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THE DEVELOPMENT AND ORGANIZATION OF PHOTOSYNTHETIC PIGMENT SYSTEMS

Author
Schultz, Alfred Joseph

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Alfred Joseph Schultz, Jr.
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LAWRENCE RADIATION LABORATORY
UNIVERSITY of CALIFORNIA BERKELEY

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THE DEVELOPMENT AND ORGANIZATION OF PHOTOSYNTHETIC PIGMENT SYSTEMS

Alfred Joseph Schultz, Jr.

Laboratory of Chemical Biodynamics
Lawrence Radiation Laboratory
University of California
Berkeley

November 1970

ABSTRACT

The greening of flowering plants is studied through their absorption, fluorescence, circular dichroism (CD) and magnetic circular dichroism (MCD) spectra. The primary objective of these studies is to determine the arrangements of the pigments during greening.

The initial step in the greening of dark-grown plants is the light-dependent reduction of protochlorophyllide a (PChlide a) to form chlorophyllide a (Chlide a). This reaction, which involves the addition of two protons to the porphyrin ring system of PChlide a, can be studied in intact leaves, in leaf homogenates, or in a purified pigment-protein complex known as PChlide a holochrome (PCH). Improved methods are given for preparing PCH.

The PCH exhibits a complex CD spectrum in the long wavelength region (500-700 nm). This spectrum differs markedly from that of protochlorophyll a (PChl a) monomers in polar solvents, but it resembles that of PChl a aggregates in carbon tetrachloride. We conclude that PCH contains an aggregate of at least two PChlide a chromophores.

Illumination converts the PChlide a to Chlide a in the holochrome, and this is followed by a spectral shift that does not depend on light. In the presence of 2 M sucrose, which blocks the dark spectral shift, the Chlide a holochrome (CH) exhibits a double CD at the lowest energy transition of Chlide a. In the absence of sucrose, only a single CD is observed in the same wavelength region. Thus, the illuminated holochrome apparently initially contains an aggregate of at least two Chlide a chromophores. Subsequently, the chromophores disaggregate in the dark to form monomers. The added sucrose prevents this disaggregation.

Fluorescence polarization spectra support the above interpretations of the CD spectra of the illuminated holochrome. The polarization values for the CH formed in the absence of sucrose are large. They are comparable to those for pure chlorophyll a (Chl a) in viscous solvents. When the photoconversion occurs in the presence of sucrose, the observed fluorescence polarization values are only about half as great, which is consistent with the occurrence of Chl a aggregates that can delocalize the excitation among their chlorophylls.

Determination of the quantum requirements for the photoconversion is complicated by the presence of a substantial amount of inactive PChlide a which is not photoreduced. Analysis of the photochemical kinetics suggests that approximately 11% of the radiation absorbed by this inactive PChlide a is effective in stimulating photoconversion of the active PChlide a; none of the radiation absorbed by the product Chlide a is effective. The quantum requirements

for radiation absorbed by active PChlide a are calculated to be 0.48 for 630.0 nm photons, 0.60 for 640.0 nm photons, and 0.66 for 650.0 nm photons.

CD spectra of homogenates prepared from leaves grown in the dark and then given 1 hour of illumination have only a single component in the red. After 2 hours of illumination of the leaves, at least three CD components are observed at approximately 680 nm, 670 nm, and 650 nm. These increase in amplitude as the plant continues to green. Of the three components, the one at 680 nm approaches its greatest magnitude most rapidly; the one at 670 nm least rapidly. All three components are present in the CD spectra of homogenates of fully greened leaves.

These CD spectra of homogenates prepared from leaves after periods of illumination ranging from 1 to 73 hours indicate that aggregates of chlorophyll begin to form after two hours of illumination. The basic structure of the aggregate is not believed to change after it has formed during greening. At least two types of aggregates are formed, one containing chlorophyll b (Chl b) and one containing Chl a. The geometry of the Chl b aggregate differs from the geometry of the Chl a aggregate. However, in both aggregates the smallest repeating structure contains at least two chlorophyll units.

In summary, the following model is proposed. PCH contains an aggregate of at least two PChlide a chromophores. The proximity of the chromophores permits the utilization of one quantum of light for the reduction of two chromophores. After photoreduction, the aggregate of chromophores dissociates into monomers. Finally, after

about 2 hours of illumination of the leaves, other aggregates containing a repeating structure of at least two chlorophyll units form.

ABBREVIATIONS

Abs	absorbance
CD	circular dichroism
MCD	magnetic circular dichroism
ORD	optical rotatory dispersion
Chl *a*	chlorophyll *a*
Chl *b*	chlorophyll *b*
PChl *a*	protochlorophyll *a*
Chlide *a*	chlorophyllide *a*
PChlide *a*	protochlorophyllide *a*
PCH	protochlorophyllide *a* holochrome
CH	chlorophyllide *a* holochrome
PCH(suc)	protochlorophyllide *a* holochrome in buffer containing 2 M sucrose
CH(suc)	chlorophyllide *a* holochrome in buffer containing 2 M sucrose

I. INTRODUCTION

Put out the light, and then put out the light.
If I quench thee, thou flaming minister,
I can again thy former light restore,
Should I repent me; but once put out thy light,
Thou cunning'st pattern of excelling nature,
I know not where is that Promethean heat
That can thy light relume. When I have pluck'd thy rose
I cannot give it vital growth again;
It needs must wither. I'll smell thee on the tree.

Othello[69]

A scientific ideal should be the study of life while it is living. But the attainment of this ideal frustrates dispassionate science. At the present time we must often destroy the very life whose processes we wish to learn about. The two principal reasons why the ideal hasn't been achieved here, as shall be seen, are instrumental limitations and overwhelming complexity.

An investigator of living organisms can proceed along two general lines. First, after describing the unscathed organism as completely as possible, he can figuratively and/or (but usually and) literally tear it apart, studying each of these simpler parts. Second, he can put components, either the natural parts or analogs to them, together to recreate the organism or a part of it. The second course has only been partially successful, yet it has been useful in studying relationships between parts.

In this dissertation I have primarily used the latter course, but I have chosen to let the plant do the assembly of the photosynthetic pigments for me as it develops (greens). In this manner, I was able to study both the organization and development of the photosynthetic pigment systems. The former course might have been utilized in the same way, by observing the photosynthetic machinery being destroyed during leaf senescence.

Flowering plants (angiosperms) do not produce chlorophyll until they are exposed to light. (Gymnosperms, such as pines, and most photosynthetic microorganisms can produce chlorophyll in the dark.) The reason, if there is a reason, for the delay is not obvious. Until it becomes photosynthetic, the sprout is dependent upon the energy stores of the seed. It becomes photosynthetic long before greening is completed; thus, the seed energy stores are conserved by delaying photosynthetic development until the plant is in the light and photosynthesis can provide energy for its own development. Perhaps chlorophyll formation just happened to be the point where further photosynthetic development was blocked.

By simply germinating angiosperms in darkness, the greening of the seedling is prevented. Such an etiolated seedling changes rapidly when it is illuminated. In three days, the leaves unfold and expand; their color changes from pale yellow to the tere-verte of a mature plant. The green intensifies almost visibly. Obviously the chlorophyll content has increased greatly.

Microscopically, the appearance of the cell organelle related to photosynthesis, the leaf plastid, has also been transformed

dramatically. The prolamellar body, a paracrystalline structure of membranes often found in etiolated plastids, has gone. In its place are the stacked discs of the photosynthetic membranes contained within a mature chloroplast.

During these three days the seedling has undergone metamorphosis from a heterotroph, dependent upon food reserves in the seed, to an autotroph, reaping light. From the physical energy of light, the fully photosynthetic seedling produces chemical energy to make oxygen from water, and sugars and amino acids from carbon dioxide. Light energy is captured by a fragile web of pigments, the chlorophylls and carotenoids. I am concerned here with the creation of this web.

Shibata found that the absorption spectrum of an etiolated (dark grown) leaf has a maximum at 650 nm.[71] The pigment responsible for this maximum is protochlorophyllide a (Fig. II-1). When the etiolated leaf is exposed to light, the protochlorophyllide a (PChlide a) is stereospecifically reduced at positions C-7 and C-8 to form chlorophyllide a (Fig. II-1). Raising the temperature of the leaf to about 40° to 50°C for a few minutes destroys the leaf's ability to photoreduce PChlide a. Both the stereospecificity of the reduction and the destruction of reducing ability of the leaf at elevated temperatures point to involvement of an enzyme in the reaction. However, the reduction will occur at temperatures as low as -70°C. This is unusual for an enzymatic reaction. The action spectrum for the photoreaction follows the absorption spectrum of PChlide a closely, indicating that PChlide a is the photoreceptor.[77]

After the PChlide a has been extracted from the leaf with an organic solvent, it can no longer be photoreduced to form chlorophyllide a (Chlide a). PChlide a can be extracted into aqueous media complexed with a protein.[48,67,78] The PChlide a-protein complex has been named protochlorophyllide a holochrome. Protochlorophyllide a holochrome (PCH) has a molecular weight of approximately 500,000.[67] Apparently, each holochrome unit of molecular weight 500,000 contains at least two PChlide a chromophores.[67] PChlide a in the holochrome can be reduced upon illumination to form Chlide a.

