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Ora Dobjinsky Canaani
(Ph. D. thesis)

October 1975

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BIOCHEMICAL AND SPECTROSCOPIC STUDIES OF PROTOCHLOROPHYLLIDE HOLOCHROME AND ITS ROLE IN THE FORMATION OF PHOTOSYNTHETIC MEMBRANES  

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BIOCHEMICAL AND SPECTROSCOPIC STUDIES OF PROTOCHLOROPHYLLIDE HOLOCHROME AND ITS ROLE IN THE FORMATION OF PHOTOSYNTHETIC MEMBRANES

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October 1975

ABSTRACT

The assembly of the photosynthetically active membrane of chloroplasts is triggered by Chl synthesis. This process takes place when dark-grown seedlings are subjected to illumination. It involves a photoreduction of PChlide to Chlide at a protein site. The photoconversion can be studied using an extracted pigment-protein complex called PChlide-holochrome (PCH).

We have studied the subunit structure of PChlide-holochrome and Chlide-holochrome by spectroscopic and biochemical methods. It was found that PCH, a pigment-protein complex of 600,000 daltons, can be dissociated irreversibly to a 300,000 daltons subunit in 0.06% Triton X-100. After dissociation has taken place, the remaining 600 Kd protein is not in equilibrium with the 300 Kd subunit and exhibits a reduced stability and photoconversion ability together with a blue shift in the
absorption maximum of PChlide to 632 nm. On the other hand, the 300 Kd subunit retains all of its photoconversion activity, is very stable and absorbs maximally at 639 nm. Circular dichroism, fluorescence and fluorescence polarization reveal that PChlide and Chlide pigments are aggregated (probably as dimers) in the 300 Kd PCH. A non-linear development of the CD signal with extent of photoconversion was observed for the 300 Kd PCH. This kinetic behavior may indicate the presence of an intermediate state of a "PChlide-Chlide" dimer complex.

Improved methods are given for preparing PCH. The number of PChlide pigment molecules per protein of 600,000 daltons is estimated as four from stoichiometric calculations on the most purified PCH fraction. SDS-polyacrylamide gel electrophoresis on the most purified PCH shows that the holochrome is constituted from only one type of polypeptide of molecular weight 45,000 daltons to which the pigment is still attached after the protein has been dissociated.

A major dissociation occurs on the macromolecular level and is displayed in the vanishing of the 600,000 Chlide-holochrome and the appearance of Chl-holochrome of a molecular weight < 70,000 daltons. A detailed analysis of the peptide composition of the 600 Kd PCH shows that at the same time, the 45,000 daltons Chl-polypeptide is cleaved to yield a 16,000 Chl-polypeptide. These two processes are temperature dependent, are blocked at 0°C and take place at the same time as the dark spectroscopic shift (the Shibata shift).

PChlide pigment molecules are aggregated in etiolated leaves as already found from CD studies of homogenates from etiolated leaves. The newly formed Chlide exhibits a double CD characteristic of excitonically interacting Chlide molecules in close proximity to each other.
However, after the dark spectroscopic shift has taken place in vitro, in the homogenate, the Chlide aggregate appears to be dissociated, as evidence by the disappearance of the double CD signal. We investigated the CD spectra of homogenates of illuminated leaves. The CD spectra suggest the presence of monomeric Chl in leaves that have been illuminated for 10 mins. This period corresponds to the completion of the Shibata shift and points to the fact that the dark spectroscopic shift represents the disaggregation of pigment molecules, which is in agreement with the dissociation of PCH observed in vitro, both on the macromolecular and on the polypeptide level and also with the disaggregation of Chlide pigment molecules, in vitro. However, after 2 1/2 hours illumination, the Chl molecules become aggregated again as evidenced by the CD spectrum, and at the same time, the commencement of photosynthetic activities takes place.

The photoconversion reaction of etiolated bean homogenate has been studied at low temperature. It takes place at -125°C but is not completed at this temperature. We looked at the EPR spectrum at low temperatures of etiolated bean leaves in an attempt to discover an intermediate species in the photoconversion reaction. A free radical was discovered upon illumination of etiolated leaves, however its chemical nature or its possible role still remain unknown. It is not clear that it is involved in the photoconversion of PChlide to Chlide. The photoenzyme, PCH, can carry out its photochemical activity in a freeze dried pellet, namely in a solid matrix.

Finally, the mature photosynthetic membranes of spinach, barley and barley mutant (lacking Chl b) were studied by absorption and CD at room temperature and at liquid nitrogen temperature. The CD spectrum
of normal spinach chloroplast fragments at 23°C closely resembles that of normal barley. It is characterized by two minima at 654 nm and at 686 nm, and a maximum at 670 nm. However, at liquid nitrogen temperature troughs at 640, 653 and 676 nm and a maximum at 667 nm are observed. The higher resolution obtained at liquid nitrogen temperature points to a more complex geometry of Chl pigment molecules than that revealed by room-temperature CD studies. However, the CD spectra of chloroplast fragments of a barley mutant which lacks Chl b, are almost identical at room temperature and at liquid nitrogen temperature. A double CD signal appears for the barley mutant, and is characterized by a maximum at 670 nm and a large minimum at 686 nm. The minimal size that is expected for the aggregate is a dimer. However, the large amplitude of the Chl a CD suggests a more extensive aggregate than a dimer.

Kenneth Sauer
I wish to express my gratitude to Professor Kenneth Sauer for his sharp and helpful criticism, his patience and understanding during my career as a graduate student. Especially I am indebted to him for the scientific independence he allowed me to develop. His enthusiasm, knowledge and confidence were very rewarding in my scientific career.

I also want to especially thank my friends Paul Hartig, Ruth Alscher, Danny Michaelson and Agatha Wong for their support, many productive discussions, their warm friendship, and for sharing with me good times and bad.

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To all the people in our research group for their comradery and interest.

Most of all to my husband Eli for sharing with me the ups and downs of graduate school, for his help and care during these years.

This work was supported, in part, by the U. S. Energy Research and Development Administration.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Abs. max.</td>
<td>Absorption maximum</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>Chl a</td>
<td>Chlorophyll a</td>
</tr>
<tr>
<td>Chl b</td>
<td>Chlorophyll b</td>
</tr>
<tr>
<td>PChl</td>
<td>Protochlorophyll</td>
</tr>
<tr>
<td>PChlide</td>
<td>Protochlorophyllide</td>
</tr>
<tr>
<td>Chlide</td>
<td>Chlorophyllide</td>
</tr>
<tr>
<td>PCH</td>
<td>Protochlorophyllide holochrome</td>
</tr>
<tr>
<td>CH; Chl-H;</td>
<td>Chlorophyllide holochrome</td>
</tr>
<tr>
<td>RuDP-Case</td>
<td>Ribulose diphosphate carboxylase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>LDAO</td>
<td>Sodium lauryl dimethylamine oxide</td>
</tr>
<tr>
<td>SDBS</td>
<td>Sodium dodecyl benzyl sulfate</td>
</tr>
<tr>
<td>CDM</td>
<td>Carboxy dismutase</td>
</tr>
<tr>
<td>Kd</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>DEAE</td>
<td>Di ethyl amino ethyl</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>PCH-P</td>
<td>Protochlorophyllide holochrome protein</td>
</tr>
<tr>
<td>CH-P</td>
<td>Chlorophyllide holochrome protein</td>
</tr>
<tr>
<td>C-Band</td>
<td>Ribulose diphosphate carboxylase band</td>
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Abbreviations (continued)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>H-band</td>
<td>Holochrome band</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylen diamine tetraacetic acid</td>
</tr>
<tr>
<td>δ-ALA</td>
<td>δ Aminolevulinic acid</td>
</tr>
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Dedication

To my parents, whose years of love, concern, and encouragement will not be forgotten.
CHAPTER I

INTRODUCTION

A major question in biology today is differentiation. At the tissue or cellular level, differentiation has been defined as increasing specialization. The process is extremely complex and can be understood on the molecular level as the sequential synthesis and activation of specific enzymes at specific points in time. Very little is known about the regulation of synthesis of specific proteins during development. In order to elucidate mechanisms of cell differentiation, a well-defined biological system in which molecular events can be consistently analyzed is necessary. One such system of interest is that of the etioplast to chloroplast transformation, where differentiation can be studied as it occurs in etiolated plants exposed to light.

A. Plastid Development during Growth in Continuous Darkness

When plants are grown in the dark, they do not develop chloroplasts, are devoid of chlorophyll, and do not carry out photosynthesis. During the differentiation of the meristematic cells into leaf cells, the relatively undifferentiated proplastids develop into etioplasts with characteristic paracrystalline bodies. These paracrystalline bodies are termed prolamellar bodies.

The early observations concerning etioplast development are summarized by Gunning and Jagoe (1965). Using electron microscopy, it was found that the relatively undifferentiated proplastids contain just a
few single thylakoids. The first sign of the formation of the crystalline body is the development of connecting arms between these thylakoids. As differentiation proceeds, a lattice with a regular crystalline-like form appears. Gunning and Jagoe (1965) present a model of the prolamellar body as a framework of tubules lying in the three major axes of a simple cubic lattice.

A description of prolamellar body formation was given by Von Wettstein (1958, 1967). According to Von Wettstein, discrete vesicles produced by the inner membrane of the plastic envelope accumulate within the plastids and fuse to form the tubules of the prolamellar body. However, these vesicles are generally considered to be artifacts due to senescent dark grown leaves and the method of fixation with KMnO₄ which is discussed in detail in the next paragraph.

Criticism for much of the early work centers around preparation of tissue for electron microscopical examination involving fixation with KMnO₄. It is well established that some fine details of structure are altered by such a fixation procedure. Fixation of biological material with glutaraldehyde seems to better preserve certain cellular structures.

Weier and Brown (1970), using the glutaraldehyde fixation, give a most extensive description of ultrastructural changes during the development of the etioplast in the dark. In two-day old seedlings of beans, the plastids are small and contain much starch. No prolamellar bodies are observed. The only membranes appear to be segments of lamellae which are often attached to the inner membrane of the plastid envelope. By three to six days, the plastids enlarge and one or more
prolamellar bodies are observed. The prolamellar bodies are continuous with several peripheral lamellae. The peripheral membranes arise from the inner membrane of the plastid envelope and are sometimes continuous from the envelope into the prolamellar bodies.

The peripheral lamellae appear as sheets of porous membranes, while the prolamellar body consists of a system of interconnected tubules. Weier and Brown suggest that the prolamellar bodies are formed by the contraction of the porous peripheral lamellae and the formation of interconnecting tubules perpendicular to the peripheral lamellae. The fusion of the peripheral lamellae into the prolamellar body interconnecting tubules occurs around the pores linking each lamella to the one above it and to the one below it. The basic unit of the prolamellar body is a six-pointed star formed by the tubules which lie in the plane of the porous lamellae. The tubules form a hexagonal pattern and are joined to similar hexagonal patterns, above and below, by vertically arranged tubules which form rectangular patterns in vertical planes. This view of the prolamellar body basic structure and the way it is formed is shared by Wehrmeyer (1965), Ikeda (1968), and Bradbeer et al. (1974). It is in contrast to the cubic model presented by Gunning and Jagoe (1967). Furthermore, and others Weier and Brown do not find any evidence to suggest a relationship between ribosomes and the formation of the pores in the lamellae.

The quantitative aspect of plastid development during growth in darkness was studied by Bradbeer et al. (1974). They find a 41-nm repeat distance for the hexagonal units and a minimum diameter of 11.8 nm for the tubules. Based on these measurements and assuming a hexagonal model, they calculate that 1 μm³ of prolamellar body should
contain 44 \( \mu m^2 \) of membrane, which is very close to the result obtained by Gunning and Jagoe for their cubic model. This value is used to calculate the membrane equivalent at various developmental stages.

Bradbeer et al. point out that in their electron micrographs, the thylakoids which connect to the margins of the prolamellar body are arranged normal to its surface. This observation does not appear to be consistent with the interpretation of Weier and Brown, that the prolamellar body is formed by parallel thylakoids, each of which gives rise to the configuration of the tubules. It seems that the exact manner of the condensation of the thylakoids to give rise to the prolamellar body has not yet been satisfactorily explained.

B. Ultrastructural Changes during Greening and Formation of Chloroplasts

When etiolated plants are exposed to light, substantial structural changes occur, which lead to the conversion of the etioplasts into chloroplasts. These changes consist of increases in the amount of membrane and especially the amount of appressed thylakoids. Robertson and Laetsch (1974) quantified these developmental changes by determining the percentage of appressed thylakoid membrane relative to total membrane present at each stage of development.

Five different regions of barley leaf were examined by Robertson and Laetsch (1974). Owing to the activity of the leaf basal meristem, these five regions represented tissue of five different ages in a single barley leaf. Plastids at the lowest region of the leaf, near its base, were typical proplastids and contained almost no membranes in the dark. Yet, plastids at the top of the leaf were larger and more numerous and contained well-developed prolamellar bodies. There
were usually a number of single thylakoids extending into the stroma from the periphery of the prolamellar bodies. Small overlaps appeared in the primary thylakoid layers of these plastids in the dark.

To simplify the complex process of metamorphosis of an etioplast into a chloroplast that is triggered by light, Robertson and Laetsch (1974) divided the developmental changes into five stages. In the first stage the plastid has a few single thylakoids in the stroma. Small overlaps corresponding to approximately 6% appression appear in the second stage. The third stage is characterized by a few small grana with about 28% appression. Larger and more numerous grana corresponding to 56% appression indicated the fourth stage. Stage five was classified as the fully differentiated chloroplast with large grana stacks containing approximately 71% appression. Plastids in each region of the barley leaf reach full development, stage five, at different times. Etioplasts at the top of the leaf require only 12 hr illumination to reach full development. At the base of the leaf, plastids require 48 hr. Robertson and Laetsch (1974) reached the conclusion that all regions in the leaf go through similar stages of development but do not develop at the same rate. Younger regions require longer periods of illumination than older ones to form fully developed chloroplasts.

Many investigators (Gunning and Jagoe, 1965; Weier et al., 1970) observed that in the first stage, the order and regularity of the crystalline center in the prolamellar body is largely lost, although the continuity of the membrane surface is retained. This new stage has been called by Weier et al. (1970) a reacted prolamellar body. However, many profiles of prolamellar bodies in dark grown leaves do not show
a crystalline form. In some leaves, crystalline and non-crystalline profiles can appear in the same cell.

The crystalline prolamellar bodies occur not only in etioplasts that have not received any illumination, but also in etioplasts that have been returned to the darkness following five minutes illumination at 750-100 fc (Gunning and Jagoe, 1967). The crystalline prolamellar body can also reform in light of low intensity of 100 fc accompanied by chlorophyll synthesis and grana formation (Henningsen and Boynton, 1970; Weier et al., 1970). Bradbeer et al. (1974), using high light intensity, e.g. 505 fc, found that the loss of crystalline arrangement was complete within one to two hours after the beginning of illumination. But in the samples fixed after 7.5 hours of high intensity illumination, a small number of crystalline prolamellar bodies were found in the plastid section. However, recrystallization of prolamellar bodies is less prominent at higher light intensities, and no prolamellar bodies were found in 10 hour samples or in any subsequent samples. The rate of membrane dispersal is lower under low light intensity than is observed in similar leaves illuminated with high light intensity. Crystalline prolamellar bodies are much more abundant in leaves greened under low light intensity (Henningsen and Boynton, 1970).

However, prolamellar bodies also appear in light grown plants until the terminal phases of chloroplast development (Laetsch and Price, 1969). The bundle sheath cell and mesophyll cell chloroplasts of light grown sugar cane leaf from a region of the leaf containing 50% of its maximal chlorophyll have a well-developed grana and a prolamellar body connecting grana stacks (Laetsch and Price, 1969). Sometimes the paracrystalline structure of the prolamellar body is still visible. These observations point to the fact that the prolamellar body takes part in the development of plastids of both light- and dark-grown plants.
It seems that if the necessary constituents for building normal chloroplast membranes are provided by the photoreactions, the structures of the reacted prolamellar body will untangle to form normal lamellae and grana. The prolamellar body is the visualization of accumulated membrane materials constantly produced by the inner membranes of the envelope. These invaginated membranes form grana and stroma thylakoids in the light, stroma thylakoids and prolamellar bodies in the dark.

The quantitative aspect of plastid development in the light was studied by Bradbeer et al. (1974). The loss of prolamellar bodies is accompanied by an increase in thylakoid area. Between 9 and 10 hours of illumination there is an increase in thylakoid area from 20.5 to 42.0 \( \mu m^2/plastid \). It was calculated from the prolamellar body model that 1 \( \mu m^3 \) of prolamellar body contains 44 \( \mu m^2 \) of membrane. The total internal plastid membrane increases from 289 \( cm^2/leaf \) in 14 day-old dark-grown bean leaves to 2890 \( cm^2/leaf \) after 160 hours of illumination.

Initial studies on crude preparations of isolated etioplasts (Klein and Poljakoff-Mayber, 1960, 1961; Boardman and Wildman, 1962) showed that the internal ultrastructure of the organelles was relatively unchanged by the isolation procedure. These workers and, more recently, Wellburn and Wellburn (1971), showed that etioplasts were capable of development upon illumination, and exhibited similar ultrastructural changes observed in etiolated leaves exposed to light.

C. Spectroscopic Changes in Etiolated Leaves during Greening

The etioplasts are devoid of chlorophyll, but they do contain a small amount of a yellow-green pigment called protochlorophyllide. Upon illumination of the dark-grown plants, protochlorophyllide is rapidly converted to chlorophyllide \( \alpha \), which is then esterified with phytol alcohol. After a short lag phase, typically 1 hour, there is
a rapid synthesis of chlorophyll a, and subsequently chlorophyll b is formed. The photosynthetic activities appear after several hours of greening.

Protochlorophyllide is in a different state in vivo than when it is extracted into organic solvents. In the natural state, protochlorophyllide is complexed with protein. The absorption spectrum of protochlorophyllide in ether was measured by Koski and Smith (1948). The principal absorption maxima are at 623 nm and 432 nm, with specific absorption coefficients of 39.9 and 325.5 liter gm⁻¹ cm⁻¹, respectively. Both the position of the maxima and the specific absorption coefficients vary with the nature of the solvent. Protochlorophyllide shows a strong orange-red fluorescence when dissolved in a polar solvent. The fluorescence spectrum was measured by French et al. (1956). The fluorescence maximum is at 631 nm for protochlorophyll in ether.

