quickly as possible (approx. 30 min). The red absorption maximum occurred at 677 nm and was associated with a single, asymmetric trough at 681 nm in the CD spectrum. These spectra, together with the corresponding spectra of protochlorophyllide holochrome (sucrose) and chlorophyllide holochrome (sucrose), are shown in Fig. 3 for the long wavelength region. A marked difference between the CD spectra of chlorophyllide holochrome and chlorophyllide holochrome (sucrose) is apparent. After 1 h at 5 °C and 15 min at 22 °C, the absorption maximum had shifted to 674 nm and the CD trough to 679 nm (not shown). The absolute magnitudes of both features had decreased 11 % and the width of the CD trough had increased slightly. A similar decrease in absorbance accompanying the dark shift toward the blue was reported by Schopfer and Siegelman⁹. We found no isosbestic point accompanying this shift in our preparations of chlorophyllide holochrome.

Homogenates prepared from post-etiolated leaves. Studies of the absorption and CD spectra of homogenates of greening leaves, which are able to accumulate chlorophyll far beyond that possible in the holochrome, provide an extension of the observations of the holochrome preparations already described. Continuity with the chlorophyllide holochrome spectra (Fig. 3) is indicated by the rather close similarity of the CD spectra, in particular, to those of homogenates prepared from leaves following 1 h of illumination (Fig. 4A, dashed curves). In the homogenates the red absorption maximum occurs at 672 nm and a single, asymmetric trough is observed in the CD spectrum at 678 nm. Its amplitude, relative to the absorption peak, is about 40 % less than in the chlorophyllide holochrome preparation, however.

After 2 h illumination of the leaves, the homogenate CD spectrum (Fig. 4A,

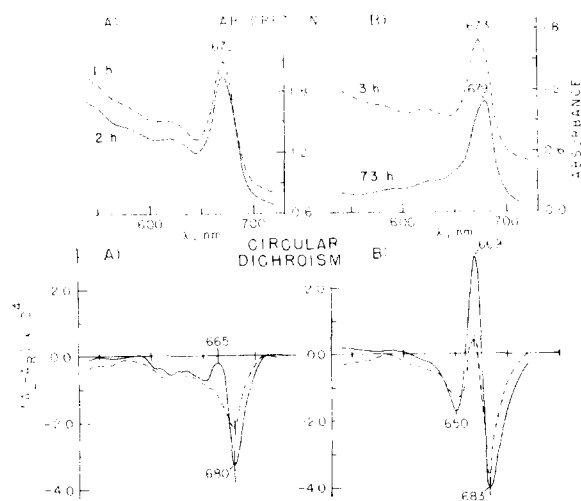


Fig. 4. Absorption (upper) and CD (lower) spectra of homogenates of greening bean leaves. Optical path: 10.0 mm. Times of prior illumination of the leaves are indicated on the absorption spectra. Straight vertical lines on the CD spectra indicate approximate noise levels.

solid curve) begins to show changes which develop progressively upon further illumination. A relative peak appears at 665 nm which grows larger after 3 h illumination. Finally, after 73 h illumination, the peak has grown further and shifted to 669 nm, and a trough has appeared at 650 nm (Fig. 4B). Intermediate spectra (not shown) were observed at other periods in this process. In addition to the substantial increase in total absorbance, the red absorption maximum has shifted to 679 nm after 73 h illumination.

The absorption and CD spectra of 6-day-old etiolated bean seedlings which were then illuminated for 73 h (Fig. 4B, solid curves) closely resemble those of chloroplast fragments isolated from mature barley leaves (Fig. 5, dashed curves). The wavelengths of the peaks and troughs differ by at most 3 nm in the CD spectra of these two different preparations from different plants. This is true even at shorter wavelengths not shown in Fig. 4.

Chlorophyll b-minus barley mutant. Some understanding of the origin of particular features in the CD spectra can be gained by comparison of the properties of chloroplast fragments isolated from normal barley with those of a mutant that