A number of shifts of the red absorption maximum occur in the absorption spectrum of an etiolated leaf after phototransformation of the PChlide a at room temperature. First, the absorption maximum shifts from 650 nm to 678 nm. This shift is very rapid, less than 4 msec.[54] Then, the maximum shifts from 678 nm to 684 nm. The shift from 678 nm to 684 nm will take place in the dark. It is completed in 6 to 10 min at 2°C.[12,22] Another dark shift from 684 nm to 673 nm follows that from 678 nm to 684 nm. The shift from 684 nm to 673 nm was first described by Shibata.[71] It will also take place in the dark. At 22°C it requires 10 to 15 min to be completed. The origin of neither of these dark shifts is known. Although the later shift may be related to the addition of the phytyl group which is completed within one hour after the beginning of illumination.[76,100,104]

For about 2 hours after the beginning of illumination, the chlorophyll content of an etiolated leaf remains approximately constant. It then begins to increase rapidly. The increase continues for about two or three days, reaching a final value about two orders

of magnitude greater than the initial value. At about the same time that the rapid synthesis of chlorophyll begins, the red absorption maximum of leaves also begins to shift toward longer wavelengths. The shift continues for about a day until the maximum is at 678 to 679 nm, which is where it is located in mature plants.

Within about ten minutes after the initiation of illumination, traces of chlorophyll b (Chl b) appear.[72] Chl b is identical to chlorophyll a (Chl a) except that the methyl group on C-3 is replaced by a formyl group, -CHO (Fig. II-1). The Chl b content of the leaf increases more rapidly than the Chl a content.[72] By the time the leaf has matured, there is about 1/3 as much Chl b as Chl a.

Losev and Gurinovich were unable to detect energy transfer from Chl b to Chl a in homogenates of greening leaves until after 2 hours of illumination.[51]

The carotenoid content of an etiolated leaf, in terms of weight, is on the order of 17 times its protochlorophyll and protochlorophyllide content.[44] The carotenoids of etiolated seedlings are predominantly xanthophylls. After beginning illumination, carotenoids are rapidly synthesized as the seedling's chlorophyll content increases.[24] A mature chloroplast contains about 3 times as much carotenoid as an etiolated plastid (etioplast).[44] The major carotenoids of green leaves are β-carotene, lutein, violaxanthin, and neoxanthin.[24]

Losev and Gurinovich found that in greening seedlings energy transfer from carotenoids to chlorophyll a developed later than energy transfer from Chl b to Chl a.[51]

Smith first detected oxygen evolution from dark grown barley after a 30 min exposure to light.[79] However, others have found that photosynthetic oxygen evolution and carbon dioxide fixation begin between 10 and 16 hours after the beginning of illumination.[4,13]

I sought to answer a number of questions. First, how the photosynthetic pigments are arranged in a mature leaf. Second, how the arrangement of the pigments develops during the greening of the leaf. Third, how are the chromophores arranged on the holochrome. And fourth, what the mechanism of the photoreduction of PChlide a is.

II. ABSORPTION AND CD SPECTRA OF GREENING LEAVES

"When we study material things of whatever nature, as regards their forms and the repetition of their identical parts, we soon recognize that they fall into two large classes of which the following are the characters: Those of the one class, placed before a mirror, give images which are superposable on the originals; the images of the others are not superposable on their originals, although they faithfully reproduce all the details. A straight stair, a branch with leaves in a double row, a cube, the human body - these are of the former class. A winding stair, a branch with the leaves arranged spirally, a screw, a hand, an irregular tetrahedron - there are so many forms of the other set. The latter have no plane of symmetry."

Pasteur[52,59]

A. Introduction

Most natural organic molecules and macro-molecular structures are dissymmetric. Clearly a probe which could not only detect this dissymmetry, but also determine the geometry of molecules and their relationship to one another, would be extremely useful. One such probe is circular dichroism, the difference in absorption of left and right circularly polarized light.

1. CD of Chlorophyll Aggregates

Optical activity is commonly thought of as a means of detecting an asymmetric carbon atom such as is found in an amino acid. However, it will detect dissymmetry in an arrangement of molecules as

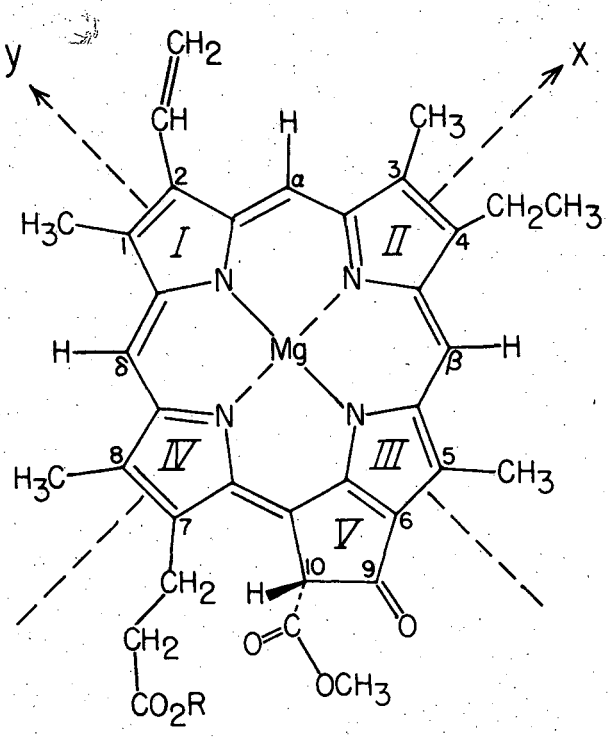
well, even if the individual subunits comprising the structure are completely symmetric.

Pasteur illustrated how a whole can be dissymmetric even when the components are symmetric with the example of a spiral staircase:

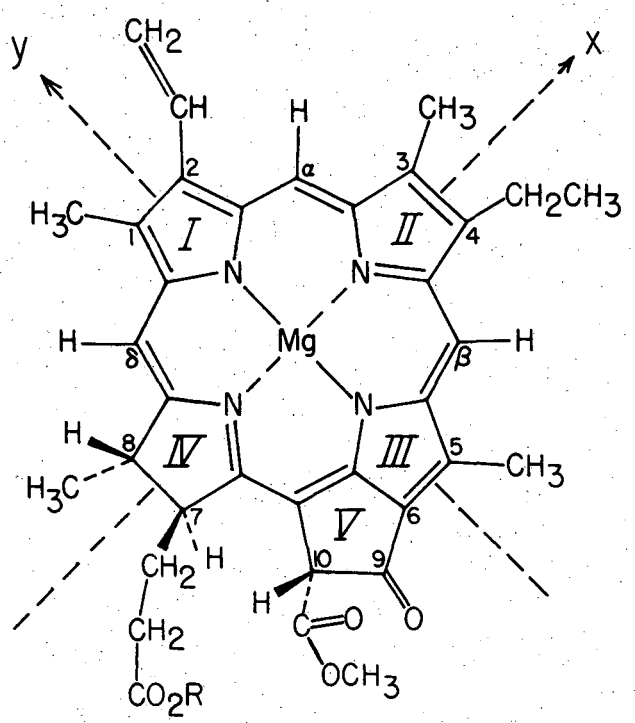
> "Imagine a spiral stair whose steps are cubes, or any other objects with superposable images. Destroy the stair and the dissymmetry will have vanished. The dissymmetry of the stair was simply the result of the mode of arrangement of the component steps." Pasteur[52,60]

Chlorophyll molecules are not completely symmetric. Chlorophyll _a_ possesses three asymmetric carbon atoms in its ring structure (C-7, C-8, and C-10), as well as two asymmetric carbon atoms in the phytyl esterifying group (see Fig. II-1), but Pasteur's illustration is still relevant. The dissymmetry of an aggregate of chlorophyll molecules can be much greater than that of an individual chlorophyll molecule. This can be illustrated by using CD strength as a rough measure of dissymmetry. The CD of Chl _a_ crystals is approximately five times greater than the CD of Chl _a_ dimers in carbon tetrachloride solution,[19] which is in turn approximately three times larger than the CD of Chl _a_ monomers.[38] Thus the dissymmetry of these chlorophyll aggregates, like that of Pasteur's spiral stair, could come mostly from the way the individual chlorophyll molecules are arranged.