In vivo, in etiolated leaves of angiosperm seedlings, three protochlorophyllide forms have been identified by their absorption and fluorescence maxima at -196°C (Kahn, Boardman and Thorne, 1970; and Dujardin and Sironval, 1970). The first, P650 (absorption maximum at 650 nm) is transformed rapidly by light to chlorophyllide (Shibata, 1957). The second, P637, occurs to a lesser extent in etiolated leaf, but is more predominant in etiolated leaf extracts. It is also phototransformable to chlorophyllide (Kahn et al., 1970). The third species, P628, occurs only in low concentrations in etiolated leaves. It is not directly phototransformed to chlorophyllide, but, under some circumstances, seems to act as a precursor of P650 (Granick and Gassman, 1970). The same absorption bands are also observed at room temperature, but they are enhanced and better resolved at low
temperature (-196°C). P650 and P637 can be converted to P628 by heat, acid, and freezing-and-thawing (Dujardin and Sironval, 1970; Gassman, 1973).

The fluorescence spectrum of etiolated leaves at -196°C exhibits only two maxima, F655 and F630. However, the excitation spectrum of F655 shows peaks at 650 nm and shoulders at 638-639 nm. Boardman et al. (1970) concluded that the fluorescence emitted at 655 nm originates from P650, but it is activated by light absorbed both by P650 and P637. Energy is transferred from P637 to P650 with high efficiency. Fluorescence emitted at 630 nm originates from P628.

Protochlorophyllide is converted to chlorophyllide via several spectroscopic intermediates. One of the current views on the terminal steps in the photoreduction of protochlorophyllide to chlorophyllide was formulated by Litvin and Belyaeva (1971), by Thorne (1971), and by Mathis and Sauer (1973).

According to Litvin and Belyaeva (1971), an intense short time (2-4 msec) irradiation (200 W/m²) at 20°C or lower temperatures, gives rise to the first intermediate, C684/676, which has an absorption maximum at 676 nm and fluorescence maximum at 684 nm at -196°C. This intermediate is unstable, short-lived and transforms in continued irradiation (17 msec at 20°C), to C690/680. The last reaction of the transformation of C684/676 to C690/680 does not occur in the dark. In the dark, however, C684/676 decays to a different species, C675/670. This product, C675/670, is very stable and was identified as an esterified Chlide form as shown by thin-layer chromatography on cellulose. Only upon further illumination does C684/676 transform to C690/680, which suggests a second photochemical reaction. The kinetics
of the photoconversion of P655/650 (Pchlide with absorption maximum at 650 nm and fluorescence maximum at 655 nm), as measured by the changes of fluorescence intensity of the transforming forms, follows very closely the theoretical curve for two consecutive first order photochemical reactions. Thorn's (1971) measurements of dark ethanol extractions of leaves indicate a requirement of two quanta to give a molecule of C684/678, compared with one quantum for a molecule of the intermediate C674/668. This is in accord with two photochemical reactions in sequence.

Following the second photochemical reaction, C690/680 is dark shifted very rapidly to C695/685. Finally, C695/685 is transformed in the dark to C683/670. The last dark shift, discovered by Shibata (1957), occurs in a time of the order of 30 min at a temperature higher than 0°C. The dark shift involves first the shift in emission maximum from F693 to F683, followed by a doubling of fluorescence emission. Based on these observations, Thorne (1971) suggested that the Shibata shift involves two distinct processes in sequence. The rapid shift in absorption maximum from 678 nm to 684 nm has been attributed to environmental changes (Gassman et al., 1968) as well as to protein relaxation in the absence of any physical environmental change (Thorne, 1971). The second dark shift has been variously ascribed to metabolic chemical changes and orientation changes (Thorne, 1971), to phytlation (Sironval et al., 1965), and also to disaggregation of Chlide from the protein complex (Butler and Briggs, 1966; Bogorad et al., 1968) and, on the other hand, to disruption of Chlide aggregates (Butler and Briggs, 1966; Mathis and Sauer, 1973).
Mathis and Sauer (1973) observed essentially the same intermediates in the photoconversion of protochlorophyllide to chlorophyllide at 0°C. They proposed an interesting model which is based on the presence of dimers of protochlorophyllide, absorbing at 650 nm. Protochlorophyllide dimers are reduced stepwise to dimers of chlorophyllide absorbing at 678 nm, in two successive light reactions. The intermediate mixed protochlorophyl-chlorophyllide dimer absorbs at 676 nm and dissociates much faster to monomers than the chlorophyllide dimer. The last slow dissociation is regarded as the cause of the Shibata shift. Accordingly, the following model is presented:

\[ \text{P-P650} \xrightarrow{\text{hv}} \text{P-C676} \xrightarrow{\text{hv}} \text{C-C678} \xrightarrow{\text{dark}} \text{C-C684} \]

\[ \text{P+C672} \xrightarrow{\text{dark}} \text{2C672} \]

\[ \text{P635} \xrightarrow{\text{hv}} \text{C672} \]

A different model was suggested by Nielsen and Kahn (1973) and by Thorn and Boardman (1972). Photoconversion of PChlide to Chlide is viewed as an intrinsically first order photochemical process, in which excitation transfer between PChlide and Chlide acts as the complicating factor (this model is discussed in detail in Section F). The same view is shared by Vaughan (1975). Measurements of Chlide fluorescence excited at 670 nm were found to be in good agreement with Chlide absorption throughout the photoconversion process. It was concluded that the intrinsic quantum yield of Chlide fluorescence is constant during the course of photoconversion in a sucrose holochrome preparation (Vaughan and Sauer, 1974). A constant quantum yield of
fluorescence is in contrast with a model in which closely coupled dimers of Chlide exist (Mathis and Sauer, 1972). Such dimers would be expected to display a fluorescence efficiency different from that of the monomers (i.e., mixed PChlide-Chlide dimers) present at partial conversion. Changes in the shape or position of the absorption band during photoconversion are also absent (Mathis and Sauer, 1972). However, the CD spectrum offers a strong evidence for an excitonically paired PChlide photoconverting to an excitonically paired Chlide (for a detailed discussion, see Section G). In conclusion, the exact mechanism in which PChlide is photoconverted to Chlide has not yet been satisfactorily explained.

The assignment of absorption and CD maxima to PChlide or Chlide does not give us information with regard to their molecular nature. They could reflect different pigment-protein or pigment-pigment interactions.

D. The Spatial Location of Protochlorophyllide in the Etiolated Leaf

The location and distribution of PChlide in etioplasts was investigated by several groups of researchers (Boardman and Wildman, 1962; Boardman and Anderson, 1964; Klein et al., 1964). They suggested the association of PChlide with the prolamellar bodies through phase contrast and fluorescence microscopic observations of bean plastid suspensions. Phase contrast revealed the presence of dark bodies within the etioplasts, which were the exclusive areas of red fluorescence when irradiated with short wave blue light, and seemed to be identical with prolamellar bodies seen in electron micrographs.
Boardman (1962; 1964) isolated a phototransformable PChlide-protein complex of MW $6 \pm 0.5 \times 10^5$ and a molecular diameter of 100 to 110 Å. The tentative identification of the PChlide-protein complex as a constituent of the tubular membranes of the prolamellar body is based on Kahn's observations. Kahn (1968) observed red fluorescence in isolated prolamellar bodies and, more importantly, the presence of regularly arranged macromolecules in the tubular membranes. These macromolecules seem to be close-packed with an axial center-to-center distance of 80 Å. Similar or identical macromolecules can be seen in non-tubular membrane segments which are continuous with tubular membranes. They have an average diameter of about 100 Å. Because the isolated PChlide-protein complex has a diameter of 100 to 110 Å, it may be the macromolecule observed in the prolamellar body and so an integral part of these membranes. However, some etiolated tissues do not have prolamellar bodies but do have PChlide.

E. The Molecular Properties of Protochlorophyllide Holochrome

The phototransformation of PChlide to Chlide requires a specific pigment-protein complex, the PChlide holochrome (Smith, 1948). It was first extracted into aqueous solution by Krasnovsky and Kosobutskaya (1952), and Smith and Benitez (1953). The biochemistry and physiology of PChlide holochrome have been extensively reviewed by Boardman (1968), Kirk (1967), and more recently by Rebeiz and Castelfranco (1973).

PChlide holochrome is photoreduced by the addition of two hydrogens trans to one another at the 7 and 8 positions of the pyrrole ring IV of the porphyrin macrocycle to produce Chlide holochrome. This transfer is irreversible, and both the nature of the transfer mechanism and the
identity of the reductant are unknown. Evidence in the literature for quinones, reduced pyridine nucleotide, or ascorbic acid in either partially purified holochrome or intact leaves may imply the presence of several reductants associated with the protein (Robbelen, 1956; Rudolph and Butatsch, 1966; Oku and Tomita, 1970). The possible role of a protein thiol group in the photoconversion has also been proposed (Boardman, 1962). However, no conclusive evidence has been presented for its actual participation in the reaction. Recently, Griffiths (1974) suggested NADPH as the hydrogen donor for PChlide photoreduction. He obtained a fraction of isolated etioplasts which show a high level of chlorophyll formation under a series of light flashes. If these etioplasts are water lyzed, a very low level of chlorophyll is obtained. However, if the sample is incubated in the presence of an NADP regenerating system, a greatly increased absorption due to chlorophyll at 675 nm is accompanied by a decrease in the absorption due to PChlide at 633 nm. Kransnovsky et al. (1970) were able to achieve the chemical photoreduction of PChlide to chlorophyll in solutions of ascorbic acid in pyridine at 20°C, with a yield of about 12%.

Smith and coworkers (1960) partially purified PChlide holochrome. They showed that the holochrome is a protein-PChlide complex which needs no external cofactor for the light-mediated reduction to Chlide holochrome. It was found to have a sedimentation coefficient of $S_{20,w} = 18$ and a molecular weight of $600,000 \pm 50,000$. The specific activity of the highest purity PChlide holochrome was found to be equivalent to 0.24 g of protein per μmole PChlide; thus the aggregate of molecular weight about 550,000 contains at least two PChlide
chromophores (Schopfer and Siegelman, 1968). PChlide holochrome ran as two separate peaks on agarose column, of MW 550,000 and 300,000. The behavior of PChlide holochrome on agarose column suggested an aggregating-disaggregating system.

The chain of reactions occurring during photoconversion can be monitored by the change in the absorption maximum. The number of intermediates observed in the photoreduction of PChlide holochrome is smaller than in an etiolated leaf, and they also occur at shorter wavelengths. However, it is assumed that the same processes take place in both. In PChlide holochrome the photoconversion can be schematically represented in the following way:

\[
\text{P639} \xrightarrow{\text{hv}} \text{C678} \xrightarrow{\text{dark}} \text{C672}
\]

Using flash spectroscopy, it was shown that the light induced absorption change was completed in less than $10^{-5}$ sec at 0°C (Schopfer and Siegelman, 1969). The dark transformation takes about 30 min at 12°C. There is no concomitant shift of the Soret band. This wavelength shift was found at all stages of the purification carried out by Schopfer and Siegelman (1969). The dark shift is inhibited below 0°C or in the presence of 2 M sucrose.

Circular dichroism measurements and fluorescence spectroscopy studies point to the presence of PChlide in an aggregated state. The exact number of chromophores per protein macromolecule is still a matter of controversy. Circular dichroism measurements done on PChlide holochrome indicate a dimer of PChlide molecules as the basic unit (Schultz and Sauer, 1972). On the other hand, fluorescence
spectroscopy studies at -196°C performed by Kahn et al. (1970) indicate a basic aggregate of at least 4 chromophores per unit. Thorne (1971) suggested an aggregate of 20 chromophores per unit. His estimate is based on calculations of energy transfer between PChlide to Chlide in etiolated bean leaves at -196°C. However, the exact number of PChlide molecules per protein molecule is still a matter of controversy. Recent studies (Vaughan and Sauer, this thesis) indicate that there are at least 4 chromophores per 600,000 MW protein.

The presence of an aggregate of PChlide molecules does not seem to be a prerequisite for the photoreduction of PChlide to Chlide. Henningsen and Kahn (1974) have isolated a subunit of PChlide holochrome of molecular weight about 170,000 from bean leaves and another one with a molecular weight of 63,000 from barley leaves. Fluorescence measurements at liquid N₂ temperature, the kinetics of photoreduction and circular dichroism experiments at 20°C executed on the barley subunit indicate the presence of only one PChlide molecule per 63,000 molecular weight protein molecule. Both subunits are obtained with the aid of a detergent saponin, and exhibit the photoreduction of monomeric PChlide to Chlide. Barley PChlide subunits absorbing at 644 nm were indistinguishable by Sephadex gel filtration from their immediate photoproduct absorbing at 678 nm. However, a shift in the absorption maximum from 678 nm to 672 nm correlated with changes in the circular dichroism spectrum at 580 nm to 590 nm (Forster et al., 1971) and, more importantly, on the molecular level, with a change in the molecular weight from 63,000 to 29,000 (Henningsen et al., 1974). It was suggested that the change in molecular weight reflects the dissociation
of the Chlide holochrome into a Chlide a carrier protein complex and a photoenzyme, before the incorporation of chlorophyll into the lamellar membrane. A similar observation was also reported by Bogorad, Laber and Gassman (1967). They isolated a small molecular weight Chl-protein in a sucrose density sedimentation experiment. However, this pigment-protein complex was not further characterized.

F. Kinetics and Quantum Yield of Photoconversion of PChlide to Chlide a

The kinetics of the phototransformation were studied in leaves and in the PChlide holochrome. Smith and Benitez (1954) found that in barley leaves the kinetics of transformation are consistent with a second order reaction, but the temperature coefficients were low for a bimolecular collision reaction. The rate of transformation was found to be directly proportional to light intensity, showing that the apparent bimolecular reaction was not dependent on the collision of two photochemically excited molecules. The transformation was completely inhibited at -196°C. It occurred at -70°C, but it was not complete at this temperature. Both the rate and the extent of photoconversion increased as the temperature was increased to 40°C. The dependence on temperature shows that the photoconversion is not a purely photochemical process, but that some thermochemical steps are also involved. In PChlide holochrome, the rate of phototransformation was found to be independent of the initial concentration of PChlide and not influenced by the viscosity of the medium. These results suggest that the transformation reaction does not involve a bimolecular collision process. Boardman (1962) postulated that the phototransformation involves a restricted collision between photoactivated PChlide
molecule and hydrogen or electron donor, in which both are integral parts of the holochrome macromolecule. First order kinetics would be expected. However, the kinetic data obtained by Boardman (1962) did not follow simple first order kinetics. The transformation data fit the sum of two first order reactions. In order to account for this type of kinetics, it was proposed that either the PChlide holochrome is a mixture of two types of complexes—each of which contains one PChlide molecule per protein molecule—or there are two PChlide molecules per protein molecule, and the rate of transformation of one PChlide is different from the rate of transformation of the second PChlide.

Sironval et al. (1968) presents a different view. He found that the fluorescence of etiolated bean leaves could be related to first order kinetics at 647 nm, but at 630 nm or shorter the photoconversion appeared to follow the sum of two first order processes. Thorne and Boardman (1972), using 630, 640, and 647 nm light, observed a second order kinetics when 75% of the total PChlide was converted in whole bean leaves. Their work implies that the occurrence of energy transfer between excited PChlide and newly formed Chlide leads to the apparent second order kinetics by way of competition between the energy transfer process and the photoconversion reaction. The energy transfer process was also implicated in the dynamic model set forth by Nielsen and Kahn (1973). The kinetics of photoconversion was investigated in barley leaves and in preparations of PChlide holochrome subunits from barley. It was found that, in barley leaves which exhibit energy transfer in the macromolecular aggregate of chromophore molecules at -196°C, the
-19-

kinetics depart from first or second order. However, in barley sub-units prepared with the aid of a detergent saponin, and in which the absence of energy transfer indicated monomeric PChlide, the kinetics is first order.

The dynamic model describing the photoconversion of PChlide to Chlide is based on the assumption that the basic process of transformation follows first order kinetics. The dynamic model implies the occurrence of four steps in the photoconversion process:

1) PChlide + hν → PChlide*, 2) PChlide* + 2H → Chlide, 3) PChlide* → PChlide + lost energy, and 4) PChlide* + Chlide → PChlide + Chlide*.

Steps 1) through 3) are first order with respect to PChlide* (excited PChlide). Step 4) describes the energy transfer from PChlide* to newly formed Chlide, is not first order, and is assumed to compete effectively with the reactions leading to the loss of PChlide*.

Fluorescence measurements conducted by Vaughan and Sauer (1974) suggest that energy transfer takes place from PChlide to Chlide at room temperature in crude bean holochrome preparations. The occurrence of energy transfer at room temperature offers strong support for the dynamic model presented by Nielsen and Kahn (1973). It was found that Chlide fluorescence excited at 587, 638, 640 and 650 nm rose more rapidly than did the absorption curve. This difference was greatest at 640 nm, the absorption maximum of PChlide. However, fluorescence excited at 670 nm coincided with the absorption at 678 nm. Excitation at 670 nm caused no photoconversion and therefore was absorbed solely by Chlide. These results suggest that Chlide fluorescence excited at wavelengths shorter than 660 nm is dependent not only on the concentration of Chlide, but also on the concentration of excited PChlide.
transferring energy to newly formed Chlide.

The quantum yield for the photochemical transformation was measured in bean PChlide holochrome by Smith and French (1958, 1959). The wavelengths used were 642 nm and 644 nm. Four independent experiments yielded the values 0.652, 0.478, 0.703, and 0.610, with an average of 0.6. These determinations were based on the assumption of second order kinetics for the overall process. Using an initial rate based on first order kinetics, Nielsen and Kahn ( ) calculated a value of 0.43 for the quantum yield in dark grown barley leaves and isolated barley PChlide holochrome subunit. The agreement between the two studies, regardless of the model employed in the calculations, is fairly good. It seems that the photoconversion is a one-quantum process with an overall efficiency of about 0.5. In particular, this is consistent with the model of Mathis and Sauer (1972) where only one of the two PChlide molecules in the holochrome is photoconverted with high efficiency.