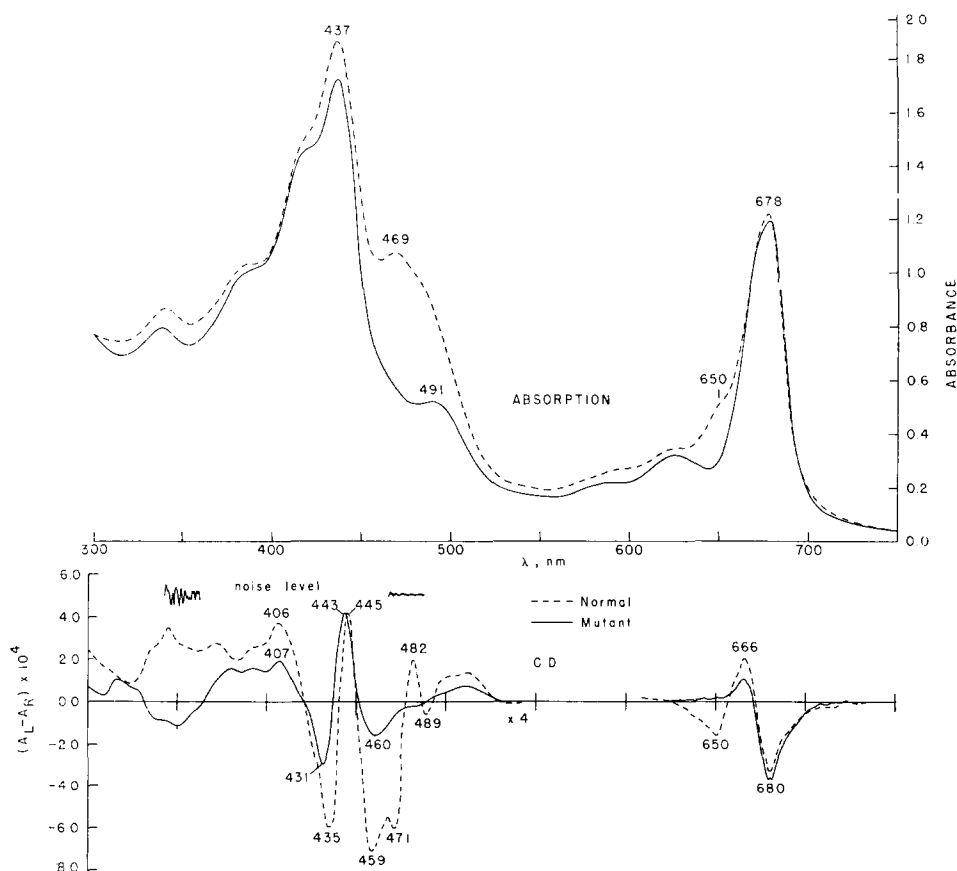


Fig. 5. Absorption and CD spectra of chloroplast fragments isolated from normal barley (---), and from a barley mutant (—) that lacks chlorophyll *b*. The CD curves from 300 to 550 nm are shown at $4 \times$ the amplitude indicated by the scale at the left (We are indebted to Dr E. A. Dratz for recording these CD spectra.)

is missing chlorophyll *b*^{17,24}. This comparison, which is illustrated in Fig. 5, indicates that the 650-nm trough and part of the peak at 666 nm arise from chlorophyll *b*. Even more dramatic differences occur in the blue in the region of the Soret band of chlorophyll *b* near 470 nm.

Chlorophyll *b* is known to be synthesized with a delay of several hours relative to chlorophyll *a* in post-etiolated seedlings⁷. Thus, the changes in the CD spectra of the homogenates of leaves between the 2nd and 73rd hour of illumination (Fig. 4) may arise largely from the increase of chlorophyll *b* synthesis during this interval. The CD spectrum of the homogenate after 2 h illumination is not unlike that of the barley mutant *minus* chlorophyll *b*, but it remains to be seen whether the state of chlorophyll *a* also undergoes further modification during the later stages of greening.

Turbidity effects absent in CD spectra. The turbidity of the suspensions appears not to have distorted the CD spectra. We deliberately added scattering substances such as serum albumin or talcum powder to achieve a similar turbidity in solutions of (+)-camphorsulfonic acid or sonicated chloroplast fragments. This increased the noise level somewhat, but had no significant effect on the CD peak positions, amplitudes or shapes, even at turbidities of 1.0 measured in the Cary 14 spectrometer. The insensitivity of our CD measurements to pronounced light scattering of the sample results partly from the placement of the sample directly in front of the end-window photomultiplier²⁰. In addition, and by contrast with the situation

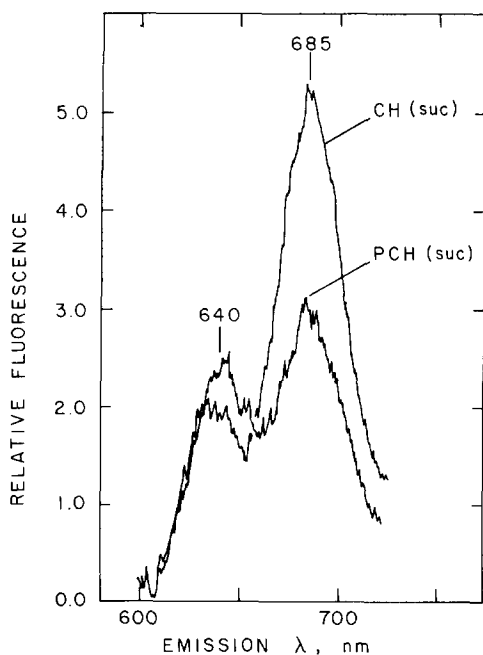


Fig. 6. Fluorescence emission spectra of protochlorophyllide holochrome (sucrose) and of chlorophyllide holochrome (sucrose) at 7°C. Excitation wavelength, 440 nm. Monochromator bandwidths, 20 nm. The protochlorophyllide holochrome (sucrose) spectrum exhibits a peak at 685 nm owing to a small amount of chlorophyllide formed by the exciting light during the process of obtaining the emission spectrum.

in polypeptide solutions²⁷, our materials probably do not consist of a homogeneous distribution of the CD-generating chromophoric groups (chlorophyll molecules) throughout the scattering particles (protein matrix). Thus the CD bands and the differential scattering of circularly polarized light appear to be largely separable effects in our systems, and we do not see the distortions reported by Urry and Krivacic²⁷.