The spectra of such aggregates will differ from that of the monomer in other ways also. In general, each of the monomer transitions will be split into a number of components which is equal to or



PROTOCHLOROPHYLL-A



CHLOROPHYLL-A

Fig. II-1. Molecular structures of PChl a (or PChlide a) and Chl a (or Chlide a) showing the positions of the asymmetric centers. For PChl a or Chl a, R = $C_{20}H_{39}$; for PChlide a or Chlide a, R = H (from Houssier, C. and Sauer, K.[38])

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less than the number of molecules in a unit cell* of the aggregates.[35]

The CD spectrum of an aggregate of identical units can be decomposed into a nondegenerate (single) component and a degenerate (multiple) component. The nondegenerate component arises from the intrinsic optical activity of the monomer. On the other hand, the degenerate component originates from the coupling of the transition oscillators in different molecules of the aggregate. The decomposition of the CD spectrum of an aggregate can be seen in Fig. II-5.

The spectral splitting, the strength of the absorption components, and the strength of the CD components (rotational strength) depend upon the geometrical arrangement of the transition moments of the molecules in the aggregate, i.e., upon the geometry of the aggregate. This dependence can be shown for a dimer using the equations of Tinoco,[93]

$$D_{\pm} = \mu^2 \pm \vec{\mu}_1 \cdot \vec{\mu}_2 \qquad \text{(II-1)}$$

$$R_{\pm} = \mp \pi \omega_o (\vec{R}_{12} \cdot \vec{\mu}_1 \times \vec{\mu}_2) \qquad \text{(II-2)}$$

$$\omega_{\pm} = \omega_o \pm V_{12}/hc \qquad \text{(II-3)}$$

$$V_{12} = \frac{1}{R_{12}^3}\left[\vec{\mu}_1 \cdot \vec{\mu}_2 - \frac{3(\vec{R}_{12}\cdot\vec{\mu}_1)(\vec{R}_{12}\cdot\vec{\mu}_2)}{R_{12}^2}\right] \qquad \text{(II-4)}$$

where $D_{\pm}$ are the dimer dipole strengths, $R_{\pm}$ are the dimer rotational

---

*The unit cell is defined as the smallest repeating unit making up the aggregate, i.e., the smallest unit containing translationally nonequivalent molecules.

strengths, $\vec{\mu}_1$ and $\vec{\mu}_2$ are the electric dipole transition moments of monomer 1 and monomer 2, ω_0 is the monomer transition frequency in cm^{-1}, $\omega_\pm$ the same for the two dimer components, $\vec{R}_{12}$ is a vector connecting the centers of the two molecules in the dimer, and V_{12} is the interaction energy resulting in the splitting.

2. CD of Mature Normal and Mutant Barley

Thus far, no artificial models have been made which are photosynthetically active. Turning now to natural systems which can evolve oxygen when provided with an oxidant, such as ferricyanide, and light, Fig. II-2 shows the CD and absorption spectra of chloroplast fragments prepared essentially by the method of Park and Pon[57] of normal barley, (Strain: Lyon), and a mutant of normal barley lacking Chl b, (Strain: Chlorina).[32,33] Considering the mutant first because it is simpler, lacking Chl b, we see that its CD at wavelengths longer than 600 nm is similar to that of Chl a in carbon tetrachloride (see Fig. IV-4) in the same wavelength region, except that the signs of the components are reversed. A dimer that is a mirror image of the Chl a-CCl_4 dimer might produce such a CD spectrum.[18,19] A larger aggregate with 2 molecules per unit cell arranged as a mirror image of the solution dimer might also. The interactions between chlorophyll-chlorophyll molecules produce CD whose number of components is typically equal to or less than the number of molecules in a unit cell of the aggregate.[35]

Chlorophyll-protein or chlorophyll-lipid interactions, or interactions between two different chlorophylls (such as a, b), on the other hand, produce only single CD bands and, if present, would only

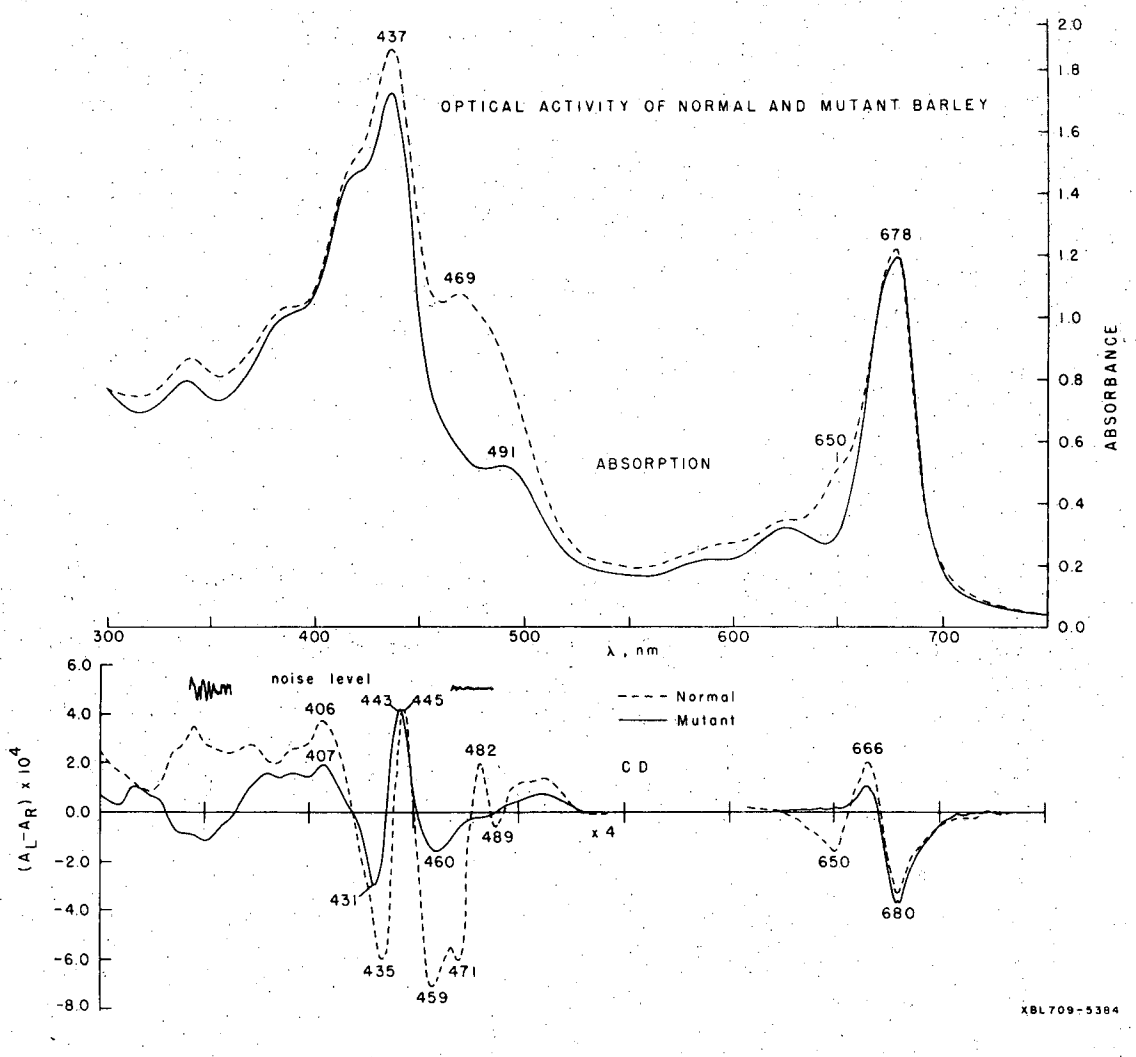

Fig. II-2. The optical activity of chloroplast fragments of normal barley (--------) and a barley mutant (————) which lacks Chl b. Optical pathlength, 1.00 cm. (I am indebted to E. Dratz for recording the CD spectra shown above.)

increase or decrease the asymmetry of the observed double CD components.[18,19] These are essentially solvent effects changing the magnitude and apparently sometimes the sign of the intrinsic optical activity of the monomer (nondegenerate component of the CD).

The sign of the nondegenerate CD component of solution dimers appears to be of opposite sign to that of the mutant barley chloroplast fragments.