G. Circular Dichroism Studies on the Structure and Photochemistry of Protochlorophyllide Holochrome

Circular dichroism is defined as the difference in absorption of left and right circularly polarized light. An asymmetric system will display optical activity, and therefore exhibit circular dichroism. Circular dichroism is a sensitive measure of the geometrical arrangement of molecules in a disymmetric aggregate system. The quantum mechanical treatment that describes the resonance interaction between excited states of weakly coupled aggregate systems is the exciton
model (Kasha, 1963). According to this model, instead of locally exciting each individual molecule in the aggregate, a collective excitation is described for the system, generating an exciton band. The width of the exciton band is dependent on the oscillator strength of the corresponding electronic transition in the unit molecule, inversely proportional to the cube of the intermolecular distance, and a function of the geometrical relationship between transition dipoles in the unit molecules. For an aggregate of $N$ molecules, the exciton band will consist of $N$ discrete exciton states. Allowed electric dipole transitions will take place to exciton states equal or smaller in number to the number of molecules per unit cell. As a result of exciton splitting, there are pronounced spectral effects of molecular aggregates compared with the individual molecules. The absorption band for the optical transition in question will be split into a number of components smaller or equal to the number of molecules per unit cell. The exciton contribution to the rotational strength is conservative (Tinoco, 1962), meaning that the rotational strength must sum to zero over the exciton band. Thus, in a dissymmetric aggregate system, each transition to an exciton state will have an opposite rotational strength and therefore circular dichroism with an opposite sign. The rotational strength which can be measured experimentally from the area of the corresponding circular dichroism band is proportional to the distance between the monomer dipoles and the angular relationship between them. Hence, circular dichroism can provide information, in principle, on the geometry of the system.
Pigment aggregation is the origin of band splitting of PChl and Chl pigments. The CD spectrum of PChl dimers in CCl₄ exhibits strong evidence of this band splitting. In contrast to the CD of monomer PChlide in ether, which shows a positive peak at 624 nm and a negative peak at 600 nm assigned to the Q_y (0-0) and Q_x (0-0) transitions respectively, the CD of PChl dimer exhibits three peaks, a trough at 603 nm, a positive peak at 622 nm and another trough at 628 nm. The chlorophyll a CD spectrum changes from a single trough at 657 nm, for Chl a monomer in ether, to a large positive peak at 677 nm and a small negative peak at 659 nm for Chl a dimers in CCl₄ (Houssier and Sauer, 1970).

The CD spectrum of PChlide holochrome extracted from dark grown bean leaves resembles the CD exhibited by a PChl dimer in CCl₄. However, all the peaks appear to be shifted to the red. The troughs occur at 613 nm and 647 nm while the relative maximum occurs at 637 nm. PChlide holochrome is very stable in the presence of 2 M sucrose, and upon illumination it is phototransformed to Chlide holochrome with an absorption maximum at 678 nm. The dark shift to 674 nm is blocked in 2 M sucrose solution (Schultz and Sauer, 1972). The CD spectrum of Chlide holochrome in 2 M sucrose exhibits red band multiplicity, a large positive peak at 678 nm and a small negative peak at 687 nm. This spectrum is different from that of either Chl a monomer in ether or Chl a in CCl₄, where the signs and relative magnitude of the two long wavelength components are reversed. When PChlide holochrome is phototransformed to Chlide holochrome in the absence of sucrose, at 0°C, the red absorption maximum occurs at 677 nm and is associated with a single, asymmetric trough at 681 nm in the CD spectrum. After 1 hr at
5°C, the absorption maximum shifted to 674 nm, the CD trough to 679 nm, and decreased in magnitude. No isosbestic point was found to accompany this shift.

Schultz and Sauer (1972) proposed a model based on the presence of two closely interacting PChlide molecules on the holochrome particles, giving rise to a dimer of Chlide molecules upon illumination. Chlide dimer is very stable in 2 M sucrose, but undergoes dissociation in the absence of sucrose, to yield Chlide monomers.

The effect of high concentration of sucrose on the spectral properties of PChlide and Chlide holochrome was further investigated by Mathis and Sauer (1972). It was found that while the CD spectrum of PChlide holochrome was identical in the presence or absence of high sucrose concentration, the Chlide holochrome existed in two different conformations giving rise to markedly different CD spectra. In the presence of sucrose the CD spectrum was identical to the one observed by Schultz and Sauer (1972). It has a large positive peak at 676 ± 1 nm and a small negative peak at 689 ± 2 nm. In the absence of sucrose, concomitantly with the dark shift in the absorption maximum, there is a progressive decrease in the amplitude of the CD signal until eventually it disappears. This is in contrast to the CD spectrum obtained by Schultz and Sauer (1972). However, at temperatures below 0°C, the CD signal in the absence of sucrose is stable and exhibits a large negative peak at 685 nm, and small positive peak at 674 nm. The change from one conformation to the other is reversible.

During photoconversion, the absorption spectrum shifts regularly, but there is no change in the shape of the absorption band and no indication of an intermediate species. However, the CD does not
-24-
evolve linearly with percent photoconversion. In high sucrose concentration, when photoconversion is lower than 20%, a single positive CD peak appears at 682 nm. Upon further illumination, a double CD develops characteristic of fully converted material. In the absence of sucrose, at low temperature again, the CD does not evolve linearly but, in contrast to a high sucrose sample, the single positive CD peak at 682 nm is absent. Analysis of these spectra revealed only two components. The model proposed by Mathis and Sauer claims that a dimer of PChlide molecules is photoreduced by two successive photochemical reactions. The single positive CD peak indicates an intermediate form of mixed PChlide-Chlide which upon further illumination transforms to a Chlide dimer exhibiting a double CD. The Chlide holochrome so produced can exist in two forms, the one in sucrose is stable and the other one in the absence of sucrose is unstable and undergoes dissociation yielding two non-interacting Chlide molecules.

In the PChl subunit from barley, the dark shift from 678 nm to 672 nm is accompanied by a decrease in 580 nm absorption and in 590 nm dichroism (Foster et al., 1972). All of these changes follow first order kinetics with a half-life time of 15 min at 23°C. The rates are strongly dependent on viscosity and temperature. It is proposed that the Chlide holochrome is in an unstable conformation and the spectral shifts follow the conformational relaxation of the holochrome to a more stable state.

The CD spectrum of bean and barley PChlide holochrome saponin subunit at the red region of the spectrum are characterized by a broad negative band which is usually resolved into two components at 635 nm and 615 nm. Upon photoconversion, this peak disappears, and
the newly formed Chlide holochrome subunit shows a positive peak at 680 nm. Following the dark shift, the positive peak at 680 nm disappears and is replaced by a positive component at 672 nm and a negative component at 660 nm. Henningsen et al. (1973) attribute this CD signal to aggregation among particles.

The absence of exciton interaction and first order kinetics in barley holochrome subunits suggests a single PChlide molecule per protein subunit of 63,000 MW. However, the photoconversion for bean holochrome subunit of 170,000 MW departs from first order kinetics in bean PChlide holochrome extracted with 3.6% saponin. So the possibility still exists that the bean PChlide saponin subunit contains more than one PChlide molecule.

H. Molecular Weights of Protein Subunits of the Lamellae of Etioplast and Chloroplasts

A solubilization of etioplast and chloroplast membranes is obtained with an anionic detergent, sodium dodecyl sulfate (SDS). The qualitative analysis of solubilized lamellar proteins can be performed by disc electrophoresis in polyacrylamide gels. Electrophoresis performed on solubilized spinach, wheat and barley chloroplasts yielded similar patterns. About 10-17 components are observed (Remy, 1971; Machold, 1971).

A current problem that has been intensely investigated, is whether the exposure of etiolated material to light leads to a de novo synthesis of new proteins (Rhodes, 1963; Drumm, 1970) or whether etiolated plastids contain all or at least a large part of the proteins
necessary for building the lamellae and grana (Collot et al., 1970). If the latter case prevails, the structural changes might be due to a reorganization of the existing proteins rather than to a de novo protein synthesis.

Remy (1973) observed no qualitative difference between etioplast and chloroplast proteins by gel electrophoresis. The difference seemed to be only quantitative. During chloroplast development, a large increase in a protein of MW 23,500 takes place. This protein becomes a major one in mature chloroplasts. Some other proteins of smaller molecular weight decrease in their concentration. The ten thylakoid polypeptides observed by Remy (1973) are spread in molecular weight from 81,000 for component 2 to 14,200 for component 10. Polyacrylamide electrophoresis of etioplast membranes shows that sugars are linked to the structural protein of lowest MW of 10,000 (Lagoutte and Duranton, 1972). However, the resolution of the polyacrylamide disc electrophoresis technique is not sufficient to detect a de novo synthesis of protein among the large number of etioplast and developing chloroplast proteins present.

Thornber et al. (1967) found that the bulk of the chloroplast lamellar material is composed of two major chlorophyll proteins, complex I and II, and two proteins which migrate between them, one of which is pigmented. A green complex III and yellow complex IV are free pigments. Complex I of MW 100,000 was found to be enriched in Chl a, and identified as part of the PS I chlorophyll protein. Complex II of MW 35,000 was found to be enriched in Chl b and was identified as part of the PS II chlorophyll protein.
Labeling experiments with δ-[3H] aminolevulinic acid (ALA) and SDS polyacrylamide electrophoresis on etiolated membranes of Zea mays suggested that PChlide is associated with two polypeptide chains of MW 21,000 and 29,000. Chlorophyll in the developing chloroplast membranes was found to be associated with four polypeptide chains of MW 21,000, 25,000, 29,000 and 70,000. When the etiolated plants are exposed to light, PChlide is converted to Chl. The newly formed Chl remains associated with polypeptide chains of MW 21,000 and 29,000. However, there is a separate pathway where Chl is associated with polypeptide chains of MW 25,000 and 70,000 (Guignery et al., 1974).

In this thesis, the role of PChlide holochrome in the development of the photosynthetic membrane is studied in detail, with special attention to the subunit structure of the holochrome. The interactions among the pigments associated with the holochrome are investigated using absorption and CD. The photoconversion reaction is studied at low temperature and for freeze-dried preparations in a solid pellet. Evidence is presented identifying the only polypeptide of the holochrome of 45,000 daltons and its cleavage after photoconversion as correlated to the spectroscopic dark shift.
CHAPTER II

ABSORPTION AND CD SPECTRA OF GREENING BEAN LEAF HOMOGENATES, OF NORMAL SPINACH, AND OF BARLEY MUTANT AT ROOM TEMPERATURE AND AT LIQUID NITROGEN TEMPERATURE

A. Introduction

A most striking phenomenon in plant physiology is the greening of cellular and etiolated plants. During this period substantial sub-cellular structural changes occur, which lead to the conversion of the etioplasts into chloroplasts. This topic is discussed in detail in Chapter I (B,C). The progress of greening has been studied by Schultz and Sauer (1972) using spectroscopic methods. They monitored the changes in absorption and CD spectra of greening leaf homogenates as they develop progressively with illumination. Our observations confirm these results, and we have extended their study to a shorter period of illumination.

We have also studied the optical properties of photosynthetically active chloroplast membranes of normal spinach and of a mutant of normal barley lacking Chl b. The absorption and CD spectra of normal and mutant barley at room temperature (23°C) were reported by Schultz and Sauer (1972). The results described in this chapter corroborate the previous studies and extend them to liquid nitrogen temperature. At this temperature, the spectra are expected to be resolved better because of the restricted mobility of the molecules. Therefore, more information may be obtained about the spatial relationship between
Chl a and Chl b molecules in normal spinach and between Chl a molecules in the barley mutant.

B. Materials and Methods

Red kidney beans (Phaseolus vulgaris L.) were grown 11 ± 2 days on vermiculite in the dark at 22°C. The leaves (50 to 100 g) were illuminated for various times and then homogenized. The homogenates were prepared according to the method described by Schultz and Sauer (1972).

Spinach (Spinacia oleracea, var.), barley (Hordeum vulgaris) and barley mutant lacking Chl b (H. vulgaris, strain Chlorina) were grown outdoors in vermiculite. Leaves were homogenized for 30 sec in 0.02 M Tris buffer (pH 8.0), containing 0.5 M sucrose and 0.01 M NaCl. The brei was sonicated (Biosonik sonicator) for 10 sec at maximum power. The strained, sonicated homogenate was then centrifuged for 10 min at 14,000 g in a SS-34 rotor with a Sorvall centrifuge, after which the supernatant was recentrifuged at 100,000 g for 30 min using a #60 Ti rotor in a Spinco Model L-2 ultracentrifuge. The sediment was recovered and resuspended in about 1 ml of 0.02 M Tris buffer (pH 8.0). Finally, the resuspended sediment was clarified by centrifugation at 14,000 g for 10 min. This method for the preparation of chloroplast fragments was adapted from the procedure reported by Park and Pon (1963).

Absorption spectra of the various preparations were measured in a 1 cm cuvet using a Cary Model 14 spectrophotometer. CD spectra were measured in a Jasco J-20 spectrophotometer.

For the liquid nitrogen temperature measurements, a special dewar was used, designed by J. M. Olson. The sample consisted of a suspension of chloroplast fragments in 60% glycerol in water. A clear glass was obtained upon immersing the sample rapidly in liquid nitrogen.
C. Results

(1) CD of greening leaf homogenates. Figures 1 and 2 depict the absorption and CD spectrum of a homogenate prepared from dark grown bean leaves illuminated for 10 min and another sample illuminated for 2-1/2 hr at the intensity of 90 ± 10 ft-candles at room temperature. After 10 min of illumination, the absorption maximum was located at 672 nm. A single trough appeared at 664 nm in the CD spectrum.

The CD minimum at 664 nm did not correspond to the absorption maximum at 672 nm. However, the absorption maximum of Chl a monomers in ether is at 661 nm and the CD spectrum is characterized by a single trough at 657 nm (Houssier and Sauer, 1970). The optical activity of a homogenate from leaves that received 10 min illumination resembled very closely that of Chl a monomers in ether both in the sign and the shape of the CD signals, except that in the homogenate the absorption and CD peaks are red shifted by 7 to 10 nm.

Mathis and Sauer (1972) observed a single positive CD peak at 682 nm as an intermediate in the photoconversion of excitonically paired PChlide molecules to excitonically paired Chlide molecules. This peak was interpreted as due to a mixed PChlide-Chlide dimer. However, it has an opposite sign to Chl a monomers in ether and is red shifted by 25 nm. This could be due to strong pigment-pigment interactions.

After 2-1/2 hr of illumination, the absorption maximum has shifted to 674 nm and the CD spectrum had a more complex nature. It consisted of a large trough at 679 nm, a positive peak at 664 nm, and a small minimum at 650 nm. The CD spectrum obtained by us after
Figure 1. The absorption spectrum of homogenates from dark grown bean leaves illuminated for 10 min or 2-1/2 hr at 90 ± 10 ft-candles, at 23°C in 1-cm pathlength.
Figure 2. The CD spectrum of homogenates from dark grown bean leaves illuminated for 10 min or 2-1/2 hr at 90 ± 10 ft-candles, at 23°C in 1-cm pathlength.
2-1/2 hr illumination was similar in its shape and the position of the peaks to the one obtained by Schultz and Sauer (1972) after 2 hr of illumination. The magnitude of the CD signal and the corresponding absorption after 10 min and 2-1/2 hr illumination are compared in Table II-1.

<table>
<thead>
<tr>
<th>Time of illumination</th>
<th>Absorbance (A)</th>
<th>CD (AA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min</td>
<td>0.30</td>
<td>-0.6 x 10^{-4} (664 nm)</td>
</tr>
<tr>
<td>2-1/2 hr</td>
<td>0.52</td>
<td>-1.8 x 10^{-4} (679 nm)</td>
</tr>
</tbody>
</table>

The results in Table II-1 suggest that although the magnitude of the absorption has increased by a factor of only 1.7, the amplitude of the CD signal has increased by a factor of 3. The presence of multiple numbers of peaks of opposite signs in the CD spectrum, and the nonlinear increase in the intensity of the CD signal, points to an exciton interaction among a number of closely situated Chl molecules in homogenates of leaves illuminated for 2-1/2 hr. On the other hand, a single CD peak in leaves illuminated for 10 min represents the possibility of Chl monomers.

(2) CD of mature spinach, barley and mutant barley at room temperature and at liquid nitrogen temperature. The absorption and CD spectra of normal spinach at room temperature and at -196°C are shown in Fig. 3. The absorption maximum was located in both at 678 nm. A shoulder at 650 nm was prominent in the absorption of normal spinach but was missing
Figure 3. The optical activity of chloroplast fragments of normal spinach at 23°C (optical pathlength, 1.00 cm) and a suspension in 60% glycerol at -196°C (optical pathlength, 0.20 cm).
in the barley mutant (Figure 4). This shoulder is due to the absorption of Chl b. The CD spectrum of normal spinach chloroplast fragments at 23°C closely resembles that of normal barley. It was characterized by two minima at 654 nm and at 686 nm, and a maximum at 670 nm.

Barley mutant, deficient in Chl b, had an asymmetric CD signal featuring a large trough at 685 nm and a small positive peak at 672 nm. My results are compared with those obtained by Schultz and Sauer (1972) in Table II-2.

Table II-2. Peak Positions and CD Amplitudes for Normal Spinach and Barley Mutant at Room Temperature

<table>
<thead>
<tr>
<th>Sample</th>
<th>Abs. max.</th>
<th>CD Peaks and ΔA x 10^4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinach*</td>
<td>678 nm</td>
<td>654 (-1.4); 670 (+1.8); 686 (-2.2)</td>
</tr>
<tr>
<td>Barley**</td>
<td>678 nm</td>
<td>650 (-2.0); 666 (+2.0); 680 (-3.5)</td>
</tr>
<tr>
<td>Barley mutant*</td>
<td>678 nm</td>
<td>672 (+0.9); 685 (-3.2)</td>
</tr>
<tr>
<td>Barley mutant**</td>
<td>678 nm</td>
<td>666 (+1.0); 680 (-3.5)</td>
</tr>
</tbody>
</table>

*This work

**Schultz and Sauer (1972)

There is a general agreement between the results obtained here and those of Schultz and Sauer (1972). A similar ratio of intensities of maximum and minima is found in both cases, yet the peaks in this study are found consistently at about 5 nm longer wavelength. These measurements were extended to liquid nitrogen.

The absorption maximum of a suspension of spinach chloroplast fragments in 60% glycerol was at 673 nm, hence slightly blue-shifted
Figure 4. The optical activity of chloroplast fragments of a barley mutant which lacks Chl b at 23°C (optical pathlength, 1.00 cm) and a suspension in 60% glycerol at -196°C (optical pathlength, 0.20 cm).
compared to room temperature spectrum (Fig. 3). The absorption peak became narrower, with more pronounced shoulders at 650 nm and 620 nm.