Fluorescence measurements

Emission spectra. The measurement of fluorescence spectra of protochlorophyllide holochrome and protochlorophyllide holochrome (sucrose) is complicated by the efficient phototransformation caused by the excitation light. The fluorescence spectra at 7 °C were measured with no more than 12 % accompanying transformation through the introduction of neutral density filters (transmission 0.008) into the excitation beam. Typical fluorescence emission spectra for protochlorophyllide holochrome (sucrose) and chlorophyllide holochrome (sucrose) are shown in Fig. 6.

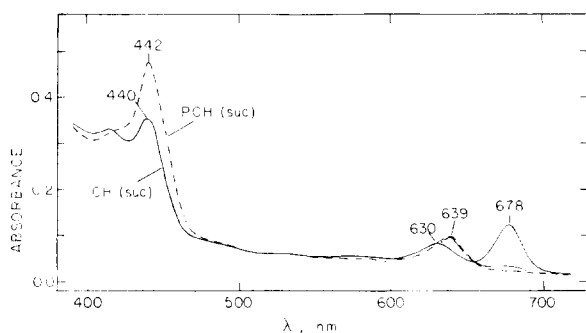


Fig. 7. Absorption spectra of protochlorophyllide holochrome (sucrose) before fluorescence emission spectrum in Fig. 6 (---), protochlorophyllide holochrome (sucrose) after fluorescence emission spectrum (—) and chlorophyllide holochrome (sucrose) after complete photo-conversion (—). Optical pathlength, 1.0 cm.

The corresponding absorption spectra are shown in Fig. 7. The extent of transformation of protochlorophyllide holochrome (sucrose) during the fluorescence measurement is estimated from the absorbance at 678 nm due to chlorophyllide. Comparison of Figs 6 and 7 shows that the chlorophyllide formed initially has a substantially greater fluorescence efficiency ($> 3\times$ at 685 nm) than does the chlorophyllide in the fully transformed chlorophyllide holochrome (sucrose).

The shorter wavelength emission peak of protochlorophyllide holochrome (sucrose) occurs at 642 nm, whereas that of the residual inactive protochlorophyllide in chlorophyllide holochrome (sucrose) occurs at 637 nm. The difference results from the loss of the weakly fluorescent, active protochlorophyllide in the latter material and perhaps, in part, from some decrease in self-absorption within the sample. Based on the corresponding absorbance changes, the uncorrected relative fluorescence efficiencies of inactive and active protochlorophyllide in protochlorophyllide holochrome (sucrose) are in the ratio 3.3:1.

The emission spectrum of this same sample of chlorophyllide holochrome (sucrose) measured using unattenuated excitation light is shown in Fig. 8. In addition

to the maxima at 637 (inactive protochlorophyllide) and 684 nm (chlorophyllide), there appears a distinct shoulder at 745 nm. The latter is undoubtedly a vibrational component ($o \rightarrow \tau$) of the chlorophyllide emission.

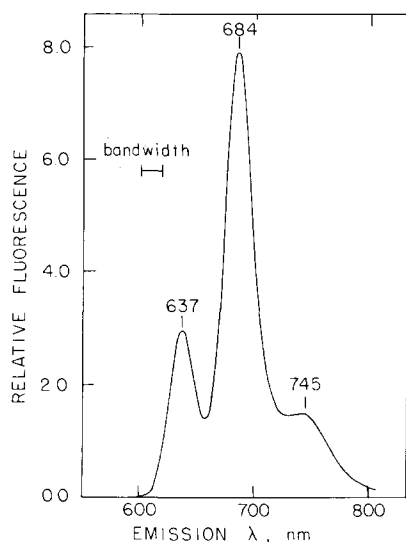


Fig. 8. Fluorescence emission spectrum of chlorophyllide holochrome (sucrose) at 7 °C. Excitation wavelength, 440 nm; excitation intensity $125 \times$ greater than that used in Fig. 6. Monochromator bandwidths, 18 nm.

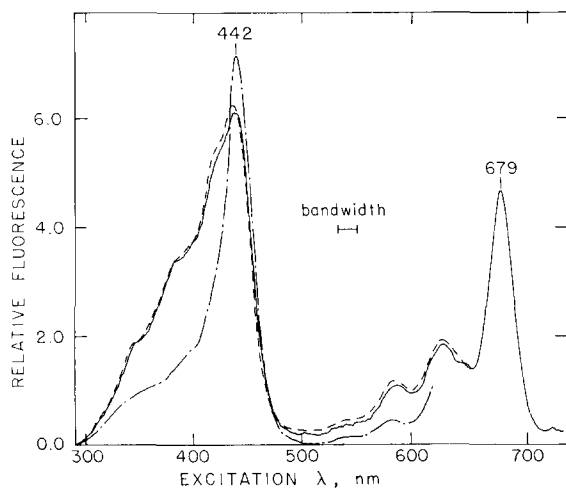


Fig. 9. Fluorescence excitation spectra of chlorophyllide holochrome (sucrose) at 7 °C at the emission wavelengths 637 nm (---), 685 nm (-.-), and 745 nm (—). Monochromator bandwidths, 16 nm. Instrument gain has been adjusted to facilitate comparison of the spectra.