There are many constituents in the chloroplast fragment systems: chlorophylls, carotenes, cytochromes and other electron transport molecules, lipids and proteins.[56,105] The chlorophylls are the only constituents absorbing significantly at wavelengths greater than 550 nm.

The spectra of normal barley compared to those of mutant barley clearly show the contributions of Chl b. From a comparison of the CD in the red, one can see that Chl b has first a positive, then a negative component, a feature which resembles that of Chl b dimers in carbon tetrachloride.[19] In the blue region of the spectrum, the difference between the normal and mutant CD is extremely complex. Monomeric chlorophylls have at least two electronic transitions, so close it is difficult to resolve them, in the blue.[38] This complicates interpretation of the Soret region of any chlorophyll spectra, especially those where each of the electronic transitions might be split. Making an approximate comparison between the two CD spectra, we find a small maximum at 500 nm, a small minimum at 489 nm, a maximum at 482 nm, a large minimum at 471 nm with about the same depth as the minimum in the red at 650 nm, a small maximum at 450 nm,

a large minimum at 435 nm, and a maximum at 406 nm. The first three features are interesting because carotenoids absorb more strongly in that region than Chl b, suggesting either that a particular type of optically active carotenoid is missing from the mutant or that the presence of Chl b induces optical activity in a carotenoid which is present in the mutant.

The CD of Chl b dimers in carbon tetrachloride has an approximately symmetric positive, negative degenerate CD component at red wavelengths.[18,19] At blue wavelengths another degenerate component, again approximately symmetric, is negative then positive and of about 4 times greater magnitude than the red component. Sauer found that different growth conditions can alter the ORD of spinach chloroplast fragments.[63] The normal and mutant barley were grown under the same conditions in a phytotron (growth chamber), but Highkin and Frenkel found that the mutant grew less rapidly than the normal barley.[33] I have also grown these plants outdoors. Under these conditions, sometimes the normal plant was taller and possessed more foliage than the mutant, but at other times the situation was reversed. The point is that the photosynthetic pigment systems of the mutant and normal barley might respond differently to the same environment, producing differences in the spectra not directly related to Chl b.

3. CD of Greening Leaves

In these experiments, the CD spectra were measured during the development of the chloroplasts. The chlorophyll concentrations in mature lamellae are greater than 0.1 M; the concentration at the beginning of greening is about two orders of magnitude

less than 0.1 M. The concentration remains about constant for approximately the first two hours of illumination. This so-called lag phase then ends, and chlorophyll synthesis becomes very rapid. CD spectra could be expected to reveal whether the concentration of chlorophyll was changing in the lamellae (or were the number of lamellae just increasing); and if associations between molecules are not present from the beginning, when they form and whether they change in character during the course of greening.

To reduce scattering to acceptable levels for the measurement of CD spectra, it was necessary to make homogenates of the leaves. The absorption changes of these homogenates, as greening proceeded, agreed with those of whole leaves.[28]

B. Experimental

Red kidney beans (Phaseolus vulgaris L.) were treated with the seed disinfectant and protectant Arasan (powder form; Dupont) at the rate of 0.35 gm per 500 gm of seeds. Vermiculite (plaster aggregate), soaked in tap water for at least a day (until it no longer floated) and drained shortly before, was placed in 7 cm x 20 cm x 45 cm wooden flats sterilized by autoclaving. Autoclaving the wooden flats seems to be the most important procedure to prevent the appearance of fungus while the beans are germinating. The seeds were scattered thickly (about 1/2 inch apart) on top of the vermiculite, then covered with about 1 cm more of firmly packed vermiculate. The flats were subsequently placed in a metal cabinet that was washed with methanol between uses. It was immediately closed, and the

cracks were made impervious to light, but not air, with black cloth and black masking tape. The flats remained in the sealed cabinet 9 ± 2 days.

After 9 ± 2 days in the dark, the plants were illuminated for different durations under fluorescent room lights (G.E. Warm White) with an intensity of 90 ± 10 fc. The two primary leaves (the first two leaves to form) were harvested toward the ends of the periods of illumination. To obtain the shortest illumination periods (those about 1 hour in duration), the flats of bean sprouts were removed from the dark, and their leaves picked as rapidly as possible, placing them in a beaker or on a wet paper towel exposed to the same illumination as the seedlings. As soon as the picking was completed, I immediately weighed the leaves, recorded their illumination time, then homogenized them. In one instance, after homogenization and straining, the extract was frozen at -15°C and stored for ten days. It was then thawed and the preparation completed. The leaves were homogenized for 30 sec in a Waring blender with 4 ml of 0.05 M (K) PO_4 (pH 8.0) buffer per gm of leaves. Everything after picking and weighing was done at ice temperature. Next, the homogenate was wrung through 8 layers of cheesecloth, and sonicated (Biosonik sonicator) for 10 sec at maximum power. The strained, sonicated homogenate was then centrifuged 10 min at 14,000 g in a #40 rotor with a Spinco Model L-2 ultracentrifuge, after which the supernatant was recentrifuged at 100,000 g for 30 min using the same equipment. The sediment was recovered and resuspended in about 1 ml of 0.05 M (K) PO_4 buffer (pH 8.0). Finally, the resuspended sediment was again

centrifuged at 10,000 g for 10 min to remove any undispersed sediment (see Fig. II-3).

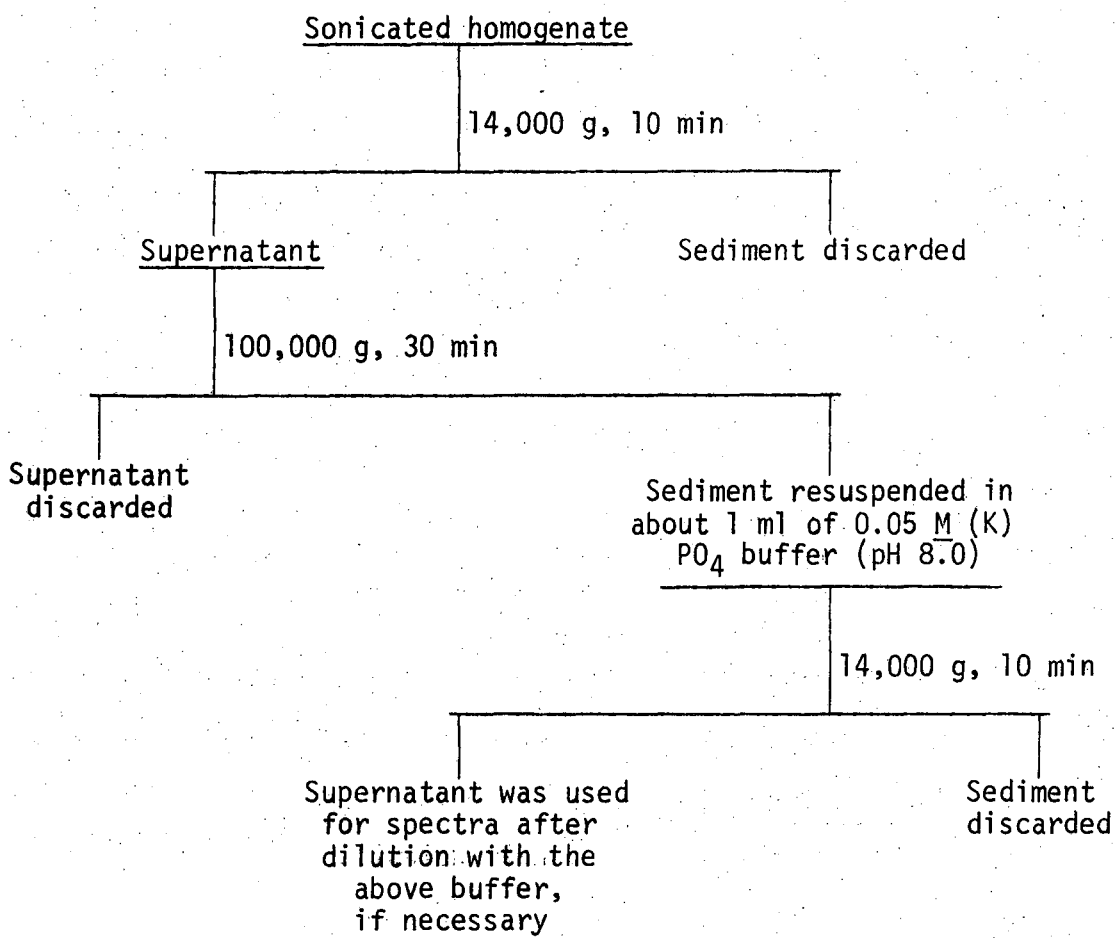

Fig. II-3. Flow scheme for the preparation of homogenates of greening bean leaves.