Troughs at 640, 653, 676, and 696 nm, a maximum at 667 nm, and a small minimum at 696 nm were seen in the CD spectrum at liquid nitrogen temperature. The band width of the CD features narrowed appreciably compared to the room temperature signal, and was resolved to additional components. The shoulder that appeared at room temperature at 640 nm developed into a trough at 640 nm, and a small minimum appeared at 696 nm. The peaks were slightly shifted to shorter wavelength in contrast to the room temperature spectrum.

In the barley mutant, the absorption maximum occurred at 672 nm (Fig. 4). The band width decreased compared to that at 23°C, yet the absorption envelope did not show more structure at low temperature. In the CD spectrum at liquid nitrogen, we observed a positive peak at 670 nm, a large trough at 684 nm, and a very small trough at 697 nm. The CD pattern at low temperature was similar to that obtained at 23°C although it had a smaller band width. The relative amplitudes of the CD peaks are summarized in Table II-3.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Abs. max.</th>
<th>CD Peaks and ΔA x 10⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinach</td>
<td>673 nm</td>
<td>640(-0.48); 653(-1.1); 667(+1.05); 676(-2.1);</td>
</tr>
<tr>
<td>Barley mutant</td>
<td>672 nm</td>
<td>670(+2.8)</td>
</tr>
</tbody>
</table>

The liquid nitrogen CD spectrum of normal barley is illustrated in Figure 5. The general character of the CD spectrum was very similar to
Figure 5. The CD spectrum of chloroplast fragments of normal barley at -196°C in 60% glycerol. Optical pathlength, 0.20 cm.
that of spinach chloroplast fragment suspensions at -196°C (Fig. 3). The peak positions for normal barley were identical to those for spinach except for the trough at 667 nm (spinach) that appeared at 680 nm (barley).

D. Discussion

(1) The state of the pigment after a short illumination of dark grown leaves. Etiolated leaves contain PChlide holochrome. The first reaction that occurs upon shining light on dark grown plants is the photoconversion of PChlide to Chlide. The same reaction is carried out also in the isolated PChlide holochrome. The CD evidence suggests that PChlide which is aggregated and probably dimeric in the holochrome is photoconverted initially to a Chlide dimer. Following illumination, a dark shift takes place in the absorption band to shorter wavelength simultaneously with a disappearance of the double CD signal. This observation was interpreted as a dissociation of Chlide dimer into a monomer (Schultz and Sauer, 1972; Mathis and Sauer, 1972; this work, Chapter III). The dark shift in absorption is also observed in intact leaves (Shibata, 1957), where it is complete in 10-15 min at 23°C.

We found that a homogenate prepared from etiolated bean leaves after 10 min illumination showed an absorption maximum at 672 nm, indicating a completion of the Shibata shift. The single CD trough at 664 nm suggests the presence of Chl as a monomer at this stage of development. This observation of Chl monomers in the leaf corroborates the results obtained with the in vitro system, the Chlide holochrome. Previous work (Schultz and Sauer, 1972) indicates that the monomeric phase continues up to one hour after illumination. The CD spectra for
longer times of illumination, e.g. 2-1/2 hours, point to the fact that Chl is then in an aggregated form. This stage may correspond to the appearance of primary lamellae in the developing chloroplasts (Gunning and Jagoe, 1965). However, the latter work was done on oats.

(2) The geometry of Chl a and Chl b in mature spinach and barley mutant. In vitro measurements of the CD of Chl a and Chl b in most solvents show extremely small, nearly unmeasurable, optical activity. However, in carbon tetrachloride, where Chl a and Chl b solutions contain 85% dimer, an enhanced CD is observed. Chl a and Chl b dimers show very similar CD spectra consisting of a double CD signal that reverses sign close to the center of the absorption band. This behavior is due to the degenerate exciton interaction of the long wavelength transition moments of the two monomers in the dimer. The degenerate exciton splitting observed in the CD spectrum indicates that the monomer transition moments are not parallel, or exactly perpendicular in the dimer, nor are the Chl rings coplanar (Tinoco, 1963). Increasing Chl aggregation leads to increasing red shift of the absorption. A 10 to 15 nm red shift for the dimer relative to the monomer is observed in carbon tetrachloride. The monomer peak of Chl a at 666 nm is shifted in Chl a microcrystals by 80 nm to 745 nm (Dratz, Schultz and Sauer, 1966).

In order to gain a better understanding of the molecular architecture of pigments in the mature chloroplast membrane, we looked at the CD spectra of mature spinach barley and barley mutant (lacking Chl b) at room temperature and at liquid nitrogen temperature. The CD spectra at 23°C as well as in liquid nitrogen temperature showed
that Chl a part of the CD spectrum in barley mutant appeared to be identical to the Chl a region in the normal barley and in normal spinach. The CD signal due to Chl a was characterized by a large double CD component, as seen in the dimers and the crystal. The presence of the double CD component in the Chl a absorption region is a good evidence for Chl a - Chl a interaction. Chl-protein or Chl-lipid interaction could lead only to single CD bands and if present would only change the extent of asymmetry of the observed double CD component. We can also infer from the presence of a double CD that the interacting Chl a are not coplanar and the Chl a transition moments are not parallel or exactly perpendicular in the chloroplast fragments. Chl a monolayers have absorption maximum at 680 nm. The red shift that gives rise to the double CD in chloroplast fragments is about the same as the red shift in the Chl a monolayer. The aggregated Chl a could therefore be arranged as a monolayer. The large amplitude of the Chl a CD in chloroplast fragments suggests a more extensive aggregate than a dimer. However, the minimal size that is expected for the aggregate is a dimer where its geometry could lead to larger rotational strength than that of the solution dimer. Even at liquid nitrogen temperature there was no splitting of the absorption band. More information was gained from the CD spectrum of normal spinach and barley at the Chl b region at liquid nitrogen temperature. A degenerate component in the chloroplast fragments CD was seen in the difference between normal and the b-free mutant CD curves at 23°C.

It was suggested (Schultz and Sauer, 1972) that the double CD in the Chl b region indicates the presence of at least a dimer of Chl b. However, at -196°C, a greater resolution was obtained in the CD spectrum
of spinach and normal barley chloroplast suspension. We observed the presence of three separate peaks, e.g., two troughs at 640 and 653 nm and a maximum at about 667 nm. The presence of three peaks indicates that there are at least three Chl b molecules interacting excitonically with each other, and that the geometrical arrangement of Chl b in the chloroplast lamellae is probably different from that of Chl a. However, Chl b aggregation may be more extensive than a trimer. The exact geometrical relationship among these interacting pigment molecules can be better studied on isolated Chl-protein subunits.
CHAPTER III

ABSORPTION AND CD SPECTRA OF PROTOCHLOROPHYLLIDE HOLOCHROME AND ITS SUBUNITS, BEFORE AND AFTER PHOTOCONVERSION

As Introduction

The assembly of the photosynthetically active membrane is usually triggered by Chl synthesis. This process takes place when dark-grown seedlings are subjected to illumination. It involves a photoreduction of PChlide to Chlide at a protein site. One can study the photoconversion using either an etiolated leaf or an extracted pigment-protein complex called protochlorophyllide-holochrome (PCH).

The chain of reactions occurring during photoconversion is monitored by the change in the absorption maximum. Thus, in PCH:

\[
P\text{Chlide } 639 \xrightarrow{hv} \text{Chlide } 678 \xrightarrow{\text{dark } 20 \text{ min}} \text{Chl } 674
\]

Abs. max. 639 nm 678 nm 674 nm

The kinetics of the photoreduction of PCH was investigated by Boardman (1962). His data could not distinguish between the following cases: a) a sum of two first-order photochemical reactions (parallel reactions), and b) two consecutive first-order photochemical reactions.

Case a) can be accounted for by assuming two different types of PCH. Case b) may involve interaction between two PChlide molecules, such that there is a different probability of reaction for the second molecule once the first has been reduced.
Absorption and, especially, CD spectra reflect the interaction between chromophore molecules in close proximity. Exciton interactions result in absorption and CD splittings into N-components, where N is the number of interacting molecules in a repeating unit. Thus one may use this powerful technique as a fingerprint for the organization of pigment molecules and, in principle, for finding their orientations.

The initial experiments of Schultz and Sauer (1972) demonstrated exciton interactions in PCH suggesting the presence of at least a dimer of PChlide on the protein molecule. Further experiments were carried out by Mathis and Sauer (1972) on the kinetics of photoconversion. The change in the amplitude of the CD signal Δθ/θ_max in the Chl absorption region, displayed a non-linear relationship to the change in absorption A/A_max in the same region, and exhibited kinetics indicative of two first-order consecutive reactions. When the CD signal was followed during the progress of the reaction as a function of percent of conversion, a single peak appeared at an intermediate conversion. The single peak in CD implied a monomeric form and could be explained by the absence of exciton interaction between two non-identical molecules.

The following model was presented:

\[ P-P \xrightarrow{\text{hv}} P-C \xrightarrow{\text{hv}} C-C \xrightarrow{\text{dark}} C + C \]

\[(P = \text{PChlide}; \ C = \text{Chlide})\]

Schopfer and Siegelman (1968) used a highly purified PCH. From the profile of PCH elution on an agarose column, they determined that PCH has a MW of 550,000 and is in a dynamic dimer-monomer equilibrium with its subunit of MW ~300,000. The stoichiometry of PChlide to
protein for the 500,000 protein was PChlide:protein = 2. A ratio of PChlide:protein = 4 was obtained by Kahn (1970) based on fluorescence studies at low temperature.

Henningsen and Kahn (1971) reported the isolation of a subunit in the presence of a detergent-saponin. Saponin-PCH appeared to be photoconvertible. The absence of energy transfer at low temperature suggested that the subunit of MW ~150,000 contains just one PChlide per protein. The CD spectra of Saponin-PCH before and after photoconversion were characterized by Henningsen, et al. (1973), for bean and barley homogenates. In this chapter, we have depicted the sedimentation behavior and optical properties of the different pigment-protein complexes of PCH having molecular weights of 600,000, 300,000 and 150,000 daltons isolated from bean leaves.

B. Materials and Methods

Saponin-PCH was isolated according to Henningsen and Kahn (1971).

(1) Sucrose gradient centrifugation. Saponin-PCH containing 0.5% saponin in 0.1 M tricine-Na, pH = 8.5 was centrifuged in gradients of 10% to 30% sucrose including 0.5 ml 70% sucrose as a cushion. Centrifugation lasted for 9 hr at 41,000 RPM in the SW-41 rotor of the Beckman ultracentrifuge (Model L-2), at 4°C. One ml samples were loaded on the gradients. After centrifugation, the gradients were collected in 20 fractions of 0.6 ml each. Every fraction was illuminated and analyzed by absorption at 678 nm for photoconvertibility. Sucrose gradients were calibrated with an internal marker, hemoglobin (64,500 daltons).
(2) Gel chromatography. Agarose 1.5 M, 200-400 mesh was poured into a 4.0- x 70- cm column and equilibrated with 0.01 M tricine buffer pH = 8.0 containing 0.1 M KCl at 4°C. The flow rate was maintained at 1 ml/min. Twelve ml samples were layered on the column. Eluate was collected in 3.6 ml fractions and analyzed for its absorption at the red spectral region (600-700 nm).

The void volume \( (V_0) \) of the column was determined with blue dextran 2000. Protein standards such as bovine serum albumin (68,000 daltons), catalase (native: 240,000; subunit: 60,000), aldolase (150,000), pepsin (35,000) were measured spectrophotometrically at 280 nm, hemoglobin (64,000) at 530 nm and Cyt c (12,400) at 414 nm.

Absorption and CD of PCH and its various subunits were measured in a Cary Model 14 and in Jasco J-20 spectrophotometers, respectively. The samples were placed in 1-, or 5-cm cuvet at room temperature or at 5°C.

(3) Extraction of PCH (600 Kd) and its subunit (300 Kd). Dark grown bean leaves 12 ± 2 days old were homogenized in 0.05 M Tris buffer pH = 8.5, containing 0.002 M MgSO₄, 0.001 M EDTA, 25% (v/v) glycerol and 0.06% Triton X-100. The crude extract was further purified according to the scheme in Table III-1.

C. Results

(1) Optical properties and sedimentation behavior of saponin-PCH. The flow chart for the preparation of saponin-PCH is shown in Table III-1. Typical final yields varied from 20% to 40%. 
Table III-1. Isolation of Saponin-PCH

Flow Chart

- Crude Extract (#1) -
  20% (w/v) $(NH_4)_2SO_4$
  1 hr, 16,000 g, pH 8.5

- Supernatant (#2) -
  38% (w/v) $(NH_4)_2SO_4$
  1 hr, 16,000 g, pH 8.5

- Supernatant Discarded (#3) -
  Pellet Resuspended
  0.5% (w/v) saponin (#4)
  1 hr, 20,000 g

- Supernatant (#5) -
  Discarded

- Supernatant (#7) -
  Pellet
  Discarded (#6)
  1.5 hr, 100,000 g

- Supernatant (#9) -
  Saponin-PCH

- Pellet Discarded (#8) -
  Discarded
Yields Obtained for Saponin-PCH

<table>
<thead>
<tr>
<th>Step</th>
<th>PCH Units (VxA₆₃₉)</th>
<th>% Yield</th>
<th>Convertibility (A₆₇₈/A₆₃₉)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>28.3</td>
<td>100</td>
<td>1.59</td>
</tr>
<tr>
<td>#2</td>
<td>26.6</td>
<td>94</td>
<td>1.87</td>
</tr>
<tr>
<td>#3</td>
<td>0.8</td>
<td>2.8</td>
<td>0.5</td>
</tr>
<tr>
<td>#4</td>
<td>7.35</td>
<td>26</td>
<td>1.86</td>
</tr>
<tr>
<td>#5</td>
<td>6.6</td>
<td>23.3</td>
<td>1.8</td>
</tr>
<tr>
<td>#6</td>
<td>2.0</td>
<td>7.1</td>
<td>1.67</td>
</tr>
<tr>
<td>#7</td>
<td>5.9</td>
<td>20.8</td>
<td>1.8</td>
</tr>
<tr>
<td>#8</td>
<td>0.85</td>
<td>3</td>
<td>1.67</td>
</tr>
<tr>
<td>#9</td>
<td>5.4</td>
<td>19.1</td>
<td>1.8</td>
</tr>
</tbody>
</table>

The sedimentation pattern of saponin-PCH was studied on a 10% to 30% sucrose gradient in a velocity sedimentation experiment (Fig. 6). Calibration of the gradient with hemoglobin gave an estimate of 150,000 to 200,000 daltons, molecular weight, for the major peak of photoconvertible PCH. This major peak corresponds to the subunit obtained by Henningsen and Kahn (L971). Aggregates of higher molecular weight were also observed along the gradient. The absorption spectra of saponin-PCH before and after photoconversion are shown in Figure 7.

Illumination at the intensity of 90 + 10 ft. candles was sufficient to photoconvert the PChlide absorbing at 642 nm to Chlide absorbing at 678 nm. A dark shift in the absorption maximum from 678 nm to 672 nm, followed the photoconversion. The half-life time
Figure 6. Saponin-PCH centrifuged in the dark in 10-30% sucrose gradient. Photoconvertible samples were measured as absorption at 678 nm.
Figure 7. Absorption of saponin-PCH (-----) and saponin-CH after 1 min illumination (----) and saponin-CH after 4 min in the dark (-----) at 23°C. Optical pathlength, 1.00 cm.
for the dark shift was 4 min at 23°C, and the absorption maximum after 4 min was located at 675, as shown in Figure 7. The CD of saponin-PCH was characterized by a broad trough at 600 to 650 nm (Fig. 8). After illumination, the broad trough vanished and a positive peak appeared at 685 nm due to newly formed Chlide. This result is in agreement with that reported by Henningsen et al. (1973).

(2) Comparison between the 600 and the 300 Kdalton pigment-protein complexes. The purification scheme for the isolation of PCH (600 Kd) and its subunit (300 Kd) was based on that reported by Schopfer and Siegelman (1968). A higher yield (25%) was achieved in this work by reducing the pH in the DEAE-cellulose step from 8.0 to 7.3. The yields obtained after each step are summarized in Table III-2. In some experiments, the DEAE-cellulose step was omitted to facilitate the procedure. Figure 9 depicts the elution profile of PChlide-protein complex on agarose 1.5 M column. Two major peaks were observed. These peaks were estimated as 6000,000 ± 100,000 and 300,000 ± 75,000 daltons according to the calibration of the column with protein standards (Fig. 10).

The ratio between the two species varied from one experiment to another. In some experiments, the 300 Kd component appeared only as a shoulder.

In order to check the possibility of an aggregation-disaggregation equilibrium, the peak fractions of 600 Kd and 300 Kd were collected separately, concentrated against powdered sucrose for 24 hr and re-chromatographed separately on agarose 1.5 M. Figures 11 and 12 show the elution patterns of rechromatographed 600 Kd and 300 Kd separately.
Figure 8. CD spectra of saponin-PCH (dark) and saponin-CH after 2 min illumination, measured at 23°C. Optical pathlength, 1.00 cm. Saponin-PCH was resuspended in 0.1 M tricine buffer pH = 9.0, 0.1 M NaCl, 0.001 M EDTA, and 0.6% saponin.
Table III-2. Holochrome Purification Scheme for the Isolation of 600 Kd and 300 Kd Pigment Protein Complexes

<table>
<thead>
<tr>
<th>Step Description</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>100%</td>
</tr>
<tr>
<td>17% polyethylene glycol, 1 hr centrifugation at 48,000 g</td>
<td></td>
</tr>
<tr>
<td>Pellet</td>
<td>70%</td>
</tr>
<tr>
<td>Supernatant discarded</td>
<td></td>
</tr>
<tr>
<td>resuspended in tricine 0.01 M</td>
<td></td>
</tr>
<tr>
<td>KCl 0.2 M, pH 8.0</td>
<td></td>
</tr>
<tr>
<td>centrifuged 1/2 hr at 48,000 g</td>
<td></td>
</tr>
<tr>
<td>Pellet</td>
<td></td>
</tr>
<tr>
<td>Supernatant, made 0.2 M KCl</td>
<td>50%</td>
</tr>
<tr>
<td>Hydroxyl Apatite, washed with 0.2 M KCl, pH 8.0, eluted with 0.10 M K(PO₄)₃, pH 8.0</td>
<td></td>
</tr>
<tr>
<td>concentrated against powdered sucrose, dialyzed against K(PO₄)₃, 0.03 M, pH 7.3</td>
<td></td>
</tr>
<tr>
<td>DEAE Cellulose</td>
<td>30%</td>
</tr>
<tr>
<td>eluted with 0.03 M - 0.35 M K(PO₄)₃, pH 7.3</td>
<td></td>
</tr>
<tr>
<td>linear gradient</td>
<td></td>
</tr>
<tr>
<td>Agarose</td>
<td>25%</td>
</tr>
<tr>
<td>eluted with tricine 0.01 M, KCl 0.1 M, pH 8.0</td>
<td></td>
</tr>
</tbody>
</table>
Figure 9. Chromatography of PCH on agarose 1.5 M (4 x 70 cm). Flow rate = 1 ml/min. $V_0 = 285$ nm. Absorbance at 639 nm (PChlide) is shown.
Figure 10. Calibration curve of agarose 1.5 M (4 x 70 cm). Void volume $V_0 = 285$ ml. Flow rate = 1 ml/min.
Figure 11. Chromatography of 600 Kd PCH on agarose 1.5 M (1.8 x 70 cm).
The absorbances at 280 nm (protein) and 440 nm (PChlide) are shown.
Figure 12. Chromatography of the 300 Kd PCH subunit on agarose 1.5 M (1.8 x 70 cm). Flow rate = 0.2 ml/min. \( V_0 = 60 \) ml. The absorbances at 280 nm (protein) and 440 nm (PChlide) are shown.
Each of them appeared as a single peak with its own original molecular weight. This observation suggests the absence of an aggregation-disaggregation equilibrium of each species by itself.