Excitation spectra. The excitation spectra of chlorophyllide holochrome (sucrose) fluorescence measured at 637, 685 and 745 nm are shown in Fig. 9. The emission at the latter two wavelengths results from identical excitation spectra, whereas the fluorescence at 637 nm has a distinctly different excitation spectrum. These

observations confirm the association of the 745-nm shoulder with the chlorophyllide emission. They also argue against the occurrence of substantial excitation transfer from inactive protochlorophyllide to chlorophyllide in chlorophyllide holochrome (sucrose) at 7 °C. By application of the method of matrix rank analysis of Weber²⁸ to the spectra of chlorophyllide holochrome (sucrose) shown in Figs 8 and 9, we find that there are two, but not three, distinct fluorescent components in the completely converted holochrome at 7 °C. These components are protochlorophyllide (inactive) and chlorophyllide.

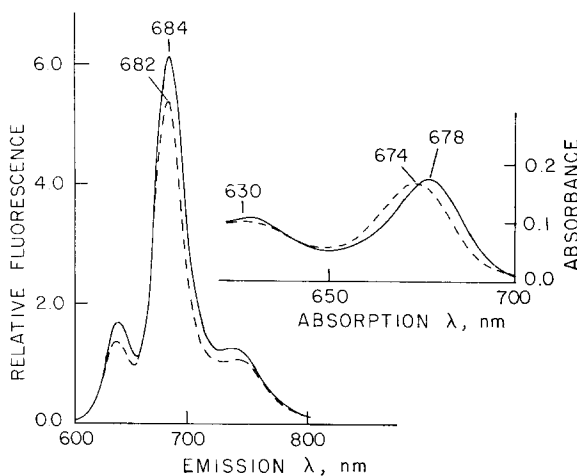


Fig. 10. Absorption and emission spectra of chlorophyllide holochrome at the beginning (—) and at the end (---) of the dark shift following photoconversion. Excitation wavelength, 440 nm.

In the absence of sucrose, the transformed chlorophyllide holochrome undergoes a dark shift in the absorption maximum from 678 to 674 nm during about 20 min at room temperature. This absorption shift and the concomitant change in the fluorescence emission spectrum are depicted in Fig. 10. The decrease in magnitude by 8 % in the emission spectrum is accompanied by a small decrease in the absorption and is probably not significant.

Polarization. The fluorescence polarization of chlorophyllide holochrome was studied using a fully converted sample with A (1 cm) = 0.15 at the red maximum. Although the sample was maintained at about 5 °C, the dark shift of the absorption from 677 to 673 nm continued during the course of the measurements. The polarization values, $p = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$, for emission at 680 nm are plotted as a function of excitation wavelength in Fig. 11A. Because there was no measurable change in the polarization values during the course of the dark shift, the measurements at different times are not distinguished from one another on the plot. The p values and their dependence on excitation wavelength agree generally with those obtained for chlorophyll *a* in viscous solvents; however, there are significant differences among the several published polarization spectra²⁹⁻³².

The fluorescence polarization spectrum of chlorophyllide holochrome (sucrose) under the same conditions is shown in Fig. 11B*. In the wavelength regions

* The presence of the asymmetric sucrose molecule introduces some rotation of the plane of polarized light. This was calculated to be less than 2° under the conditions of the experiment.

where comparisons can be made, the p values of chlorophyllide holochrome (sucrose) are approximately half those of chlorophyllide holochrome. The wavelength dependences are qualitatively similar, however.

The polarization of the protochlorophyllide holochrome (sucrose) fluorescence at 642 nm was measured using neutral density filters (transmission 0.008) in the excitation beam in order to retard the photoconversion. With the excitation wavelength at 440 nm, p values of $+0.06 \pm 0.14$ were observed. The large uncertainty resulted from the low signal level under these experimental conditions. Protochlorophyll *a* in mineral oil exhibits a polarization of $+0.23$ at the corresponding wavelengths²².