The spectra of these 14,000 g to 100,000 g fractions were recorded on a Cary Model 14 spectrophotometer and a Cary Model 60 polarimeter with a Cary 6001 circular dichroism accessory modified by the

installation of a red-sensitive phototube (R136, Hamamatsu TV Co., LTD and obtained through Cary Applied Physics Corp.). The calibration of the Cary CD instrument was checked with a 1 mg/ml solution of d-camphorsulfonic acid. The Cary 6040022 thermostatable sample cell (optical pathlength: 1.00 cm) was modified to fit the CD accessory. All CD spectra were recorded with the leaf homogenates at 0 to 5°C. The spectra have all been normalized to an absorbance (less turbidity) at the red maximum of 1.00. The actual absorbances (less the turbidities) ranged from 0.56 to 1.13. The absorbance less the turbidity was measured from a straight line drawn under the red maximum tangent to the absorption spectrum.

C. Results

In order to simplify Fig. II-4, only the spectra from 550 nm to 720 nm of the leaves homogenated after receiving 1, 2, 3 and 73 hr of illumination have been shown. Spectra at 5/6, 4, and 13 hr were also recorded. They will be considered in the text. In addition, spectra of all the samples were recorded at wavelengths less than 550 nm, where the spectra of the 13 and 73 hr samples closely resembled that of mature normal barley chloroplast fragments (see Fig. II-2). The other leaf homogenates (5/6, 1, 2, 3, 4 hr) had to be diluted to reduce their total absorbance to a level which would permit spectra to be recorded in this wavelength region. It was felt that this decreased signal/noise too much to make the spectra of the 5/6, 1, 2, 3, 4, hr leaf homogenates meaningful at wavelengths less than 550 nm.

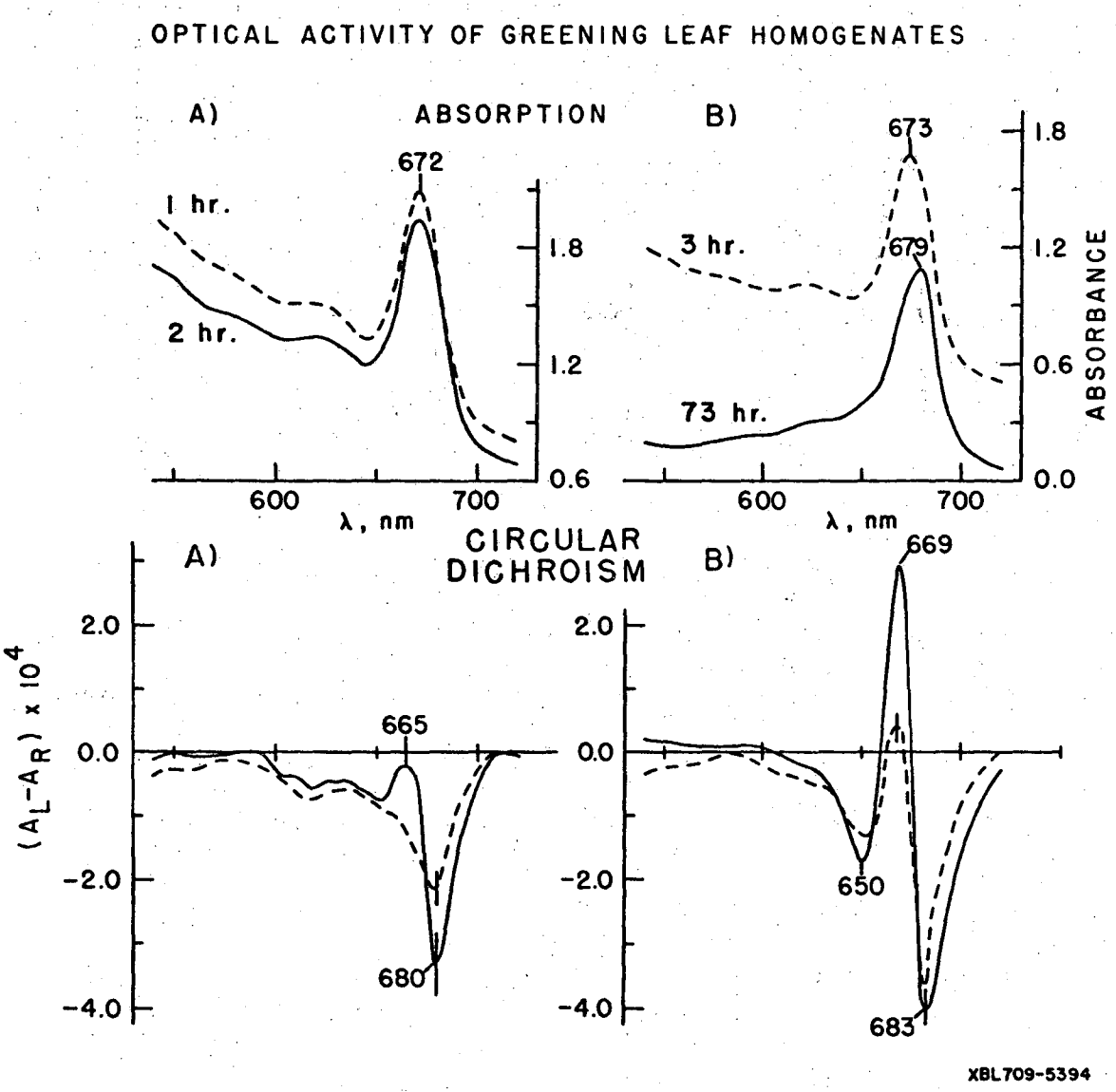

Fig. II-4. The optical activity of homogenates of greening bean leaves. Optical path: 1.00 cm. A) 1 hr. of illumination of the leaves: (-----); 2 hrs. of illumination: (———). B) 3 hrs. of illumination: (-------); 73 hrs. of illumination: (———). Straight vertical lines on CD spectra show approximate noise levels of the CD spectra.

The spectra of leaves which received only 5/6 or 1 hr of illumination (Fig. II-4) apparently had only single negative CD bands centered at about 678 nm. However, the absorption bands were at 671 and 672 nm. When the crude homogenate from leaves illuminated 1 hr was frozen and thawed, the red CD component shifted 10 nm toward the blue to 668 nm, while the absorption band shifted only 1 or 2 nm to 670 nm. At the completion of a second hour of illumination the CD spectrum had undergone changes which continued to develop for another 71 hours. In particular, the minimum in the CD originally present and now at 680 nm had deepened, a maximum had begun to form at 665 nm and a minimum may have begun to emerge at 650 nm (Fig. II-4). The absorption spectrum had changed very little. The position of the maximum was at 671 nm.

One should remember that in all cases the leaves were homogenized immediately after the specified illumination.

After 3 hours in the light, a second minimum had clearly developed in the CD at 650 nm, the maximum had strengthened and was now located at 668 nm, and the first minimum had deepened only slightly (less than 10%) and was now at 682 nm. The absorption peak had begun a red shift, now being located at 673 nm. The CD of the homogenate prepared from leaves illuminated for 4 hours was very similar to that of the 3 hour sample with the exception that the maximum at 668 nm had again increased. The shift of the absorption maximum toward lower energy continued; it was now at 675 nm. After 13 hours one finds that the first 3 bands have continued to grow. The absorption maximum is now at 678 nm. Finally, the CD of a sample obtained

from leaves illuminated for 73 hours (Fig. II-4) is very similar to that of mature barley chloroplast fragments containing the normal amounts of Chl a and Chl b (Fig. II-2). The absorption spectra are likewise nearly the same.

In summary, where there was originally only a single CD band, at least three form at approximately 680 nm, 670 nm, and 650 nm after 2 hours of illumination. These three all increase without any discontinuities as the plant continues to green. Although they increase at different rates, the 680 nm band approaching its greatest magnitude most rapidly and the 670 nm band band least rapidly.

D. Discussion

1. Monomeric Chlorophyll a in Leaf Homogenates

The earliest CD spectra (see Fig. II-4: 5/6 hour, 1 hour) are very similar to those of monomeric Chl a.[38] However, the magnitude of the least energetic Chl a CD band is three or four times greater than it is in organic solvents. This is probably due to an enhancement of the intrinsic chlorophyll optical activity by the electrostatic field from the surrounding molecules, e.g., protein.[91,92] There is also a greater disparity between the position of the CD and absorption peak than in organic solvents. That is, the CD minimum and the absorption maximum are respectively at: 657 nm and 661 nm in ether, 662 nm and 666 nm in carbon tetrachloride with 0.5% ethanol, and 678 nm and 671 to 672 nm in the homogenate. The 0.5% ethanol

breaks up dimers. Freezing and thawing shifts the absorption to 670 nm and the CD to 668 nm, as well as increasing the magnitude of the CD slightly (about 25%).