The 600 Kd component isolated from the agarose column had an absorption maximum at 632 nm. Only 20% of the PChlide was photoconverted to Chlide absorbing at 673 nm. The 600 Kd pigment-protein was very unstable, and lost its photochemical activity in 3 hr at 20°C. The 300 Kd had an absorption maximum at 639 nm. Fifty-five percent of the PChlide was photoconvertible to Chlide absorbing at 678 nm. The 300 Kd PCH complex was stable for at least two days at 20°C. The CD spectra of both the 600 Kd and the 300 Kd species were characterized by pronounced minima at 613 nm and 642 nm, and a relative maximum at 632 nm (Fig. 13). After complete photoconversion, these peaks vanished in both species. No CD signal was apparent for the 600 Kd CH (20% maximal photoconversion); however, a large double CD showed up for the 300 Kd CH (55% maximal photoconversion). The double minima in the CD spectra of the 300 Kd and 600 Kd are a direct good evidence for PChlide-PChlide interaction in a pigment aggregate. It seems that both the 300 Kd PCH and the 600 Kd PCH possess at least two PChlide molecules each in close proximity, leading to exciton splitting. The large double CD signal observed for the 300 Kd after complete photoconversion indicates that the newly formed Chlide is also aggregated and at least two Chlide molecules are excitonically interacting with each other. The CD signals observed by us have the same shape and peak positions as reported for etiolated leaf homogenates (Mathis and Sauer, 1972). The development of the CD signal as a function of the
Figure 13. The CD spectra of 300 Kd and 600 Kd PCH in the dark at 5°C. Optical pathlength 5.00 cm. Absorbance at 639 nm was 0.3.
extent of photoconversion of PCH (300 Kd) is illustrated in Figure 14. At 11% and 25% conversion, the amplitude of the CD signal in the 600-650 nm range was reduced, yet no CD signal was noticed in the 650-700 nm range. At 55% conversion, the double minima at 613 and 642 nm vanished and a double CD with a maximum at 673 nm and a minimum at 686 nm appeared, due to newly formed Chlide. We did not observe a linear change of the CD signal with the extent of photoconversion. The evidence presented here suggests that in the 300 Kd holochrome a dimer of PChlide is photoconverted to a dimer of Chlide molecules. The double CD Chlide signal was vary stable at 5°C, but decreased to about 40% of its original size after 1/2 hr at 38°C (Fig. 14).

D. Discussion

The apparent molecular weight (150,000 - 200,000) for saponin-PCH isolated from etiolated bean leaves and determined by a velocity sedimentation experiment on a sucrose gradient is in good agreement with the value of 170,000 reported by Henningsen et al. (1974) which was determined by gel filtration on Sephadex G-100. Upon photoconversion of saponin-PCH to saponin-CH, the absorption maximum shifted from 642 nm to 678 nm, and the amplitude of the CD trough in the 600-650 nm range vanished. No exciton splitting was detected. A dark spectral shift from 678 nm to 672 nm followed the photoconversion. These findings corroborate the studies reported by Henningsen et al. (1973, 1974), who also found that the photoconversion departs from first order kinetics in bean PCH extracted with 3.6% saponin. The kinetic data
Figure 14. The CD spectra of 300 Kd PCH at different extent of photoconversion. Temperature = 5°C; optical pathlength = 5 cm; absorbance (639 nm) = 0.3.
and the CD evidence obtained so far leave in question the number of PChlide molecules per bean saponin-PCH subunit (170,000). It is not clear that there is only one PChlide molecule per PCH subunit.

The two PCH pigment-protein complexes of 600 Kd and 300 Kd molecular weight were first detected by Schopfer and Siegelman (1968) by gel filtration on agarose column. They interpreted the diffuseness of the PCH band on polyacrylamide gel columns as an indication of an aggregating-disaggregating system. We isolated the 600 Kd and 300 Kd using an agarose column. After incubation of 24 hr, each species was rechromatographed and each appeared as a single peak with its own original molecular weight. These results argue against the occurrence of an aggregation-disaggregation equilibrium. We suggest that the subunit (300 Kd) is produced irreversibly from the 600 Kd protein. Once made, it does not reassociate easily to form the 600 Kd protein.

The two species were compared with respect to their optical properties and their ability to photoconvert PChlide to Chlide. The subunit (300 Kd) was more stable against denaturation and had a higher phototransformation activity (55% maximal conversion) than that of the 600 Kd (20% maximal conversion). The CD spectra of both materials exhibit features characteristic of exciton splitting involving at least two PChlide molecules. They were similar in shape and in the position of the minima for both species. We cannot distinguish between a case of two identical 300,000 subunits with two PChlide molecules on each or one 300,000 subunit with a dimer of PChlide molecules and another subunit with no pigment molecules. The spectrum of the 300 Kd protein after complete phototransformation
showed a double CD with a crossing at 680 nm. It is proposed from the spectra that a dimer of PChlide is photoconverted to a dimer of Chlide molecules.

Additional evidence for the presence of at least two Chlide molecules in close proximity comes from fluorescence studies on the 300 Kd PCH during photoconversion. The time course of Chlide fluorescence excited at 640 nm (absorption maximum of PChlide) did not correspond to the intrinsic Chlide fluorescence excited at 670 nm (Vaughan, 1975). Higher level of Chlide fluorescence was obtained when excited at 640 nm due to the fact that some of the energy absorbed by PChlide was transferred to the newly formed Chlide. In another experiment, Chlide fluorescence polarization excited at 670 nm was measured during photoconversion. The polarization decreased from a value of 0.24 at 0% conversion to 0.05 at 100% conversion. The fluorescence and fluorescence polarization results indicated that PChlide and Chlide pigments were aggregated (probably as dimers) in the 300 Kd holochrome, allowing energy transfer from PChlide to Chlide (Vaughan, 1975).

A non-linear development of the CD signal with the extent of phototransformation was observed for the 300 Kd PCH. This kinetic behavior may indicate the presence of an intermediate state of a "PChlide-Chlide" dimer complex, in agreement with Mathis and Sauer (1972). However, we did not observe the single CD peak at 682 nm that they reported for this mixed dimer.
CHAPTER IV

ABSORPTION AND EPR SPECTRA OF ETIOLATED LEAVES, AND THEIR HOMOGENATES AT LOW TEMPERATURE AND IN DRY STATE

A. Introduction

The temperature dependence of the production of Chlide from PChlide in etiolated barley leaves during illumination was described by Smith and Benitez (1954). The transformation was completely inhibited at -195°C, it occurred slowly at -70°C, but it was not complete at this temperature. Boardman (1962) studied the temperature dependence using preparations of PChlide-protein complex and obtained very similar results.

The existence of two successive light reactions in the photoconversion was postulated by Litvin and Belyaeva (1971). The first light reaction produced the intermediate C684/676 (Chlide with absorption maximum at 676 nm and fluorescence maximum at 684 nm), which acted as a substrate for the second light reaction in the formation of C690/680 (absorption maximum at 680 nm and fluorescence maximum at 690 nm). The first intermediate C684/676, also called P688-676 by Sironval and Kuyper (1972), exhibited energy transfer from PChlide to Chlide, and therefore was described as a unit containing a mixture of PChlide and Chlide molecules in variable proportions. These authors distinguished between two kinds of reductions of PChlide in the leaf. The reductions of the first kind were defined as those occurring below
-100°C and even at liquid N₂ temperature. They were regarded as involving only one Chlide molecule in an aggregate of n PChlide molecules. The reduction of only one molecule gave rise to the intermediate P688/676, containing one Chlide molecule and (n-1) PChlide molecules. The reductions of the second kind were defined as those taking place only above -80°C. They were considered to involve the remaining (n-1) PChlide molecules which were reduced as illumination proceeded. It was suggested that the reductions of the second kind required a light triggered conformational change in the protein matrix which could take place only above -80°C.

In other experiments, Sironval and Kuyper (1972) found that after initial photoconversion at -125°C, and some period (20 min) in the dark at this temperature, additional phototransformation and reduction have taken place in the dark, as evidenced by increased Chlide fluorescence at -196°C and also by direct extraction of the pigments. These results led Sironval and Kuyper to believe that some unknown step (x) existed between the absorption of radiation and the reductions since the reductions appeared to be separable from the absorption of radiation. Litvin and Belyaeva (1972) also suggested one of the possibilities for C684/676 could be that it is a half-reduced form or a stabilized radical which transformed in darkness in the leaf or during extraction of the pigment to C675/670. Rubin et al. (1962) showed that leaves in which reduction proceeded at a temperature below -100°C underwent a marked increase of low temperature emission when warmed in darkness to -80°C. On the basis of this evidence it was proposed by Rubin (1962) that a stabilized radical was created during illumination at low
temperature and was further reduced to Chlide above -80°C in darkness. On the other hand, Sironval et al. (1972) regarded this increased emission in the dark previously observed by Rubin (1962) as due to the presence of energy transfer units.

We investigated the possibility of formation of a stabilized radical as an intermediate in the photoconversion reaction in etiolated leaves using EPR spectroscopy at low temperature. A characteristic signal from divalent, unbound Mn, appeared in the EPR spectra. In addition, a free radical signal was observed at g = 2.0. However, we did not see a large consistent difference in the intensity of the signal in dark etiolated leaves as compared to illuminated etiolated leaves.

The lowest temperature at which photoconversion in homogenates prepared from etiolated bean leaves was carried out was at -125°C. Phototransformation occurred at this temperature, but was not completed.

B. Materials and Methods

(1) EPR. EPR measurements were conducted on 12 ± 2 day old etiolated bean leaves. A Varian E-3 (X band, 9.5 GHz) EPR spectrometer was used in recording the spectra of etiolated leaves.

Low temperature experiments were performed using 3 mm cylindrical (I.D.) quartz sample tubes packed with etiolated leaves in the dark. A Varian low temperature accessory (Model #E-4557-9) was used to maintain the temperature within 5°C of the nominal value. Spectra were recorded by sweeping from low field to high field with the spectrometer time constant, microwave power and scan rate as noted
in the figure legends. The irradiation of the thermally equilibrated sample took place inside the Dewar, through an unsilvered area, using polychromatic light from a Xenon lamp with a U.V. filter attached to it.

(2) Absorption. Absorption was measured in a Cary 14 spectrophotometer. The sample was placed in a lucite holder inside a Dewar. The irradiation took place inside the Dewar, after temperature equilibration, using polychromatic light from a 1000-watt projector with the appropriate Corning Glass color filters and focussed on the sample by a pair of planoconvex lenses. In some experiments, only a 125-watt projector was used. The sample consisted of a crude extract of 12 ± 2 day old etiolated bean leaves that had been dialized overnight against 0.05 M Tris buffer pH = 8.5 and concentrated against powdered polyethyleneglycol. Glycerol and 1 M KCl solution were added to the sample to a final concentration of 60% glycerol and 0.4 M KCl. This mixture yielded a clear, uncracked glass upon rapid freezing in liquid N₂. The sample compartment and the PM tube were flushed constantly with dry N₂. The reference beam was masked with neutral density filters to balance the scattering originating from a control consisting of 60% glycerol, 0.4 M KCl solution frozen to a glass at liquid N₂.

(3) PCH in a dry state. A crude homogenate of etiolated leaves was dialyzed overnight against 0.01 M Tris buffer pH = 8.0, containing 10% glycerol and then freeze-dried in a lyophilizer for 18 hr. The dry powder was pressed into a transparent pellet in a 25 ton ring press by a force of 10 tons, in the dark. The absorption difference
spectrum of an illuminated pellet and a dark kept pellet was measured in the Cary 14 spectrophotometer with a special scattering accessory. All illuminations were performed at 23°C at the intensity of 90 ± 10 ft. candles.

C. Results

(1) EPR. Figure 15 shows the EPR spectrum from etiolated leaves in the dark at -160°C. The 6-line pattern observed is a typical signal from divalent, unbound Mn, resulting from the hyperfine interaction of the unpaired electrons with the 5/2 spin of the Mn\(^{55}\) nucleus (Garrett and Morgan, 1966).

The low temperature spectrum is very similar to the spectrum of 3.6 \times 10^{-5} M MnSO\(_4\) at -196°C (Blankenship, 1975). The fourth line from the left in the Mn spectrum shown has a g value of 1.986, which is in good agreement with the g = 1.98 obtained in a calibrated Mn\(^{+2}\) EPR spectrum (Blankenship and Sauer, 1974). When the sample was illuminated at -120°C, a free radical signal appeared at g = 2.0 with a line width of 11 gauss (Fig. 16). In the same figure, the Mn\(^{+2}\) 6-line signal was also observed. Raising the temperature to 25°C followed by freezing back to -120°C caused a reduction in the size of the signal (Fig. 17). The signal had an appearance of an emission, probably because of uncritical tuning of the instrument, which might artificially produce such polarization. However, it does not affect the basic observation of a radical signal. The signal was easily saturated at a low microwave power of about 2 mW. Warming the sample from -120°C to -50°C caused an increase in the intensity of the line, while boiling the leaves or freezing and thawing for 2 to 3 times
Figure 15. EPR spectrum of intact etiolated leaves at -160°C in the dark. Instrumental conditions:
microwave power, 25 mW; modulation amplitude, 1.0 G; time constant 0.3 sec; scan rate, 125 G/min.
Receiver gain, $2 \times 10^5$. 
Figure 16. EPR spectrum of intact etiolated leaves at -120°C in the light. Instrumental conditions: microwave power, 0.63 mW; modulation amplitude, 4.0 G; time constant, 1.5 sec; scan rate, 16.7 G/min. Receiver gain, $1.25 \times 10^5$. 

$g = 2.140$

$g = 1.998$
Figure 17. EPR spectrum of intact etiolated leaves previously illuminated at -120°C. Temperature was raised to 25°C (sample melted) and recooled to -120°C in the dark. Instrumental conditions: microwave power, 0.63 mW; modulation amplitude, 4.0 G; time constant, 1.0 sec; scan rate, 16.7 G/min. Receiver gain, $1.25 \times 10^6$. 
reduced it to 1/3 of its original size. However, the significance of this free radical signal is still undetermined because it appeared both in the dark and under illumination at -196°C.

(2) Absorption. The cooling system for measuring the absorption spectrum at low temperature consisted of a Dewar reservoir filled with liquid N₂. Insulated copper tubing conducted cold N₂ stream from the reservoir to a second optical Dewar which was inserted in the Cary 14 spectrophotometer. A quartz cell (5 mm diameter) was held in place in the optical Dewar supported by a lucite rod. The temperature of the Dewar was varied by changing the level of liquid N₂ in the reservoir. An iron-constantan thermocouple attached directly to the quartz cell was employed for measuring the temperature of the sample. The thermocouple was calibrated to the accuracy of ±1°C. The absorption spectrum of a homogenate of etiolated leaves at -125°C is shown in Figure 18. A clear, uncracked glass was obtained with PCH suspension in 60% glycerol and 0.4 M KCl. The absorption maximum was located at 633 nm. Following 15 min illumination obtained from a 1000-watt projector at -125°C, about 10% of PChlide was converted to Chlide at this temperature (Fig. 19). The sample was warmed to 2°C in the dark, and about the same extent of conversion still remained. The spectrum at 2°C was nearly identical to that at -125°C. Further illumination at room temperature at the intensity of 90 ± 10 ft. candles for 1 min increased the extent of photoconversion to 30% (Fig. 20). This set of experiments was repeated at different temperatures. Photoconversion did not take place below -125°C. The results for several different low temperatures are summarized in Table IV-1.
Figure 18. The absorption spectrum of a homogenate of etiolated leaves in 60% glycerol in the dark, at -125°C.
Figure 19. Absorption of a homogenate of etiolated leaves in 60% glycerol at -125°C after 15 min illumination at -125°C.
Figure 20. Absorption of a homogenate of etiolated leaves in 60% glycerol at 23°C after illumination at 90 ft. candles for 1 min at 23°C following a previous illumination at -125°C.
Table IV-1. Low Temperature Photoconversion of PCH

<table>
<thead>
<tr>
<th>Low Temperature Illumination</th>
<th>% Conversion at Low Temperature*</th>
<th>% Conversion at Room Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>-102°C</td>
<td>15%</td>
<td>26%</td>
</tr>
<tr>
<td>-108°C</td>
<td>12.6%</td>
<td>24%</td>
</tr>
<tr>
<td>-113°C</td>
<td>8.7%</td>
<td>22.3%</td>
</tr>
<tr>
<td>-116°C</td>
<td>10%</td>
<td>30%</td>
</tr>
<tr>
<td>-124°C</td>
<td>10%</td>
<td>23.6%</td>
</tr>
<tr>
<td>-130°C</td>
<td>0%</td>
<td>30%</td>
</tr>
</tbody>
</table>

*Percent conversion is defined as the number of photoconverting PChlide molecules out of the total pool of PChlide.