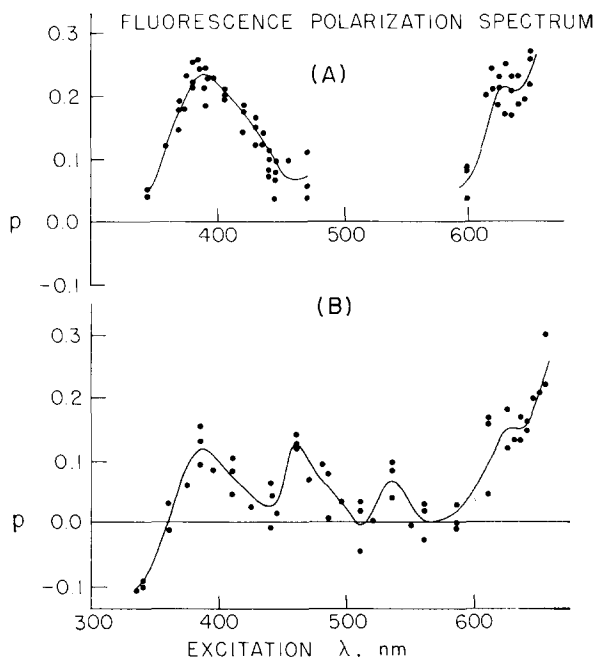


Fig. 11. Fluorescence polarization spectra at 7 °C. (A) Chlorophyllide holochrome. Emission wavelength, 680 nm. (B) Chlorophyllide holochrome (sucrose). Emission wavelength, 682 nm. Monochromator bandwidths, 25 nm.

DISCUSSION

Exciton interactions among aggregated pigments

Coupling of like electronic transitions in physically associated chromophoric molecules results in a splitting of the absorption bands into a pattern that is characteristic of the array^{33,34}. This effect is known as a weak exciton or localized exciton interaction, to contrast it with the strong or free exciton interactions typically found in molecular crystals. In general, localized exciton interactions result in band splittings into N components, where N is the number of interacting molecules in the array. Not all of the possible components need be observed, however, because selection rules governing the intensities of the transitions depend critically on the geometry of the array^{34,36}. Additional information can be gained from observation of these band multiplicities for asymmetric molecules using CD, where

not only the energies and intensities but also the signs vary among the exciton components. The origin of this interaction and its relationship to the aggregate geometry is described in an excellent analysis by Tinoco³⁷.

Chlorophyll, protochlorophyll and related pigments exhibit exciton splittings in non-polar solvents, in molecular crystals and in preparations of photosynthetic membrane fragments. These can be observed in absorption²⁵ as well as in ORD and CD spectra^{23,24}. The double CD features of the aggregated pigments are readily distinguished from the much weaker single CD components of the monomers. Furthermore, the CD and absorption spectra of dimers and higher aggregates are particularly sensitive to the relative orientations of the pigment molecule chromophores²⁴.

Fluorescence intensity and, especially, fluorescence depolarization also provide sensitive indications of pigment interactions. Latimer and Smith³⁸ observed strong fluorescence polarization, comparable to that of chlorophyll *a* in a viscous solvent, from the chlorophyllide produced via photoconversion in the holochrome. Goedheer and Smith³⁹ observed that increasing illumination of etiolated leaves produced a progressive decrease in the fluorescence polarization of the holochrome extracts prepared from the leaves. Because their results extrapolated to a value indicating 50 % of the maximum polarization at the first stage following photoconversion, they concluded that the observed depolarization resulted from rotation of the chlorophyllide molecules within the holochrome framework. This was subsequently shown not to be the case by Losev and Gurinovitch⁴⁰, who observed strong (80–100 %) fluorescence polarization in leaves at either 20 or –100 °C immediately following photoconversion. They attributed the polarization decrease upon longer illumination to excitation transfer among associated chlorophyllide molecules, rather than to rotational diffusion.

During the greening process the yield of chlorophyllide fluorescence decreases approximately 10-fold, starting at a level characteristic of chlorophyll *a* in organic solvents⁴¹. The intensity decrease presumably results from quenching processes that become more probable as the concentration and extent of aggregation of chlorophyll increases in the developing plastid.

CD and fluorescence changes accompanying greening

The CD spectrum of protochlorophyllide holochrome or protochlorophyllide holochrome (sucrose) isolated from etiolated seedlings (Fig. 1) is significantly more complex than that of protochlorophyll *a* in ether^{22,23}. Protochlorophyll *a* has two nearly degenerate electronic transitions in the red region and two more in the blue. From long to short wavelengths the signs are (+, –) in the red and (–, +) in the blue for the CD components in ether. For protochlorophyllide holochrome the sign of the longest wavelength component is negative and there seem to be at least three components in this region. One expects to see as many as four components in the long wavelength region of the dimers (each of the Q_x and Q_y transitions should be split into two components); there appears to be a strong overlap of the central pair. It seems clear that protochlorophyllide *a* does not occur as isolated molecules (*i.e.* one per holochrome protein) in protochlorophyllide holochrome; however, it will be necessary to obtain better resolution of the CD spectra in order to determine the extent of the interacting array. Measurements of CD spectra at

liquid nitrogen temperature have provided such increased resolution in the case of a bacteriochlorophyll-protein complex^{15,42}.