These CD spectra have no major degenerate components in the red bands, indicating clearly that this chlorophyll is predominantly monomeric, i.e., the chlorophylls are not close enough to interact. This agrees with the work of Butler,[15,16] who found that for the first 1.5 hours of illumination, the chlorophyll appeared as a single symmetrical absorption band at 670 nm in whole leaves. The larger CD magnitude can be explained as an electrostatic interaction of the chlorophyll with its carrier protein.[91,92]

The disparity between the CD minima and the absorption maxima of the 5/6 and 1 hour samples is not easy to explain. These spectra may contain a very small degerate component such as one can clearly see in the two hour sample. Freezing and thawing breaks these aggregates up. Butler found, using a 2.5 hour illuminated leaf, that freezing and thawing destroyed the asymmetry of the Chl a absorption band.[15,16]

Alternately, the CD could be made up of two different types of monomers (monomers in two different environments, e.g., one closely bound to a protein and one loosely bound). The one absorbing at a longer wavelength, about 680 nm, but having a greater interaction with its carrier molecule and hence a greater rotational strength. Freezing and thawing would have to alter the protein-chlorophyll bonds to make both environments the same. Pursuing this idea further, the 680 monomer might continue to exist through maturity.

However, it will be seen later that as the absorption maximum of CH shifts, the CD minimum, which is quite similar to the one being discussed here, shifts in like manner. Thus, one would be forced to postulate that the absorption of the 680 monomer shifts in the same way as the absorption of the shorter wavelength monomer, a rather unlikely postulate.

In either case, the earliest CD spectra are predominantly monomeric in character.

Phytylyzation is completed within 1 hour after the beginning of illumination.[76,100,104] In addition, the prolamellar bodies in etioplasts (chloroplast precursor) should have changed their form by this time;[44,108] in these homogenates prepared after 5/6 and 1 hour of illumination, it has probably been fragmented. Primary layers of the lamellae probably are present.[44,107] Smith[79] first detected oxygen in dark grown barley leaves after a 30 min exposure to light. Oxygen evolution at this early stage in greening suggests that little if any aggregation of the chlorophyll is necessary for this portion of photosynthesis to begin. However, this may not be photosynthetic oxygen evolution.[4,13] For example, Anderson and Boardman[4] found that 3-(p-chlorophenyl)-1,1-dimethyl urea (CMU) at a concentration sufficient to cause 100% inhibition of ferricyanide reduction by mature chloroplasts had no effect on ferricyanide reduction by plastids isolated from etiolated bean leaves which had been illuminated for 6 hours. As the plastid developed, the level of inhibition increased.

2. Formation of Chlorophyll a Aggregates and the Appearance of Chlorophyll b

At the end of two hours of illumination, one can see that the CD is acquiring a double component, possibly indicating the formation of an aggregate of Chl a.

Even after only two hours of illumination, Chl b is probably evident in the CD spectra at 650 nm (Fig. II-4). After three hours its optical activity can be clearly seen.

Butler's spectra again agree with the CD. He found that after illumination for two hours the main Chl a band of whole bean leaves had become asymmetric due to the formation of two bands at 670 nm and 680 nm at -196°C. (At room temperature, they are nearer 673 nm and 683 nm.) Freezing and thawing destroyed the asymmetry of the band. In addition, the first indication of Chl b and of light-induced fluorescence yield changes appeared at this time.[15,16] The fluorescence yield decreased slightly in response to actinic illumination. This was taken as an indication that the captured light energy was being utilized for photosynthesis. It has also been shown by fluorescence excitation measurements that energy transfer from carotene to Chl a in greening bean leaves begins between one hour and two hours after the illumination begins.[14,28] However, Losev and Gurinovich[51] could not find any indication of energy transfer from carotene to Chl a in the fluorescence excitation spectra of homogenates of greening etiolated barley leaves until 4 to 6 hours after the initiation of illumination. Losev and Gurinovich[51] did find energy transfer from Chl b to Chl a after only 2 hours of illumination.

The depolarization of fluorescence from homogenates increases as the leaves from which they are prepared accumulate chlorophyll.[30,51] The increase in depolarization of fluorescence in this case probably indicates a migration of energy between pigment molecules. Hence, the results from CD, i.e., that chlorophyll aggregates are forming, do not conflict with the increase in the depolarization of fluorescence.

The molecular ultrastructures responsible for the three CD components of mature leaf samples apparently appear within three hours after the initiation of the greening process.

3. Possible Chlorophyll Aggregates

The changes in the absorption maxima positions and the magnitude of the CD bands observed in the spectra could be caused by the formation of dimers. Such dimers, however, would probably have to be able to transfer energy between themselves, because there are 225 chlorophyll molecules present in mature chloroplast lamellae for each cytochrome f and cytochrome b_6 molecule (the photosynthetic cytochromes).[105]

Such changes could also be caused by the formation of a polymer of chlorophyll. It could be a 1, 2 or 3 dimensional polymer, although a 2 dimensional system would conform most closely with the geometry of the lamellae and also the position of the absorption maximum. Chlorophyll a monolayers absorb at approximately 680 nm in the red.[5] The intensity of the CD effects is consistent with either a 1 or 2 dimensional array geometry. Actually, as the normal and mutant barley CD demonstrated, there must be at least 2 geometries,

one for Chl *a* and one for Chl *b*. The red shift of the chlorophyll absorption maximum from 670 to 678 nm might be due to a change in environment, because the red maximum of chlorophyll solutions is dependent upon the solvent.[61,69] This bathochromism would also depend upon the nature of the polymer,[35] but again this is essentially a solvent effect.

For linear arrays most of the calculated interaction for the molecular exciton model comes from the first 8 to 10 molecules on either side of the origin.[35] In lamellae, the calculations of Hochstrasser and Kasha showed that 90% of the dipole-dipole interaction comes from molecules within a radius of 35 molecular units.[35] They say that, in general, departure from this idealized planar region would be expected to produce quantitative, but not qualitative, changes in exciton band structure. In other words, lamellae with a radius of less than 35 molecular units would have the same number CD bands, but their positions and magnitudes would be different.

The dipole-dipole sums of bulk aggregates converge slowly.[35] Crystal size and shape may have an influence on exciton band structure, at least partially caused by the slow convergence.[35] The spectra of a bulk array would probably continue to change, until its growth were completed.

In any case, the presence of two components (bands) each for Chl *a* and Chl *b*, as shown in the barley CD, means that when the molecular exciton model is applied there are at least two molecules per unit cell.[35] Each unit cell might contain a "dimer". This then would be the basic unit of the array.

Low temperature derivative spectroscopy has shown that the low energy electronic transition of green plants to actually be a sum of bands positioned at about 670 nm and 680 nm.[16]

Such multiple bands have been assigned to Chl a in different molecular ultrastructures.[16,29] Thus, one type of array of Chl a absorbed at 670 nm and another absorbed at 680 nm. However, a plausible alternative explanation is that the two bands arise from an electronic transition split by the interactions between the Chl a molecules of a single array. The crossing point of the degenerate component of the barley mutant would be crucial for deciding between these two proposals. Previously, we reported a value of 685 nm for the crossing point, which implies that the 670 nm band does not contribute to the degenerate CD.[19] However, subsequent measurements failed to confirm that finding. The value I obtain for the crossover point of the degenerate component of the CD spectrum of Chlorina 2 chloroplast fragments in Fig. II-2 is 674 nm, which is consistent with the alternate explanation that the two bands originate from a single array of Chl a. This point might vary depending upon the growth conditions of the plant, etc. Nevertheless, a variation of 11 nm appears unlikely.