These experiments suggest that photoconversion still occurs at -125°C, but it is not complete, because additional transformation took place after further illumination at 23°C. No significant increase in photoconversion was noticed when the homogenate illuminated previously at low temperature was warmed up in the dark to room temperature.

(3) Photoconversion of a dry etiolated leaf homogenate. The absorption spectrum of a dry pellet dissolved in the dark, and of the illuminated solution at room temperature are shown in Figure 21. The solution of the dry pellet exhibited 40% photoconversion of PChlide to Chlide. In another experiment, one pellet was illuminated in a dry state and then dissolved in the dark, while another one was dissolved in the light after receiving illumination in dry state. No significant difference was observed between the two spectra (Fig. 22).
Figure 21. Etiolated leaf homogenate was lyophilized. 30 mg were pressed into a pellet by a force of 10 tons. Absorption spectrum of a suspension from the dark pellet and after illumination of the suspension (absorption maximum at 673 nm).
Figure 22. The absorption spectra of 1) illuminated suspension of a dry pellet, and 2) illuminated pellet resuspended in Tris buffer. Intensity of illumination was 90 ft. candles for 1 min.
These results show that at least some step in the photoconversion reaction can take place in a pellet, namely in a solid matrix. The extent of the photoconversion in the dry pellet and in the resuspended pellet were nearly identical. However, it is still possible that the illuminated dry pellet is carrying out only a partial photoconversion leading to the formation of an intermediate species, while the whole process is completed only later after resuspension of the pellet in the buffer in the dark. To test this possibility, we looked at the difference spectrum between an illuminated pellet and a dark pellet, and measured it directly, taking advantage of the fact that the pellets were transparent, using the scattering accessory of the Cary 14 instrument. Photoconversion of PChlide to Chlide occurred in a dry pellet (Fig. 23). From these results we conclude that the whole photoconversion process can take place in a dry solid matrix.

D. Discussion

Oku and Tomita (1970) found that plastoquinone in etiolated leaves of dark grown bean plants was localized in the proplastids. They reported that plastoquinone was associated with soluble PCH and the plastoquinone to PChlide ratio was about 1.2 on a molar basis in the pigment protein complex. They suggested the possible participation of plastoquinone in the phototransformation of PChlide to Chlide. We observed the appearance of a free radical signal at \( g = 2.00 \) in the EPR spectrum of etiolated bean leaves. The signal intensity decreased appreciably upon denaturing treatments like boiling, or freezing and thawing of the etiolated bean leaves. It may be possible that this
Figure 23. Difference spectrum of an illuminated dry pellet and a dark pellet (light-dark). Illumination at 90 ft. candles, for 1 min.
signal is due to a plastoquinone radical that is formed in the leaves during illumination, and may participate as an electron donor in the photoreduction. However, the nature of this free radical and its possible role is still unknown. Another potential candidate for an electron donor is a transition metal which changes its oxidation state upon photoreduction of PChlide.

The study of photoconversion at low temperature suggests that the transformation takes place at temperatures from -70°C down to -125°C, but did not occur below -130°C in our experiments. However, it is possible that with sufficient light intensity the process would occur also at liquid N₂ temperature, as reported by Kuyper and Sironval (1972), who used a xenon lamp for sample irradiation. The phototransformation was not complete at low temperatures, which is in agreement with previous reports (Smith and Benitez, 1954; Boardman, 1962; Kuyper and Sironval, 1972). A reasonable hypothesis is that only the first photochemical step is allowed at low temperature, converting a single PChlide molecule in each pigment-protein complex, and a conformational change in the protein moiety is required for further photoconversion, as suggested by Sironval et al. (1972). We have not noticed a significant additional increase in photoreduction after initial illumination at low temperature followed by elevation of temperature in the dark, which is in contrast with the findings reported by Kuyper and Sironval (1972). Therefore, it is not likely that an intermediate is formed during low temperature irradiation and becomes converted to Chlide in the dark at an elevated temperature. We have shown that the phototransformation can be completed
in a solid dry phase to the same extent as in a buffer suspension of the pigment-protein complex.

To summarize, we found that the photoconversion reaction can take place at temperatures as low as \(-125^\circ C\), but it is not completed at this temperature. No additional photoconversion occurred in the dark after the initial photoconversion at \(-125^\circ C\). A free radical appeared in etiolated leaves upon illumination, but neither its chemical nature nor its role are known. It was shown that the photoenzyme, PChlide holochrome, can carry out the complete photoconversion reaction in a solid matrix.
CHAPTER V

THE ISOLATION AND CHARACTERIZATION OF THE MAJOR 45,000 DALTONS PCH POLYPEPTIDE BY SDS POLYACRYLAMIDE ELECTROPHORESIS AND COLUMN CHROMATOGRAPHY

A. Introduction

In vivo, three types of PChlide are distinguishable by their absorption maxima and photoconversion: a PChlide-650 and a PChlide-636, which are phototransformable, and PChlide-628, which is not (Kahn et al., 1970). Upon aqueous extraction of the pigment complexes, there is a shift in the absorption maximum from 650 nm in vivo to a 638-640 nm form which is phototransformable; and there is also a non-phototransformable pigment complex absorbing maximally at 627-628 nm in the extract. These shifts to shorter wavelengths were reported to occur also in the etiolated leaves upon freezing and thawing cycles, infiltration with HCl, and thermal denaturation. Dujardin and Sironval (1970) suggested that the PChlide-628 represents pigment molecules loosely linked to proteins, since the absorption was very similar to the absorption of PChlide monomers in organic solvents.

Chlorophyll synthesis upon illumination triggers the assembly of the photosynthetically active membrane. PCH is believed to be the site of Chl synthesis. The holochrome or one of its constituents has been visualized as a shuttling photoenzyme. According to the
model, suggested by Bogorad, Laber and Gassham (1967), the photo-
enzyme attaches to a photoinactive PChlide-630 molecule to form
PChlide-650, and catalyzes the photoreduction of the pigment. The
Chlide thus formed leaves the photoenzyme and the latter attaches
another PChlide-630 molecule, and so forth. Granick and Gassman
(1970) calculated that the loading of the photoenzyme after a flash
of light was accomplished with a half-time of about 20-50 seconds,
in etiolated bean leaves. Süzer and Sauer (1971) estimated a half-
tIME of about 2.5 min in etiolated barley. It is assumed that the
holochrome has to recycle in the photoreduction. Henningsen et al.
(1974) have isolated a photoactive subunit of PCH with the aid of a
detergent, saponin, from barley leaves of MW 63,000. Photoconversion
of the PCH subunit yields Chl-H with an absorption maximum at 678 nm.
A spectral shift from 678 nm to 672 nm proceeds after photoconversion,
and at the same time the MW of the subunit changes from 63,000 to
29,000. Henningsen et al. (1974) proposed that the Chl-H subunit
dissociates into a Chl-carrier protein complex and a photoenzyme.

Duranton et al. (1974) have demonstrated in Zea mays L. leaves
that PChlide is associated with two polypeptide chains of MW 21,000
and 29,000. Chl is associated with four polypeptide chains of MW
21,000, 25,000, 29,000, and 70,000 daltons. It was proposed that
the Chl polypeptides of 25,000 and 70,000 daltons were made in a
separate pathway.

We investigated the behavior of two different preparations of
PCH from etiolated bean leaves. The isolation of the first was based
on ion exchange chromatography and the second one was based on
differential solubility. PCH did not appear as a homogenous peak but was present as two separate species. This may reflect an equilibrium between the intact protein and its constituent subunits or the presence of two kinds of pigment-protein complexes in the leaf. We found that the major polypeptide of PCH has a molecular weight of 45,000 daltons by SDS polyacrylamide gel electrophoresis. Chlide was attached to this polypeptide.

B. Materials and Methods

Bean seedlings (Phaseolus vulgaris, variety red kidney) were grown on vermiculite 12 ± 2 days in the dark at 22°C. The leaves (50 to 100 g) were harvested and ground in a buffer containing 10 mM Tris-HCl, pH = 8.5; 2 mM MgSO₄; 1 mM EDTA and 25% (v/v) glycerol. In some cases, Triton X-100 (in final concentration of 0.06%, v/v) was added to the extracting solution, and is referred to as buffer + Triton. All manipulations were done in a cold room (4°C) under a green safe light. Two kinds of preparations were used. In the first procedure, the leaves were ground in buffer + Triton in a Waring blender, during four 60-sec intervals, separated by 5 min, in a cold room. The homogenate was filtered through 6 layers of cheese-cloth, and centrifuged at 78,000 x g in a Beckman (Model L-2) ultracentrifuge (No. 30 rotor) at 0°C for 1 hr. The supernatant was treated with a 50% polyethylene glycol-6000 solution to a final concentration of 17%, and centrifuged at 48,000 g (-2°C) in a Sorvall RC-2B centrifuge for 1 hr. The precipitate was redissolved in buffer + Triton and clarified by centrifugation at 48,000 g for 30 min.
Before redissolving, the precipitate was stored with a layer of fresh buffer + Triton for about 1 hr, to facilitate the resuspension. The clarified supernatant was adjusted to 0.2 M KCl and applied to an equilibrated hydroxylapatite column (7.5 x 20 cm). The column was washed with 2 to 3 liters of Tris buffer containing 0.2 M KCl. We found that Tris buffer containing 0.1 M potassium phosphate (pH = 8.0) was sufficient to elute the PChlide-holochrome. The flow rate was maintained at 10 ml/min. All fractions with an absorption maximum at 639 nm containing PCH were combined. This partially purified pigment-protein complex was used immediately or stored at -20°C. In some experiments, DEAE ion exchange chromatography and agarose gel filtration completed the purification. This procedure was similar to the one reported by Schopfer and Siegelman (1968). The partially purified holochrome was very stable, and maintained its photoconversion activity for at least a week at 4°C.

The second PCH preparation procedure was adapted from Akoyunoglou, et al. (1970). Etiolated leaves were homogenized in Tris buffer (same as above) and filtered through 6 layers of cheesecloth, followed by centrifugation at 48,000 g for 30 min in a Sorvall RC-2B centrifuge (SS-34 rotor). The pellet was extracted three times with Tris buffer, collecting supernatants S₁ through S₃ and a fourth time with Tris buffer + Triton. The supernatant from the fourth extraction, S₄, was used directly for polyacrylamide gel electrophoresis or was centrifuged on a sucrose gradient.

Gel electrophoresis was carried out following the Weber-Osborn procedure as modified by Neville (1971). It is also described in detail in Chapter VI (Materials and Methods).
C. Results

(1) Electrophoretic analysis of PCH fractions from a sucrose gradient. The first procedure for the purification of PCH:

```
Crude Extract
  ↓
Polyethylene Glycol Precipitation
  ↓
Hydroxyl Apatite
  ↓
DEAE Cellulose (batch)
  ↓
eluted by 0.20 M K(PO₄), pH 7.3
  ↓
DEAE Cellulose (column)
  ↓
eluted by linear gradient of 0.05 M to 0.25 M K(PO₄), pH 7.3
  ↓
Agarose Column
  ↓
eluted by 0.1 M KCl, 0.01 M tricine, pH 8.0
  ↓
Sucrose Gradient Centrifugation
  ↓
5% to 25% sucrose, 16.8 ml;
  0.7 ml of 70% cushion
```

The increase in specific activity was measured by the ratio of pigment to protein absorbance, as shown in Table V-1.
Table V-1. Increase in Specific Activity

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>$\frac{A_{639}}{A_{280}} \times 100$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyl apatite</td>
<td>0.5</td>
</tr>
<tr>
<td>DEAE-batch</td>
<td>1.1</td>
</tr>
<tr>
<td>DEAE-gradient</td>
<td>1.43 to 1.1</td>
</tr>
<tr>
<td>Agarose</td>
<td>1.4</td>
</tr>
<tr>
<td>Sucrose gradient</td>
<td>3.0</td>
</tr>
</tbody>
</table>

The distribution of PCH in the sucrose gradient was measured by absorption at 440 nm (Fig. 24). The fractions were all phototransformable. Fractions number 11 and number 12 from the sucrose gradient were combined and analyzed electrophoretically on a 7.5% polyacrylamide gel (0.1% SDS). Five major bands are observed along the gel (Fig. 25). The gels were calibrated with the following molecular weight markers: BSA (68,000), pepsin (35,000), trypsin (23,300), and Cyt c (12,400). From the calibration curve, we estimated that peak A1 is a polypeptide of MW 60 Kd; peak A2 is MW 50 Kd; peak A3 is MW 38 Kd; peak A4 is 30 Kd and peak A5 is 13 Kd. In addition to these major bands, we observed three or four bands near the origin that appear to have MW larger than 100 Kd, presumably aggregates that have not been dissociated in SDS and β-mercaptoethanol. The intensity of the Commassie blue absorbance of a band on the polyacrylamide gel is for most proteins directly proportional to the amount of protein associated with that band. The ratio of intensity of the Commassie blue absorbance between any
Figure 24. A profile of PCH absorbance at 280 nm and 400 nm along a sucrose density gradient of 5% to 25% sucrose, with a 70% sucrose cushion. The samples were centrifuged at 27,000 g for 28 hr.
Figure 25. An absorption scan of Comassie-blue at 600 nm along a 7.5% polyacrylamide gel (SDS 0.1%). Each absorption band represents a polypeptide. Sample A: a peak from the elution profile of PCH from the sucrose gradient, dissociated with 1.5% SDS, 0.04 M DTT. The sample contained 40 μg protein.
two bands (shown in Fig. 25) was not constant among the different fractions of the PCH peak on the sucrose gradient. From this result we conclude that not all the bands belong to one unique protein, and some of them were polypeptides of contaminating proteins.

(2) Separation based on differential solubility of membrane bound and non-bound proteins. Since RuDP-Case is known to be a soluble protein in mature chloroplasts, Akoyunoglu et al. (1970) assumed that it is also a soluble protein in etioplasts and, therefore, can be separated from the membrane-bound PCH by differential solubility. Following the procedure described by Akoyunoglu et al. (1970), we have extracted 12 day old etiolated bean leaves three times with Tris buffer, collecting supernatants S_1 to S_3, and the fourth time with Tris buffer containing 0.06% Triton X-100, collecting supernatant S_4. The yields of PCH and the corresponding photoactivity are summarized in Table V-2.

### Table V-2. Extraction of PCH from Etiolated Bean Leaves by Differential Solubility

<table>
<thead>
<tr>
<th>Supernatant</th>
<th>(A(639) cm x V)</th>
<th>Yield</th>
<th>Absorption ( \lambda_{\text{max}} )</th>
<th>( \frac{A_{678}}{A_{639}} )</th>
<th>Protein conc. (mg/ml)</th>
<th>Specific activity units</th>
<th>A(639) ml cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>S_1</td>
<td>13.7</td>
<td>61%</td>
<td>639 nm</td>
<td>63%</td>
<td>17.2</td>
<td>8.14</td>
<td>0.14</td>
</tr>
<tr>
<td>S_2</td>
<td>3.5</td>
<td>16%</td>
<td>637 nm</td>
<td>58%</td>
<td>3.6</td>
<td>16.7</td>
<td>0.06</td>
</tr>
<tr>
<td>S_3</td>
<td>2.3</td>
<td>10%</td>
<td>637 nm</td>
<td>53%</td>
<td>1.7</td>
<td>23.0</td>
<td>0.039</td>
</tr>
<tr>
<td>S_4</td>
<td>2.8</td>
<td>13%</td>
<td>634 nm</td>
<td>45%</td>
<td>1.0</td>
<td>48.0</td>
<td>0.048</td>
</tr>
</tbody>
</table>
Phototransformability was calculated as % photoactive PChlide from the total PChlide initially present. The protein composition of $S_1$ through $S_4$ were compared after dissociation in 0.04 M DTT and 1.5% SDS by polyacrylamide electrophoresis. The band profiles of samples $S_1$ through $S_4$ are shown in Figures 26-29. The number of bands in $S_1$ was greater than twelve and reduced gradually to six in $S_4$. The approximate mobilities for these polypeptides are depicted in Table V-3.

Table V-3. Mobility on 7.5% Polyacrylamide SDS Gels

<table>
<thead>
<tr>
<th>Peak</th>
<th>#1</th>
<th>Peak</th>
<th>#2</th>
<th>Peak</th>
<th>#3</th>
<th>Peak</th>
<th>#4</th>
<th>Peak</th>
<th>#5</th>
<th>Peak</th>
<th>#6</th>
<th>Peak</th>
<th>#7</th>
<th>Peak</th>
<th>#8</th>
<th>Peak</th>
<th>#9</th>
<th>Peak</th>
<th>#10</th>
<th>Peak</th>
<th>#11</th>
<th>Peak</th>
<th>#12</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_1$</td>
<td>0.09</td>
<td>0.22</td>
<td>0.27</td>
<td>0.31</td>
<td>0.37</td>
<td>0.42</td>
<td>0.52</td>
<td>0.54</td>
<td>0.67</td>
<td>0.73</td>
<td>0.77</td>
<td>0.86</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$S_2$</td>
<td>0.06</td>
<td>0.2</td>
<td>0.24</td>
<td>0.31</td>
<td>0.35</td>
<td>0.43</td>
<td>0.52</td>
<td>0.57</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.8</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>$S_3$</td>
<td>0.05</td>
<td>0.2</td>
<td>0.25</td>
<td>0.31</td>
<td>0.35</td>
<td>0.43</td>
<td>--</td>
<td>0.54</td>
<td>--</td>
<td>--</td>
<td>0.76</td>
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<tr>
<td>$S_4$</td>
<td>0.135</td>
<td>0.225</td>
<td>--</td>
<td>0.30</td>
<td>--</td>
<td>0.393</td>
<td>0.5</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.76</td>
<td>--</td>
<td></td>
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</table>

(3) Identification of the major holochrome polypeptide. $S_4$ was centrifuged in a sucrose density gradient. PCH was distributed along the gradient in a peak with a sedimentation coefficient of 18S and also in peaks corresponding to smaller molecular weight. PChlide was phototransformable to Chlide. Fractions E and F from the 18S peak were analyzed by SDS polyacrylamide gel electrophoresis (Figs. 30 and 31). Aldolase and spinach RuDP-Case were dissociated in 1% $\beta$-mercaptoethanol, 2% SDS and 8 M urea and run on parallel gels with the dissociated PCH. Aldolase was used as a molecular weight marker, and RuDP-Case from spinach was used to identify the large and small subunits of this
Figure 26. An absorption scan of Commassie-blue along a 7.5% polyacrylamide gel (SDS 0.1%). Sample B: S1 supernatant dissociated with 1.5% SDS, 0.4 M DTT, containing 25 μg protein.
Figure 27. Conditions as described in Figure 26, except that the sample is $S_2$. 
Figure 28. Conditions as described in Figure 26, except that the sample is $S_3$. 
Figure 29. Conditions as described in Figure 26, except that the sample is $S_4$. 
Figure 30. An absorption scan of Commassie-blue along a 5-10% polyacrylamide stacked gel (SDS, urea). Sample E: Fraction from S4 on a sucrose density gradient, containing 20 µg protein.
Figure 31. Conditions as described in Figure 30, except that it is two combined fractions from S4 on a sucrose density gradient, containing 40 µg protein.