The fluorescence emission spectra at 7 °C shown in Fig. 6 for protochlorophyllide holochrome (sucrose), partly converted by the exciting beam, and for fully converted chlorophyllide holochrome (sucrose) exhibit little evidence of fluorescence from active protochlorophyllide. The peak at 637 nm in the fully converted material undoubtedly results from inactive protochlorophyllide absorbing at 630 nm. Even the increment at 642 nm in the partly converted protochlorophyllide holochrome probably results from the 636 nm form identified by Dujardin and Sironval⁴³ at -196 °C. A Stokes shift larger than 3 nm would be expected for the fluorescence of active protochlorophyllide, which absorbs at 639 nm. While the polarization of protochlorophyllide fluorescence in protochlorophyllide holochrome (sucrose) could not be measured accurately under the conditions of low excitation intensity necessary to retard photoconversion, the observed value $p = 0.06$ is low in comparison with the value $p = 0.23$ for isolated protochlorophyll *a* in mineral oil²². Presumably, the measurement for protochlorophyllide holochrome (sucrose) applies primarily to the fluorescence from the inactive protochlorophyllide.

Butler and Briggs⁴⁴ have attributed the two principal spectral forms of protochlorophyllide to monomeric (short wavelength) and aggregated (long wavelength) species. The low fluorescence yield of P_{639} relative to that of P_{630} at room temperature may result from quenching by the aggregates in the former case. The CD spectrum of protochlorophyllide holochrome discussed above is interpretable on this basis. In the region from 600 to 700 nm, there appears to be a (+, -) CD centered near 630 nm (Fig. 3) attributable to monomeric protochlorophyllide superimposed on a more complex CD pattern (-, at the longest wavelengths) which results from the aggregated P_{639} form.

The interpretation of the CD spectrum of chlorophyllide holochrome (sucrose) in Fig. 2 appears to be more straightforward. A double CD (-, +) appears in the long wavelength region associated with the Q_y transition. The Q_x transition in chlorophyll *a* is at appreciably shorter wavelength (approx. 590 nm), and does not contribute significantly near 680 nm. Chlorophyll *a* in ether exhibits a negative CD for the Q_y band²³, and the double CD of the solution dimers^{23,24} has reversed signs relative to those in chlorophyllide holochrome (sucrose). Again, low temperature CD spectra will aid in the analysis, but it seems clear that the chlorophyllide *a* in chlorophyllide holochrome (sucrose) is not monomeric and, if it is a dimer, the geometry is different from that in non-polar solvents.

In the absence of sucrose, the CD spectrum of chlorophyllide holochrome in Fig. 3 shows no evidence of a double CD with alternate signs in the long wavelength region. The same is true of a homogenate of postetiolated leaves illuminated for 1 h (Fig. 4). These spectra suggest a decreased chlorophyllide aggregation in chlorophyllide holochrome in the absence of sucrose and in the 1-h post-etiolated leaves. The single CD bands of these materials would be consistent with the presence of noninteracting or monomeric chlorophyllide.

The conclusions based on the CD spectra of chlorophyllide holochrome with and without sucrose are supported by the fluorescence properties of these preparations. At 7 °C we find (Fig. 11B) a relatively low polarization of the chlorophyllide fluorescence from chlorophyllide holochrome (sucrose), consistent with

the presence of chlorophyllide aggregates (dimers). By contrast, Latimer and Smith³⁸ and Losev and Gurinovitch⁴⁰ found essentially complete polarization of chlorophyllide fluorescence at the earliest stages of protochlorophyllide to chlorophyllide conversion. We conclude that the stage of the photoconversion corresponding to our chlorophyllide holochrome (sucrose) preparation was passed through too quickly to be observed by these workers.

In chlorophyllide holochrome the fluorescence polarization (Fig. 11A) is increased by a factor of about two compared with that of chlorophyllide holochrome (sucrose). The polarization of chlorophyllide fluorescence in chlorophyllide holochrome in the absence of sucrose is indistinguishable from that of chlorophyll *a* in viscous solvents³⁰⁻³². The strongly polarized fluorescence is consistent with the conclusion reached on the basis of the CD spectrum, that chlorophyllide holochrome contains essentially monomeric chlorophyllide, at least by the time these measurements can be made at 7 °C. The increase in fluorescence polarization and the changes in the CD spectra are much more direct evidence of this decrease in aggregation than were the blue shifts in absorption and fluorescence maxima, previously the only evidence available to support the proposal^{15,45}. The monomeric form of chlorophyllide in chlorophyllide holochrome is presumably responsible for the high polarization values previously reported^{38,40}, although our values of polarization excited at 405 nm are somewhat higher than those reported by Latimer and Smith³⁸.

The one observation that appears to be inconsistent with the picture of chlorophyllide disaggregation accompanying the blue shift of the absorption is the absence of a concomitant increase in fluorescence efficiency in chlorophyllide holochrome (Fig. 10). The simplest explanation for this is that the increase in efficiency has already occurred by the time we are able to make the first fluorescence measurements on chlorophyllide holochrome. Goedheer⁴¹ found the fluorescence efficiency of chlorophyllide in greening bean leaves initially to be comparable to that of chlorophyll *a* in methanol. It will be of interest to determine whether a lower value can be detected if measurements are made very rapidly following a strong flash of actinic light.

Light absorbed by active protochlorophyllide leads to the formation of chlorophyllide with a high quantum efficiency⁴⁶. The process does not follow simple first-order kinetics, but appears to result from the sum of two first-order processes⁷⁴. The underlying reason for this behavior is the subject of a subsequent study⁴⁸.