The crossing point was calculated by subtraction of a nondegenerate component having the shape and position of the absorption. The magnitude of this component was adjusted until the maximum and minimum of the degenerate component were of equal absolute height (see Fig. II-5).[18,19]

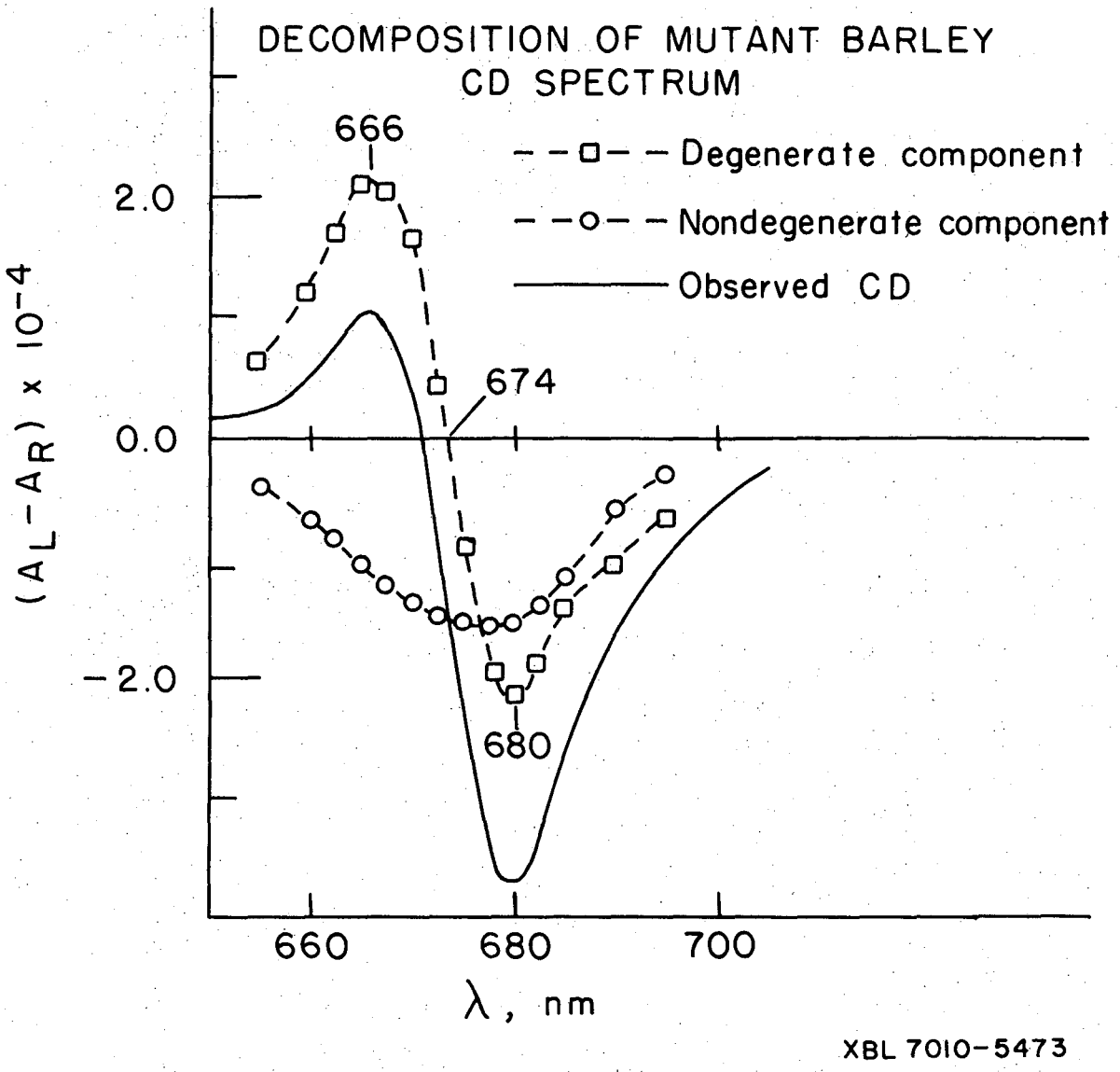

Fig. II-5. Decomposition of the CD spectrum of mutant barley chloroplast fragments into a degenerate (double) component and a nondegenerate (single) component. Optical pathlength, 1.00 cm.

4. Correlation with Microscopic Observations

The evolution of the spectra for the postetiolated state to those of the mature one prevents any strong correlations with the microscopic observations. In addition, the ultrastructural changes apparently depend upon light and growth conditions, the species, and the type of fixation.[13,44,107,108] During the first five hours of illumination, the membranous material of the prolamellar body is largely dispersed.[13,44] The development of the primary layers has been reported to begin before or at about the time that illumination starts.[44,107] Hence it probably precedes the appearance of the three CD bands. Short overlaps of the primary layers have also been reported in etiolated bean leaves.[107]

After 10 hours of illumination, there are stacks of lamellar membranes (grana) 3 or 4 layers thick.[44] Even at this time, however, the remains of a prolamellar body may still persist.[44,108] At 20 hours, the stacks have grown to 3 to 8 layers. Mature chloroplasts have 2 to 100 layers (thylacoids) per granum.[44]

The most rapid spectral changes occur between the first and fourth hour of illumination. After 13 hours they are almost complete. However, because of the variation between the ultrastructural observations of various authors,[13,44,107,108] it is difficult to make any correlations between the spectral and ultrastructural changes during greening at this time.

5. Possible Scattering Effects on the CD Spectra

The one indication that there may be distortion of the CD spectra by light scattering is the changes that occurred upon freezing and thawing a homogenate prepared from leaves which had been illuminated

1 hour. This brought about a blue shift of the lowest energy CD band, as well as its intensification. Because the transforming activity of PCH is destroyed or substantially reduced by freezing and thawing unless it is protected by glycerol or sucrose,[17,28,84] the most likely explanation is that the binding of the pigment to its carrier molecule has been altered. Repeated attempts to produce alterations in the CD spectra of samples such as chloroplast fragments and d-camphorsulfonic acid by increasing the scattering of the solution through the addition of substances such as albumin (in the visible region only), coffeemate, or talcum have failed to do anything, except increase the noise level of the spectra. The scattering was increased by an optical density of about 1.0 at the wavelength that the CD was measured. In addition, the 5/6 hour and 1 hour samples have virtually the same CD even though the turbidity of the former is 30% greater than that of the latter.

These results are in agreement with the report of Lenard and Singer[49] that particular ORD features of bovine serum albumin were not affected by the presence of membrane preparations in the sample and the report of Gratzer and McPhie[26] that ORD measurements of DNA samples were not affected by the presence of colloidal sulfur suspensions in the light path.

The effects of scattering upon optical activity have received considerable attention because the negative CD band in the ultraviolet of various membrane systems is red shifted with respect to that associated with the $n\text{-}\pi^*$ transition of alpha-helical polyamino acids. The red shift has been attributed to changes in the membrane

protein structure by some authors,[31,94] and to scattering effects by others.[40,96-99]

Urry et al. found that the magnitude of the CD bands decreased as the turbidity of the sample increased. In some instances they also found a red shift of the extrema of a band. Their systems differed from mine in a number of respects. First, the scattering of their solutions was mainly from the chromophore (protein) whose optical activity they were measuring. This is important, for an optically active chromophore will differentially scatter left and right circularly polarized light.[98] On the other hand, most of the scattering, so evident in my earliest samples, was absent in holochrome preparations (see Chapter IV). Thus, the scattering was for the most part not from chlorophyll-containing particles. Second, their measurements were in the ultraviolet (and the blue for the Soret bands of heme), whereas mine were in the visible, where the scattering is less and increases less rapidly. Apparently in their samples most like mine, the CD distortions were least. These were mitochondrial membrane fragments which sedimented at the same g force as my bean leaf homogenates.

More recently, Ottaway and Wetlaufer[55] have concluded that distortions resulting from light scattering are very small. Their analysis was based upon an extension of the classical treatment of Rayleigh scattering to include the asymmetric interaction of an optically active chromophore with the incident light. In particular, they found that the expected scattering contribution for particles with a radius of a micron is at most only about 10^{-3} of the true ellipticity. However, the ellipticity they used in calculating

this example may be much greater than the ellipticity of bean leaf homogenates.

6. Conclusions

The optical activity of greening leaves points to the formation of at least an aggregate of Chl *a* and an aggregate of Chl *b* very early in greening. Once formed, although they may be extended, the nature of their unit structure changes little, if at all.