GEL ELECTROPHORESIS OF PURIFIED PCH

% ABSORPTION AT 600 nm

GEL LENGTH (cm)
contaminating protein. The mobilities of fractions of S₄ from the sucrose gradient after SDS polyacrylamide gel electrophoresis are shown in Table V-4.

### Table V-4. Mobility of Fractions of S₄ from the Sucrose Gradient Run on Polyacrylamide Gels (SDS, Urea)

<table>
<thead>
<tr>
<th>Gel</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction E</td>
<td>0.228</td>
<td>0.385</td>
<td>0.785</td>
</tr>
<tr>
<td>PCH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction F</td>
<td>0.226</td>
<td>0.397</td>
<td>0.731</td>
</tr>
<tr>
<td>MW markers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aldolase</td>
<td>---</td>
<td>0.388</td>
<td>---</td>
</tr>
<tr>
<td>RuDP-Case</td>
<td>0.225</td>
<td>---</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Peaks F1, E1 and peaks F3, E3 had very similar mobilities to those of the large and small subunits of RuDP-Case, respectively. Peak E2, F2 appeared to possess the same mobility as the MW marker aldolase (40 Kd) and was later identified as the major constituent of PCH. The ratio of intensity between peaks F1 and F3 to F2 was larger than the ratio of E1 and E3 to E2. From this result we conclude that the three bands did not belong to one unique protein. Fraction E appeared to be enriched with E2 polypeptide compared to fraction F from the sucrose gradient.

In order to find the ratio between the amount of protein E2 identified as the polypeptide of PCH and the amount of protein E1 + E3 identified as RuDP-Case large and small subunits respectively, the following assumptions were made: 1) The area under the peak is directly proportional to the amount of protein in this band, which is
justified for most proteins. 2) The ratio between the large (A) and small (B) subunits in RuDP-Case is one-to-one in the structure of $A_8B_8$. The ratio in molecular weights is about five-to-one (Akazawa, 1974). Therefore, the ratio of the areas of E1 to E3 is taken as five-to-one. Since the area under E1 can be measured very accurately, the area E3 is estimated from the value obtained for E1. Using these two assumptions, we found that the PCH is about 50% pure in fractions E and F.

In another experiment, purified spinach RuDP-Case was mixed with PCH fraction from the sucrose gradient and run in parallel to PCH and RuDP-Case and PCH each by itself on 50S-polyacrylamide gels. The following mobilities were observed and are depicted in Table V-5.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
<th>Peak 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCH</td>
<td>0.13</td>
<td>0.34</td>
<td>0.44</td>
<td>0.74</td>
</tr>
<tr>
<td>PCH + RuDP-Case</td>
<td>0.10</td>
<td>0.31</td>
<td>0.46</td>
<td>0.74</td>
</tr>
<tr>
<td>RuDP-Case</td>
<td>0.13</td>
<td>0.34</td>
<td>--</td>
<td>0.79</td>
</tr>
</tbody>
</table>

Peak 1 was probably due to a non-specific aggregate of the large subunit of RuDP-Case and was also reported by Nishimura and Akazawa (1974). Peak 2 and Peak 4 were the large and small subunits of RuDP-Case, respectively. The only peak that was missing in RuDP-Case sample compared to PCH sample, Peak 3, had an average mobility of 0.45.

In order to identify Peak 3 as the major polypeptide of PCH, a sample of irradiated $S_4$ containing 200 µg of protein (five times more than the usual amount) was analyzed on polyacrylamide gel. After the
electrophoresis run, the gel was scanned at 675 nm before fixation. Chlide absorption was associated with two peaks (Fig. 32). The major peak H1 had a mobility of 0.4. The minor peak H2 had a mobility of 0.8. The mobility of the major peak Gl was in excellent agreement with the mobility of peak E2, F2. These results suggest that peak E2 is a polypeptide attached to Chlide, possessing a MW ~40 Kd. The minor peak appeared to be a proteolytic product of PCH (see this chapter, Section (4)). However, in many experiments it was difficult to measure the absorption of the gel directly because of rapid diffusion from the gel and scattering of the gel itself. This problem was overcome by extracting the pigment from the gel slices, and recording the fluorescence spectrum from each extracted fraction. The method is described in detail in Chapter VI, Section (1). Immediately after photoconversion, Chlide was attached to a polypeptide of 45,000 ± 10,000 daltons. Hence, we conclude that the polypeptide of 45,000 daltons which is attached to a Chlide molecule is the major and only subunit of PCH before and immediately after photoconversion.

The number of PChlide per protein was estimated on the basis of the ratio of absorbances at 440 nm (PChlide) and 280 nm (protein) for fractions E and F. The ratios A280/A440 obtained for fractions E and F were 2.0 and 2.1 respectively. These ratios are in excellent agreement with the ratio of A280/A440 = 2.0 reported by Schopfer and Siegelman (1968) for their most purified PCH. However, we found that fractions E and F are composed from equal amounts of PCH and RuDP-Case, leading us to conclude that the most purified holochrome reported by Schopfer and Siegelman (1968) was only 50% pure, and therefore an aggregate of MW ~550,000 contains 4 PChlide chromophores.
Figure 32. A scan of absorption at 675 nm along a 5-10% polyacrylamide stacked gel (SDS, urea). Sample G: an irradiated S₄ containing 200 µg protein.
(4) Proteolytic digestion of PCH. A crude extract of dark grown bean leaves containing 1% SDS was chromatographed on Sephadex G-100 in the presence of 0.1% SDS at 27°C, for 18 hr. According to the calibration curve, the SDS-treated crude homogenate contained a PChlide associated with a polypeptide of MW about 6500. However, when a more purified preparation such as S₄, containing 1% SDS, was chromatographed under the same conditions, a major fraction of PChlide (85%) was associated with a polypeptide of MW of 50,000 daltons and a minor fraction (15%) of PChlide appeared to have a MW of 6500. A crude extract of etiolated bean leaves contains many proteolytic enzymes. It is suggested that the 6500 daltons polypeptide is a proteolytic product of PCH found exclusively in the dissociated crude extract after 18 hr at 27°C, and to a much smaller degree (~15%) in a more purified preparation, S₄. The holochrome major polypeptide, however, is of ~50,000 MW, according to Sephadex chromatography.

(5) The action of detergents on the photoactivity of PCH.

(a) SDS. Upon addition of SDS to a final concentration of 1%, the absorption maximum of PChlide-holochrome shifted from 639 nm to 632 nm. PChlide was not photoconvertible to Chlide. The CD spectrum (Fig. 33) resembles the CD of PChlide monomers in ether. The CD of PChlide monomers in ether had a trough at 698 nm and a positive peak at 620 nm (Houssier and Sauer, 1970). The SDS-treated PCH showed a trough at 610 nm and a positive peak at 635 nm. The two CD spectra appeared to have a similar shape. Yet, the peaks in SDS-treated PCH were red shifted, probably due to interactions with the protein matrix. PCH was photoconverted to CH and then treated with SDS. The absorption maximum
Figure 33. CD spectra of PCH and CH after dissociation in 1% SDS. Optical pathlength 1.00 cm; absorbance 0.20.
shifted from 678 nm to 672 nm and a single positive peak at 675 nm was present in the CD spectrum (Fig. 33). Chl monomers in ether give rise to a single negative peak at 657 nm (Houssier and Sauer, 1970). The red shift and reversal of sign can be explained as due to the interaction of Chlide with the protein environment.

(b) LDAO. LDAO was added to PCH at a concentration of 0.1%. It had no effect on the absorption maximum (639 nm), the extent of photoconversion (maximal - 75%), or the characteristic double minima in the CD spectrum of PCH. However, upon addition of more LDAO, the extent of photoconversion decreased in a linear fashion (Fig. 34). At the same time, the CD signal, due to PCH, decreased in intensity. PCH containing 1% LDAO, showed only one negative peak at 614 nm. The size of this signal was five times smaller than in the control PCH.

We conclude that both SDS and LDAO inhibit the phototransformation reaction.

(6) Binding of PCH to different cation and anion exchange resins.

(a) DEAE cellulose. PCH was bound to DEAE, a weak anion exchange resin, in the range of pH = 7.0 to pH = 8.0. It was eluted as a broad, non-symmetrical peak, as already reported by Schopfer and Siegelman (1968).

(b) Phosphocellulose and Bio Rex-70. We tried to bind the holochrome to phosphocellulose and to Bio Rex-70, both weak cation exchange resins, at pH = 6.0, 6.5, 7.0, and 7.4. PCH was not stable at pH = 6.0 and lost 50% of its phototransformability after 3 hr of incubation at this pH. It was also irreversibly bound to the cation exchange column at pH = 6.0 and could not be recovered. At pH = 6.5, the
Figure 34. The change in photoconversion as a function of % LDAO added to PCH. Percent photoconversion was measured as the percent of PChlide photoconverting to Chlide from the total PChlide in the sample.
absorption maximum shifted from 639 nm to 634 nm and 50% of its phototransformability was lost upon 12 hr of incubation at this pH. At pH = 7.4, PCH did not bind to the cation exchange resins, which indicates that at this pH the protein was negatively charged and therefore was not attracted to the column. The most suitable pH appeared to be pH = 7.0. The binding properties of PCH to Bio Rex-70 and phosphocellulose at pH = 7.0 are presented in Table V-6.

Table V-6. Binding of PCH to Bio Rex-70 and Phosphocellulose at pH = 7.0

<table>
<thead>
<tr>
<th>Type of column</th>
<th>% non-bound PCH</th>
<th>A639 x 100</th>
<th>A280 x 100</th>
<th>A639 x 100 of non-bound PCH</th>
<th>A280 x 100 of non-bound PCH</th>
<th>A639 x 100 of initial sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphocellulose</td>
<td>48%</td>
<td>1.2 x 10^{-2}</td>
<td>21%</td>
<td>2.17 x 10^{-2}</td>
<td>1.71 x 10^{-2}</td>
<td></td>
</tr>
<tr>
<td>Bio Rex-70</td>
<td>42%</td>
<td>1.0 x 10^{-2}</td>
<td>22.5%</td>
<td>2.22 x 10^{-2}</td>
<td>1.67 x 10^{-2}</td>
<td></td>
</tr>
</tbody>
</table>

A portion of PCH (~20%) was bound reversibly to the cation exchange columns, and another portion (~45%) was washed off both columns at low salt, 0.025 M phosphate buffer pH 7.0. The PChlide content of the photoenzyme that did not bind to the cation exchange resins decreased compared to the sample of complex before loading to the column, while the fraction of the PCH that was bound had a relatively higher PChlide content, as measured by the specific activity of the three samples. The reversibly bound PCH was eluted from the columns by a linear gradient of 0.025 M to 1 M phosphate buffer at pH = 7.0. The results
obtained from phosphocellulose and Bio Rex-70 suggest that there are two types of PChlide-holochromes differing in their electrical charge distribution.

(c) SE-Sephadex and QAE-Sephadex. We tried to bind PCH to a strong cation exchanger, SE-Sephadex, at pH = 7.0. There was no binding at all of PCH to this column. On the other hand, PCH bound completely to QAE-Sephadex, a strong anion exchanger at pH = 7.0. We eluted the QAE-Sephadex column in steps of 0.2 M in phosphate, from 0.1 M K(P04) to 1.0 M K(P04), at pH = 7.0. PCH was eluted from the column at 0.4 M K(P04). It was detected by PChlide absorption at 440 nm.

D. Discussion

RuDP-Case constitutes up to 50% of the total protein contained in spinach leaf extracts (Kawashima and Wildman, 1970). RuDP-Case has very similar physical-chemical properties to the holochrome. RuDP-Case has a sedimentation constant in the range of 16.0 S to 19.5 S depending on the plant that it was extracted from (Kawashima and Wildman, 1970). PCH extracted from etiolated bean leaves has a sedimentation constant of 18 S (Boardman, 1962). Both proteins bind to DEAE in the pH range from 7.0 to 8.0, indicating a similar electrical charge distribution. Therefore it is very difficult to separate RuDP-Case and PCH by the usual column chromatography techniques. However, RuDP-Case is a soluble protein easily removed from mature chloroplasts by washing the thylakoid membranes with dilute buffers (Strotmann, Hesse and Edelmann, 1973). PCH, on the other hand, is associated with the prolamellar
membrane in etioplasts, probably through hydrophobic interactions, since a much greater proportion of PCH is liberated from the etioplast membrane using detergents like Triton X-100.

Therefore, the long procedure of column chromatography that yielded at the end, five polypeptides of different proteins by SDS polyacrylamide gel electrophoresis was replaced by a procedure that involved several successive washes of etioplast fragments followed by sucrose density gradient centrifugation. In the latter procedure, polyacrylamide gel electrophoresis revealed the presence of three polypeptides. The mobilities of two of these peaks were in good agreement with the mobilities of the large and small subunits of spinach RuDP-Case. The third peak was found to be a polypeptide with a molecular weight of 45,000 daltons. Using direct absorption measurements at 675 nm, and pigment extraction, it was discovered that before photoconversion PChlide, and immediately after transformation Chlide, was attached to the 45 Kd polypeptide. Similar results were obtained also by gel filtration on Sephadex G-100 (SDS 0.1%). PChlide was associated with two peaks of molecular weights 50,000 and 6500 daltons. The small molecular weight peak was probably a proteolytic degradation product. We conclude that PCH is made from one kind of polypeptide of 45,000 daltons. Since the PCH macromolecule has a molecular weight of 550,000, it probably contains twelve polypeptides of identical size.

Using two independent methods, the ratio A280/A440 and direct estimation of the absorption of PChlide-639 per mg protein (measured by the Lowry method), we calculated the number of PChlide per protein. The number of PChlide per 550,000 daltons protein was found to be 4.
Probably, only some and not all of the 45,000 daltons are attached to PChlide pigment molecules.

Chromatographing PCH on cation exchange columns indicated that there may be two pigment-protein complexes with different electrical charge distributions. Two possible explanations can be advanced for this observation: (1) Equilibrium phenomenon: There may be an equilibrium between the intact protein and its constituent subunits. (2) Two kinds of pigment-protein complexes exist in vivo in the leaf: There may be a correlation between the known differences in absorption maxima and phototransformability and the two peaks that we have separated by an anion exchange column or by differential binding to cation exchange columns.

Successive washes of etioplast fragments systematically deplete our sample of the RuDP-Case with respect to PCH, as seen in the yields and electrophoresis profile of $S_1$ through $S_4$ samples. This indicates that PCH is more tightly bound to the etioplast membrane than RuDP-Case. The detergents SDS and LDAO were used in an attempt to isolate a photoactive PCH subunit from etiolated bean leaves. However, these detergents inhibited the phototransformation reaction.
CHAPTER VI

THE CLEAVAGE OF THE HOLOCHROME AND ITS RELATION TO

THE SHIBATA SHIFT

A. Introduction

In the previous chapter evidence linking protochlorophyllide to a basic subunit of molecular weight in the order of \((45 \pm 5)\) Kd was discussed in detail. This basic subunit was obtained by dissociating the PCH molecule with the aid of a strong detergent, 2% SDS, and under reducing conditions, 1% \(\beta\)-mercaptoethanol and 8 M urea.

In this chapter evidence will be presented which indicates that the photochemical reaction \textit{per se}, involving the addition of two hydrogen atoms to the PChlide molecule to form a Chlide molecule, does not change the holochrome either on the macromolecular or on the subunit level. However, after the Shibata shift has taken place in the dark grown bean leaf homogenate, the holochrome macromolecule of 600 Kd undergoes dissociation. The 45 Kd molecular weight peptide is cleaved to yield a 16 Kd peptide to which the Chlide molecule is still attached.

Bogorad et al. (1968) suggested that the 600 Kd holochrome undergoes dissociation both \textit{in vivo}, in illuminated bean leaves, and \textit{in vitro} in crude holochrome preparations upon illumination. These investigators did not correlate the change with the Shibata shift.
Henningsen et al. (1974) observed the cleavage of a 63,000 molecular weight photoactive Chlide subunit to a 29,000 molecular weight Chlide subunit following the dark Shibata shift in dark grown barley leaf homogenate.

The significance of these findings and the possible mechanisms to account for them will be presented in this chapter.

Materials and Methods

Bean seedlings (Phaseolus vulgaris, variety red kidney) were grown 12 days on vermiculite in the dark at 22°C. The leaves (50 to 100 g) were harvested under dim green light and ground in 1:3 ratio of buffer containing 10 mM Tris-Cl, pH 8.5, 2 mM MgSO$_4$, 1 mM EDTA, and 25% (v/v) glycerol. An extracting solution containing, in addition, Triton X-100 (0.06% v/v) is referred to as buffer + Triton. All manipulations were carried out in a cold room (4°C) equipped with a green safe light.

Two kinds of preparations were used. The first involves homogenization of the leaves in buffer + Triton, followed by polyethylene glycol precipitation, and hydroxylapatite chromatography as described in detail in Chapter V-B. This partially purified holochrome is very stable and maintains its photoconversion activity for at least one week at 4°C. It was used immediately or stored at -20°C before use. It will be referred to as PCH homogenate.

The second procedure involves homogenization of the leaves in buffer, followed by extracting three times with buffer (see above) and by one extraction with buffer + Triton. Four supernatants ($S_1$ to
S₄) were collected and were assayed for protein content and PCH activity. In some of the experiments, the harvested leaves were frozen and kept at -90°C for several weeks. The leaves were not thawed prior to homogenization but ground to a fine powder with a mortar and pestle while frozen on dry ice. The powder was transferred to a Waring blender, and all subsequent steps were the same as with fresh leaves.