Comparison of Figs. 6 and 7 indicates that the chlorophyllide formed during the initial 10% of photoconversion of protochlorophyllide holochrome (sucrose) is over three times more fluorescent than is the chlorophyllide of the fully converted chlorophyllide holochrome (sucrose) at 7 °C. This can be understood on the basis of a model in which active protochlorophyllide in protochlorophyllide holochrome (sucrose) occurs in an aggregated (dimeric?) form. If the initial stages of the photoconversion result in the transformation of only one of the aggregated protochlorophyllide (active) molecules, then the resulting chlorophyllide molecules initially occur singly and their fluorescence is not quenched. In the fully converted chlorophyllide holochrome (sucrose), the chlorophyllide molecules are now essentially all in an aggregated state and their fluorescence is partly quenched at 7 °C. The role of the sucrose is probably similar to that of glycerol in high concentrations

in preventing the subsequent rearrangements that lead to a blue shift of the absorption and fluorescence maxima⁴⁷.

With increasing illumination of post-etiolated leaves (Fig. 4) a new double CD at 669 (+) and 650(-) grows in as chlorophyll *b* is synthesized. Although the contribution of the CD of chlorophyll *a* cannot be sorted out in the spectra shown in Fig. 4, the spectrum of chloroplast fragments of the barley *mutant* minus chlorophyll *b* (Fig. 5) does exhibit a double CD at 680(-) and 667(+) nm. It is reasonable to suppose, therefore, that chlorophyll *a* and chlorophyll *b* exist in an aggregated state in the mature chloroplast. Evidence from high resolution and derivative absorption spectra has supported such a postulate⁵⁰. The shift of the absorption band from shorter to longer wavelengths^{44,51} and a concomitant decrease in the fluorescence efficiency⁴⁴ have also been used to support the proposal that monomeric chlorophyll *a* becomes aggregated during illumination periods longer than about 2 h. It seems apparent from the CD spectra that, in the mature chloroplasts, the geometry of the chlorophyll *a* aggregates (-, + CD) is different from that of the chlorophyll *b* aggregates (+, - CD). The CD spectra of the solution dimers of these two chlorophylls by contrast, exhibit the same parity (+, - CD)^{23,24}.

Model for the protochlorophyll to chlorophyll transformation. On the basis of the foregoing analysis of the CD and fluorescence spectra, we are now in a position to propose a model for the changes in pigment association accompanying greening of etiolated leaves. We will tentatively relate these changes to the absorption spectral changes first reported by Shibata¹¹. Our evidence points to five stages in the process; however, there is recent absorption and fluorescence spectral evidence for other early phases that are too rapidly passed through for us to have observed using CD spectrometry^{52,53}.

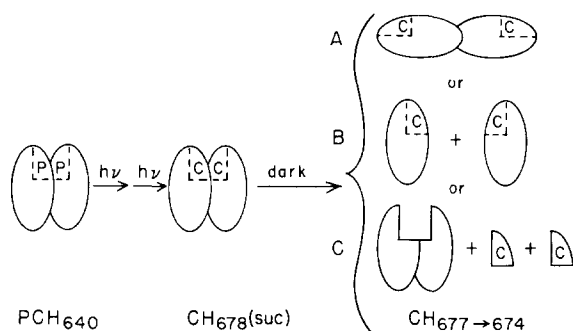
(1) Etioplasts contain both active protochlorophyllide (650 and 637) and inactive protochlorophyllide (628)¹⁰. Protochlorophyllide holochrome contains active protochlorophyllide (640) and inactive protochlorophyllide (630)⁴⁶. The CD evidence (Fig. 1) suggests that the active protochlorophyllide is aggregated (probably dimeric). Assuming that inactive protochlorophyllide does not change its state upon illumination, the evidence of Figs 2 and 3 suggests that it also is aggregated.

(2) Following illumination, Shibata¹¹ observed that the absorption band at 684 nm (678 nm in chlorophyllide holochrome) shifted to shorter wavelengths within minutes with or without further illumination. Assuming that the concentrated sucrose in our chlorophyllide holochrome (sucrose) preparation served to inhibit this dark shift, then the CD evidence of Fig. 2 and the relatively low fluorescence polarization (Fig. 11b) support the presence of aggregated (dimeric?) chlorophyllide *a* as the earliest spectral form, C684, seen by Shibata. On the other hand, we cannot yet be sure that the sucrose did not arrest the process at the still earlier stage reported by Gassman *et al.*⁵² and by Bonner⁵³.

(3) The dark shift to shorter wavelengths (673 nm in leaves; 677-674 nm in chlorophyllide holochrome) results in a simplification of the CD spectrum (Fig. 3, solid curve; Fig. 4A, dashed curve) and a 2-fold increase in the fluorescence polarization (Fig. 11A), both indicative of the loss of aggregation. At this stage the chlorophyll(ide) may be essentially monomeric; however, the wavelength difference between the absorption maxima and the CD troughs may be indicative of an inhomogeneous environment.

(4) During the second and third hour of illumination of post-etiolated leaves (Fig. 4) the appearance of a trough at 650 and a peak at 669 nm in the CD spectrum appears to indicate the appearance of aggregated chlorophyll *b*.