The procedure for isolation of S₄ is described in detail in Chapter V-B. The S₄ is of very high purity, but not very stable. It loses its activity within several hours at 4°C. The S₄ preparation was used immediately for further experiments, or made into 50% glycerol (v/v) and kept at -20°C. Absorption spectra of the various preparations were measured in a 1 cm cuvet using a Cary Model 14 spectrophotometer.

(1) Gel electrophoresis. Electrophoresis was carried out in 0.1% SDBS following the method described by Weber and Osborn (1969). The polyacrylamide gels (concentration 10% with 1 cm upper gel 5%) were run at 4°C. The gel column was 1.3 cm in diameter and 8.0 cm high. The sample contained 1 mg protein in 8 M urea, 2% SDBS, 1% β-mercaptoethanol, 10% glycerol. After electrophoresis for 4.5 hr at 3 mA/gel followed by 4.5 hr at 6 mA/gel, the gel was frozen and sliced into 3.8 mm sections. (Two identical gels were typically combined.) Each section was dispersed by extrusion from a syringe through 125 mesh nylon bolting cloth. The gel particles were then resuspended in 0.05 M Tris buffer, pH 8.0 containing 1% SDBS incubated for 4 hr at 4°C and freeze dried. The dry pellet of each tube was extracted with 90% acetone. PChlide and Chlide were detected by measuring their fluorescence in the 90% acetone extracts with a Hitachi (Perkin-Elmer) MPF-2A Fluorescence Spectrophotometer.
The gels were calibrated with respect to molecular weight using purified proteins of known size. The proteins employed were: bovine serum albumin (68,000), ovalbumin (43,000), Cyt c (12,400), myoglobin (17,800), all purchased from Schwarz/Mann, and lysozyme (14,300), trypsin (23,000) purchased from Sigma. The gels were stained overnight with 1% amido-black 10B (Baker, N. J.) in 5/1/5 methanol:acetic acid:water by diffusion. Protein was determined using the method of Lowry (1951).

(2) Sucrose gradient centrifugation. PCH homogenates containing 0.05 M glycine buffer pH 9.5 and 10% glycerol were centrifuged in gradients of 10% to 70% sucrose for 41 hr at 41,000 rpm in the SW 41 rotor of the Beckman ultracentrifuge (Model L-2) at 2°C. One ml samples containing 10 mg/ml protein were loaded on the gradients. After centrifugation, the gradients were collected in 20 fractions of 0.6 ml each. Each fraction was analyzed fluorometrically either for convertibility of PChlide to Chlide or, in preparations containing pigment which has been converted prior to centrifugation, for its fluorescence in the region of 600-700 nm.

Similar gradients containing molecular weight markers (thyroglobulin (670,000), apoferritin (450,000), γ-globulin (150,000) and bovine serum albumin (67,000)] were centrifuged in parallel with the holochrome gradients.

C. Results

(1) Location of chlorophyllide molecule on the holochrome subunit prior to and after the Shibata shift. Figure 35 shows the absorption
Figure 35. The absorption spectrum of chlorophyllide holochrome at \( t = 1 \text{ min} \) and \( t = 40 \text{ min} \), at \( 15^\circ \text{C} \).
spectrum of Chl-H homogenate immediately (1 min) after photoconversion at 22°C. The absorption maximum is typically at 678-680 nm. After 40 min at room temperature, the absorption maximum is located at 674 nm (Fig. 35).

Chl-H absorbing at 680 nm is denatured and dissociated into polypeptides in 2% SDS SDBS, 8 M urea, 1% β-mercaptoethanol and the absorption maximum is shifted to shorter wavelength at 673 nm (Fig. 36). It is then loaded on polyacrylamide gel and electrophoresis is carried out at 4°C.

Similarly, Chl-H after the Shibata shift, displaying an absorption maximum at 675 nm, is shifted to 671 nm after dissociation under denaturating conditions. A typical fluorescence spectrum of the pigment obtained from the gel after acetone extraction is shown in Figure 37. In some experiments, the fluorescence of inactive PChlide at 630 nm was observed together with Chlide fluorescence at 666 nm in the same location on the gel (Fig. 38). The pigment was identified by comparing known fluorescence spectra of PChlide and Chlide in acetone and also by the excitation spectra that were measured on each sample.

The electrophoretic patterns obtained for each dissociated sample before and after the Shibata shift are shown diagrammatically in Figure 39.

It was observed that, before the dark shift, the location of the pigment on the gel corresponds to a mobility of 0.28 ± 0.08 and molecular weight of 45,000 ± 10,000, as calculated from the calibration curve (Fig. 40). However, after the dark shift, the pigment was found at a location corresponding to a mobility of 0.63 ± 0.03 and a molecular weight of 16,000 ± 1,000.
Figure 36. The absorption spectrum of Chl-H, immediately after photoconversion, and after incubation in SDBS 2%, β-mercaptoethanol 1%, and 8 M urea.
Figure 37. An emission scan of Chlide in acetone. Chl-H was dissociated with 2% SDBS, 1% β-mercaptoethanol, 8 M urea, and run on a 10% polyacrylamide gel at 4°C. Band width for emission and excitation, 10 nm. Fluorescence excited at 430 nm.
Figure 38. An emission scan of PChlide and Chlide in acetone. Conditions as in Figure 37.
Figure 39. Schematic representation of SDBS-urea treated Chl-H on 10% polyacrylamide gels before and after the dark shift. The shaded bands depict the position of the pigment, and the respective mobility and molecular weight associated with each band.
Figure 40. Calibration curve on 10% SDBS polyacrylamide gels for the determination of the molecular weight of Chl-H polypeptide before and after cleavage.
In a sample where the dark shift was allowed to proceed only part of the way, and the absorption maximum was at 677 nm before denaturation (673 nm upon denaturation), it was found that about half of the Chlide fluorescence was associated with a molecular weight of 45,000 and the other half was associated with a molecular weight of 16,000.

The results of four experiments are summarized in Table VI-1. Each experiment represents a Chl-H sample that was incubated for a different time at 15°C after the initial photoconversion and then dissociated in SDS and β-mercaptoethanol and analyzed electrophoretically.

Table VI-1. The Molecular Weight, Mobilities, and Absorption Maxima of the Different Polypeptides before and after the Shibata Shift

<table>
<thead>
<tr>
<th>Absorption maximum</th>
<th>Mobility</th>
<th>Molecular weight</th>
<th>Incubation time at 15°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>679 nm</td>
<td>0.187 (90%)</td>
<td>57,000</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>0.875 (10%)</td>
<td>&lt;10,000</td>
<td></td>
</tr>
<tr>
<td>679 nm</td>
<td>0.3</td>
<td>40,000</td>
<td>1 min</td>
</tr>
<tr>
<td>677 nm</td>
<td>0.40 (45%)</td>
<td>31,000</td>
<td>20 min</td>
</tr>
<tr>
<td></td>
<td>0.60 (45%)</td>
<td>17,500</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.85 (10%)</td>
<td>&lt;10,000</td>
<td></td>
</tr>
<tr>
<td>674 nm</td>
<td>0.22 (5%)</td>
<td>50,000</td>
<td>40 min</td>
</tr>
<tr>
<td></td>
<td>0.66 (30%)</td>
<td>15,000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.89 (5%)</td>
<td>&lt;10,000</td>
<td></td>
</tr>
</tbody>
</table>

These results suggest that a polypeptide of molecular weight 45,000 to which the Chlide molecule is attached has been cleaved to yield a polypeptide of molecular weight of 16,000 daltons. This cleavage occurs with the same half-life time and the same temperature dependence as the dark shift. It is blocked below 0°C and is completed in about 40 min at 15°C.
A minor Chlide peak, consisting of 10% of the total Chlide on the gel and displaying a mobility of 0.86 ± 0.04 and molecular weight <10,000 is also apparent in most experiment. It is considered to be a proteolytic product of the major subunit of the holochrome (45,000 molecular weight). For further discussion of this product, see Chapter V.

In most of the experiments, except one, PChlide ran as a free pigment, with mobility identical to the bromo-phenol-blue dye. Apparently, the PChlide molecule is more easily detached from the polypeptide.

The holochrome homogenate was also analyzed for its polypeptides by gel electrophoresis. Unfortunately, the gels did not destain properly after fixing and staining. Probably, substances are present in the holochrome homogenate which interfere with the staining and destaining procedure. However, these substances are absent in the S4 PCH.

(2) The absence of the Shibata shift in the S4 Chlorophyllide holochrome. The S4 preparation, the highest purity of holochrome obtained, was used for similar experiments. Unfortunately, the S4 preparation is highly labile, and the Chlide absorption is completely lost within several hours. No Chlide was detected on the gel.

The absorption spectrum of S4 PCH immediately after photoconversion is shown in Figure 41. The absorption maximum is at 672 nm.

The absorption spectrum was measured also at partial photoconversion. It was observed that the absorption maximum is at 672 nm. There is no corresponding long wavelength form, and no Shibata shift has been observed for the S4 holochrome preparation. An identical electrophoretic pattern is seen for the following samples: 1) Dark. 2) Immediately after illumination. 3) An illuminated sample incubated for 40 min at room
Figure 41. The absorption spectrum of $S_4$ Chl-H immediately after photoconversion at 4°C.
temperature; a dark sample is shown as an example (Fig. 42). The major band in each case appears to have a molecular weight of 45,000 and occurs with a minor band of 60,000 molecular weight, according to the calibration curve (Fig. 43). They have been tentatively identified as the holochrome and the RuDP-Case, respectively. For further discussion of this point, see Chapter V.

These results suggest that $S_4$ Chl-H does not exhibit a Shibata shift, nor does it display the cleavage of the 45,000 MW peptide.

Addition of glycerol, so as to bring its concentration to 50%, has an effect on the absorption maximum of $S_4$ Chl-H. After photoconversion, the absorption maximum is found at 676 nm instead of 672 nm using low glycerol concentration. It is found that in the presence of 50% glycerol, the holochrome is more stable and the photoactivity is retained for a much longer period. A similar effect of high concentration of sucrose on the spectroscopic properties of holochrome homogenates was described by Mathis and Sauer (1972). The electrophoretic patterns of a high glycerol (676 nm form) and low glycerol (672 nm form) of the Chl-H were indistinguishable from that shown in Figure 42 for a dark $S_4$ sample.

The absorption maximum of the pigment appears to be dependent on the immediate environment of the pigment and to reflect a conformational change in the holochrome upon addition of glycerol to high concentration.

(3) Dissociation of the chlorophyllide holochrome macromolecules of 600,000 molecular weight simultaneously with the Shibata shift. The profile of PCH after centrifugation in a sucrose gradient is shown in Figure 44. The photoconvertible holochrome runs as a homogeneous peak
Figure 42. Spectrophotometric scan of SDS polyacrylamide gel electrophoresis of S4 PCH fraction, in the dark.
Figure 43. Calibration curve on 10% SDS polyacrylamide gels for the determination of the molecular weight of Chl-H. C-band locates the RuDP-Case and H-band locates the holochrome.
Figure 44. The distribution in a sucrose density gradient of photoconver-
tible PCH and inactive PCH from etiolated bean leaves. Sample is loaded
in darkness at 4°C.
and appears to have a sedimentation coefficient of 18S, by comparison of its position in the gradient to those of thyroglobulin (sedimentation coefficient 19.2S) and apoferritin (sedimentation coefficient 17.6S). However, the inactive PCH appears as an inhomogeneous peak. Approximately 70% has a sedimentation coefficient of 18S, and 30% has a sedimentation coefficient of 10S.

Illumination for 2 min at an intensity of 90 ft. candles at room temperature photoconverted all active PCH to Chl-H. The photoconverted sample was immediately loaded on a sucrose gradient. Chl-H appeared as a major peak of sedimentation coefficient of 18S and a minor peak of a smaller sedimentation coefficient of about 10S (Fig. 45).

Chl-H was allowed to complete the Shibata shift and the sample was analyzed by sucrose gradient centrifugation (Fig. 46). The 18S Chl-H macromolecule dissociated almost completely to yield a new pigment complex of a sedimentation coefficient smaller than 4.6S. The results are summarized in Table VI-2.

The sedimentation coefficient of PCH was measured by Boardman (1962). It was found to be 18S in the analytical ultracentrifuge and the molecular weight was calculated as 600,000. This result is in good agreement with the sedimentation coefficient obtained here in the sucrose gradient centrifugation. However, it is observed that upon illumination and the commencement of the Shibata shift, the 600,000 Chl-H macromolecule dissociates, and the final product is a Chlide complex of MW smaller than 70,000.
Figure 45. The distribution in a sucrose density gradient of Chl-H and inactive PCH illuminated for t = 1 min at 22°C prior to centrifugation.
Figure 46. The distribution in a sucrose density gradient of Chl-H and inactive PCH at t = 40 min at 22°C prior to centrifugation.
<table>
<thead>
<tr>
<th>Samples</th>
<th>Relative mobility</th>
<th>S value</th>
<th>MW</th>
<th>Pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pchlide holochrome</td>
<td>0.62±0.03</td>
<td>-</td>
<td>-</td>
<td>active Pchlide</td>
</tr>
<tr>
<td>Chlide holochrome</td>
<td>0.63±0.03</td>
<td>-</td>
<td>-</td>
<td>inactive Pchlide</td>
</tr>
<tr>
<td>t=2 min at 22°C</td>
<td>0.60±0.03</td>
<td>-</td>
<td>-</td>
<td>inactive Pchlide</td>
</tr>
<tr>
<td>Chlide holochrome</td>
<td>0.41±0.02</td>
<td>-</td>
<td>-</td>
<td>Chlide</td>
</tr>
<tr>
<td>t=40 min at 22°C</td>
<td>0.25±0.02</td>
<td>-</td>
<td>-</td>
<td>inactive Pchlide</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>0.68±0.03</td>
<td>19.2 S</td>
<td>670,000</td>
<td>-</td>
</tr>
<tr>
<td>Apoferritin</td>
<td>0.60±0.03</td>
<td>17.6 S</td>
<td>450,000</td>
<td>-</td>
</tr>
<tr>
<td>γ-globulin</td>
<td>0.40±0.02</td>
<td>10 S</td>
<td>150,000</td>
<td>-</td>
</tr>
<tr>
<td>BSA</td>
<td>0.37±0.02</td>
<td>4.6 S</td>
<td>70,000</td>
<td>-</td>
</tr>
</tbody>
</table>
A post-illumination dark shift in the absorption spectrum of dark grown bean leaves was discovered and characterized by Shibata (1957). The 684 nm absorption form of chlorophyll (C684), which resulted from the photoconversion of protochlorophyllide (P650) after a short illumination, slowly transformed in about 10 to 15 min in the dark to a form of chlorophyll absorbing at 672 nm (C672). The sharp isosbestic point obtained for the C684 $\rightarrow$ C672 shift indicated conversion of one definite compound to another. A similar shift in the position of the fluorescence maximum of chlorophyll (from 690 nm to 680 nm) was observed by Litvin and Krasnovsky (1957). The origin of this dark shift was unknown.

A similar dark shift is observed in chlorophyllide holochrome in vitro. Immediately after the phototransformation the principal absorbance maximum is at 678 nm, due to the newly formed chlorophyllide. The 678 nm peak shifts slowly towards shorter wavelength with a slight decrease in absorbance. At 25°C, the shift is completed in 10 to 15 min at lower temperature, 12°C, the shift requires 30 to 40 min for completion. We make the assumption that both the in vivo and the in vitro dark shifts are due to the same reaction.

Our results suggest that protochlorophyllide holochrome isolated from dark grown bean leaves is an oligomer of a polypeptide with a molecular weight of 45,000. The subunit was obtained by dissociating the protochlorophyllide holochrome with SDS, urea and β-mercapto ethanol. This treatment denatures the protein, and the 45,000 daltons subunit has lost its ability to photoconvert. Immediately after photoconversion of
the native protochlorophyllide holochrome macromolecule, the newly formed chlorophyllide holochrome appears to be of the same composition. It is composed of the 45,000 dalton polypeptide which is associated with chlorophyllide. After completion of the dark shift, the chlorophyllide holochrome yields a polypeptide of 16,000 daltons associated with chlorophyllide. These results indicate that the polypeptide of 45,000 molecular weight has undergone a cleavage to yield a smaller chlorophyllide polypeptide of 16,000 daltons. The time for completion of this reaction and the fact that it is blocked at 0°C are characteristic also for the dark spectroscopic shift.

Henningsen et al (1974), isolated an active subunit of 63,000 dalton from barley leaves with the aid of a detergent saponin. When the dark spectral shift from 678 nm to 672 nm occurred, a change in the apparent molecular weight of the chlorophyllide holochrome subunit from about 63,000 to 29,000 was observed. In holochrome extracted from bean leaves with a high concentration of saponin the spectral shift was extremely slow, and no corresponding change in the molecular weight was reported (Henningsen et al, 1974). Our experiments with S₄-chlorophyllide holochrome show the absence of both the spectral shift and the corresponding cleavage of the holochrome subunit. Apparently, the ability to carry out this reaction has been lost in this preparation.

The data of Table 4-2 demonstrate that, in the time course of the dark shift, a major disintegration occurs at the macromolecular level. The 600,000 dalton chlorophyllide holochrome with a sedimentation coefficient of 18 S dissociated to a chlorophyllide protein with a sedimentation coefficient smaller than 4.6 S. These results are in agreement with similar experiments conducted by Bogorad et al (1968).
Butler and Briggs (1966) have previously attributed the Shibata shift in leaves to a disaggregation. Pigment disaggregation was also implied from the studies of Schultz and Sauer (1972), Mathis and Sauer (1972) and on the CD spectrum of chlorophyllide holochrome. Our studies (Chapter I) confirm this observation. A double CD signal attributed to a dimer of chlorophyllide molecules disappears progressively during the course of the dark shift. It was suggested that a dimer of chlorophyllide molecules is dissociating into chlorophyllide monomers. Another feature of the CD spectrum of chlorophyllide holochrome immediately after photoconversion is a negative peak at 580 nm. This peak also vanishes in the course of the dark spectral shift (Forster et al 1971, Mathis and Sauer (1972). These experiments have been interpreted as indicating a major conformational change in the holochrome protein which in turn affects the pigment-pigment interaction. Our results support this hypothesis and develop it further to propose that the conformational change is followed by disintegration of the holochrome macromolecule and the cleavage of its chlorophyllide subunit to a smaller chlorophyllide polypeptide of 16,000 molecular weight.
References

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