(5) In the mature chloroplast the chlorophyll *a* appears to have reaggregated as well (Fig. 5, mutant), although the relative CD magnitudes suggest that the state is different from that in chlorophyllide holochrome (sucrose) (Fig. 2). This new association is accompanied by a long wavelength absorption shift to 678 nm. It is not clear whether states 4 and 5 are temporally distinct or whether they appear essentially simultaneously.



Scheme 1.

The first three stages are illustrated in Scheme 1. The protochlorophyllide holochrome, PCH_{640} , contains at least two protochlorophyllide molecules that interact sufficiently at close range to give a complex CD for the long wavelength band. These must be on the same holochrome particle. Immediately following photoconversion, the CH_{678} (suc) produced contains two strongly interacting chlorophyllide molecules, on the basis both of CD and fluorescence polarization spectra. The site of the pigment on the holochrome protein may be essentially unchanged from that in PCH_{640} . Very rapidly following photoconversion in the absence of high concentrations of sucrose or glycerol the chlorophyllide holochrome undergoes a rearrangement or dissociation. This results in the disappearance of the double CD feature, a 2-fold increase in the fluorescence depolarization and eventually a shift of the absorption and fluorescence emission maxima to shorter wavelengths. The scheme above indicates three possible explanations: (A) a rearrangement of the chlorophyllide holochrome particle leading to decreased interaction between the chlorophyllide molecules, (B) dissociation of chlorophyllide holochrome into two equivalent parts, and (C) dissociation of small chlorophyllide-protein fragments from the major protein component. The alternatives are not mutually exclusive, for structure A could precede B or C. This would account for the observation that the CD and fluorescence depolarization changes appear to occur more rapidly than does the blue shift in the absorption band. Alternative C would be consistent with the observations of Boardman¹³ and of Bogorad *et al.*¹⁴ that the final chlorophyllide-containing species has a molecular weight much different from that of the holochrome protein. In the intact etioplast, the major protein component of the holochrome would then be available for the placement of two

more protochlorophyllide molecules⁵⁴ and the chlorophyllide-protein fragments could be transferred into the newly forming lamellar structures.

Apart from the spectral shifts of Shibata¹¹, there is other published evidence to support the model presented above. The stoichiometry of the protochlorophyllide holochrome suggests that there is more than one protochlorophyllide molecule per 550000 daltons of protein^{9,10}. The ratio probably lies in the range of 2–4 protochlorophyllide *a* molecules per holochrome particle. If the protochlorophyllide holochrome really consists of two subunits, which is a possible interpretation of the chromatographic and electrophoretic analysis of Schopfer and Siegelman⁹, then it is possible that the exciton interaction occurs between protochlorophyllide *a* molecules in each subunit near the contact interface.

Kahn *et al.*¹⁰ and Thorne⁵⁵ have used evidence of excitation transfer at low temperatures to support the occurrence of interactions of as many as 20 pigment molecules in the etiolated or immediately post etiolated seedlings (States 1 and 2 in our model). It is important to recognize the differences between the probable mechanism of this excitation transfer (Förster⁵⁶ inductive resonance transfer or very weak exciton interaction) and the weak or localized exciton interaction that gives rise to the band splittings observed in absorption and CD spectra^{22,23}. Excitation transfer via the Förster mechanism can apparently occur over relatively long distances (60–80 Å)^{7,58}, whereas calculations using a point transition dipole model²⁴ indicate that the band splittings resulting from weak exciton interactions would probably be undetectable at these distances. The large differences in the sizes of the arrays calculated from excitation energy transfer resulting in fluorescence in comparison with the smaller sizes consistent with the stoichiometry and band splitting observations are therefore not necessarily incompatible with one another. They could be reconciled, for example, by a model in which protochlorophyllide *a* holochrome particles, each containing two protochlorophyllide *a* molecules interacting at the weak exciton level, are arrayed in a lattice of sufficiently close spacing so as to permit long range excitation transfer among the holochrome particles by the Förster mechanism. Evidence for the presence of such tightly organized, highly extended lattices can be seen in electron micrographs of the prolamellar bodies of etioplasts⁵⁹.

CONCLUSIONS

From the evidence of CD, absorption spectra, fluorescence spectra and fluorescence polarization of homogenates of post-etiolated bean leaves and of holochrome particles isolated from etiolated leaves, we have been able to obtain information relevant to the mutual association of protochlorophyllide *a* and chlorophyll(ide) *a* molecules. The observations suggest that the protochlorophyllide *a* molecules are initially in an aggregated, probably dimeric, state in the etioplasts, and that following illumination they dissociate to a monomeric configuration. Subsequent illumination then produces a new aggregation of the chlorophyll *a*, accompanied by the formation of aggregated chlorophyll *b*, which is characteristic of the fully developed membranes of mature chloroplasts. This new association may occur concurrently with the formation of proliferating membranes or thylakoids in the developing plastids. The relationship of this process to the development of

photosynthetic function provides a challenge to current and future research in this field.

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