III. THE PREPARATION OF PROTOCHLOROPHYLLIDE AND CHLOROPHYLLIDE HOLOCHROME

A. Introduction

An etiolated leaf contains protochlorophyllide a (PChlide a), which is photoreduced at C-7 and C-8 to form chlorophyllide a (Chlide a) when the leaf is illuminated (see Fig. II-1). This probably is the normal pathway for the biosynthesis of Chlide a in angiosperms, because PChlide can be detected in normal light grown plants. In addition, such a plant will immediately begin to accumulate PChlide when it is placed in darkness.[50,101]

PChlide a was first extracted in a form capable of being photoreduced to make Chlide a from etiolated leaves by Krasnovsky and Kosobutskaya,[48] and by Smith.[78] The former extracted the etiolated leaves with a phosphate buffer of pH 7; the latter with glycerol. Later Smith used a 0.5 M glycine buffer at pH 9.6 with 30% glycerol in a more extensive purification of "protochlorophyll holochrome", as he called the substance. It was subsequently shown to contain only the nonesterified form of protochlorophyll, i.e., protochlorophyllide (magnesium vinyl pheoporphyrin).[81] Smith's purification employed mainly ammonium sulfate fractionation. In some cases sucrose gradient centrifugation was used as a final step in the purification.[80,85] Boardman used essentially the same purification scheme.[6]

Boardman[6] calculated a molecular weight for the protochlorophyllide holochrome (PCH) of approximately 600,000 from ultracentrifugation studies. Smith's value by similar means was 700,000.[85] On the basis of one protochlorophyllide chromophore per protein, Boardman found a minimum chemical molecular weight of 1.0×10^6 to 1.7×10^6. Smith and Comber[85] reported a chemical molecular weight of the order of 1.0×10^6. Both calculations were apparently based on a spectroscopic determination of PChlide a content. The extinction coefficient used was measured by Koski and Smith.[46]

However, there seems to be some question now whether Koski and Smith measured the specific absorption coefficient of PChl a or the specific absorption coefficient of PChlide a. Koski and Smith's pigment extracted with aqueous acetone from 9 day old etiolated barley leaves was purified by repeated adsorption on sugar from petroleum ether, then adsorption from benzene and precipitation from benzene-petroleum ether. They reported that the pigment contained 2.73% magnesium, which agrees with the theoretical percentage calculated from the formula proposed for PChl a by Fischer and Oestreicher.[21] PChlide a would be expected to contain 4.47% magnesium. They gave no direct evidence for the presence of phytol. Eight day old etiolated barley (Hordeum vulgaris) leaves have been found to contain 87.5% PChlide a and 12.5% PChlide a ester.[66] Fischer and Rudiger[20] were unable to isolate phytol from PChlide a ester obtained from etiolated barley. They also reported that the seed coats of pumpkins (Curbita pepo) contained PChl a (PChlide a esterified to phytol). However, Godnev, Kaler and Rotfarb[23] reported they were able to detect phytol

after alkaline hydrolysis of PChl(ide) from etiolated barley.

Houssier and Sauer[36] isolated 4-vinyl PChl *a* (Mg-2,4-divinyl-pheoporphyrin a_5 esterified with phytol) from the seed coats of pumpkin (*Curbita pepo*) by polyethylene and sugar chromatography. The purified pigment showed no trace of colored impurity upon thin-layer chromatography. The presence of the phytol group was confirmed by silica-gel thin-layer chromatography after hydrolysis of the pigment with methanol-KOH, and also by the infrared spectrum of the pigment. Houssier and Sauer[37] obtained a molar extinction coefficient for this pigment at its red maximum (622 nm) of $\varepsilon_{622} = 22.1 \times 10^3 \frac{\ell}{\text{mole-cm}}$ in ether. Now the presence of the vinyl group on C-4 would not be expected to appreciably affect the transition at 622 nm. Thus the molar extinction coefficient of PChl *a* in ether should also be about $22 \times 10^3 \frac{\ell}{\text{mole-cm}}$. Koski and Smith determined the specific absorption coefficient of their pigment in ether to be: $\alpha_{623} = 36.9 \frac{\ell}{\text{gm-cm}}$. Thus, the molar extinction coefficient, assuming they isolated PChl *a*, would be $\varepsilon_{623} = 36.9 \times 891.5 \frac{\ell}{\text{mole-cm}} = 32.9 \times 10^3 \frac{\ell}{\text{mole-cm}}$. If, however, one assumes they isolated PChlide *a*, $\varepsilon_{623} = 36.9 \times 597 \frac{\ell}{\text{mole-cm}} = 22.0 \times 10^3 \frac{\ell}{\text{mole-cm}}$.

I shall assume that the molar extinction coefficients of 4-vinyl PChl *a*, PChl *a*, and PChlide *a* in ether at the red maximum are all $22 \times 10^3 \frac{\ell}{\text{mole-cm}}$.

In this instance, correcting for this change, one obtains 0.67×10^6 to 1.1×10^6 as Boardman's chemical molecular weight per mole of protochlorophyllide and 0.67×10^6 for Smith's. The chemical molecular weight assumes one PChlide *a* chromophore per protein carrier molecule.

Using polyethylene glycol-6000 precipitation and hydroxylapatite, DEAE-cellulose, and agarose chromatography, Schopfer and Siegelman[67] were able to obtain a purer PCH solution than either Smith or Boardman. From its elution on a calibrated agarose column, Schopfer and Siegelman[67] concluded that it consisted of two PChlide-containing species of molecular weight 300,000 and 550,000. After polyacrylamide gel electrophoresis of the PCH, they found a single diffuse band which fluoresced weakly in the red under ultraviolet light. Electrophoresis of PCH in the presence of sodium dodecylsulfate, an ionic detergent, revealed one strong sharp band and occasionally two weakly detectable slower moving bands. They concluded from these results that PCH was an aggregating-disaggregating system, which was apparently disaggregated by sodium dodecylsulfate. They reported 0.24 gm of protein per μmole of PChlide a. However, they used a millimolar absorptivity in 80% aqueous acetone of 31.1 at 626 nm, apparently based again upon the results of Koski and Smith.[46] Using a millimolar absorptivity in 80% aqueous acetone of $\varepsilon_{627} = 20.5$ (see Table VI-1, and Chapter VI-B) for PChlide a, one calculates 0.16 gm protein per mole of protochlorophyllide. Therefore, each 300,000 unit contains at least one protochlorophyllide molecule and, most probably, two; each aggregate of 550,000 molecular weight contains at least three and, most probably, four molecules of protochlorophyllide. The higher number of chromophores is more probable, because any remaining non-holochrome proteins would increase the weight of protein per μmole of chromophore, but not change the 300,000 and 550,000 molecular weights.

Schopfer and Siegelman[67] could not detect any carotenoids spectroscopically after purification. They were evident in both Smith's[85] and Boardman's[6] preparations. In fact, Schopfer and Siegelman[67] found no spectroscopic evidence for any visible pigment other than PChlide a in their purified PCH preparations.

Changing the originally reported values to conform to the extinction coefficient used here for PChl(ide) a, Smith's PCH preparations photoconverted about 33% of the total PChlide a present to Chlide a, Boardman's 33 to 40%, and Schopfer and Siegelman's, approximately 50%. Thus, Schopfer and Siegelman's PCH preparations were both the purest and the most active of any reported to date. Unfortunately, the procedure is difficult, and the yield is low, about 4%.

For spectroscopic studies, I needed a high concentration of protochlorophyllide with high activity in a solution of low turbidity, obtained in a rapid, simple manner. An absolutely pure preparation was not essential for my purposes.

B. Experimental

My methods of preparing protochlorophyllide holochrome were essentially modifications of the method given by Schopfer and Siegelman.[67,73]

Five hundred gm of red kidney beans (Phaseolus vulgaris L.) obtained at a local market was treated with 0.35 gm of the seed disinfectant and protectant, Arasan [powder form, bis(dimethylthiocarbamoyl)

disulfide] made by duPont and obtained from Asgrow Seed Co.[73] Wooden flats were sterilized by autoclaving, then filled with water-saturated vermiculite. The beans were planted in the vermiculite, then the flats were placed in a light-tight cabinet. Germination was approximately 72%. After growing in the dark for 9 days, the primary leaves of the bean sprouts were harvested. Harvesting and all subsequent isolation procedures were done with minimum exposure to a green safelight. The safelight was made from a 10 W green bulb covered with a green plastic filter selected for maximal transmission at the green minimum of protochlorophyllide absorption. A typical yield was 60 gm of leaves from 12 flats. These were homogenized with a Virtis homogenizer for 4 min with 300 ml of extraction buffer [0.1 M tris-Cl, 0.5 M sucrose, 25% (v/v) glycerol, 0.06% (v/v) Triton x-100 (alkyl phenoxy polyethoxy ethanol) obtained from Rohm and Haas Co., and 34 gm Polyclar AT (polyvinylpyrrolidone) obtained from General Aniline and Film Corp.; pH 8.0].[67,73] All buffers mentioned in this chapter had a pH of 8.0. The homogenizing vessel was surrounded with a mixture of chipped ice and dry ice to maintain its contents at about 0°C.

The homogenate subsequently was vacuum filtered through a milk filter (Rapid-Flo; Johnson and Johnson), 13 cm in diameter. The filtrate was then centrifuged at 78,000 g for one hour at 0°C in a Spinco L_2-65B ultracentrifuge (#30 rotor), (Fig. III-1). The supernatant was frozen at -15°C until the preparation was completed. This frozen crude extract was very stable.

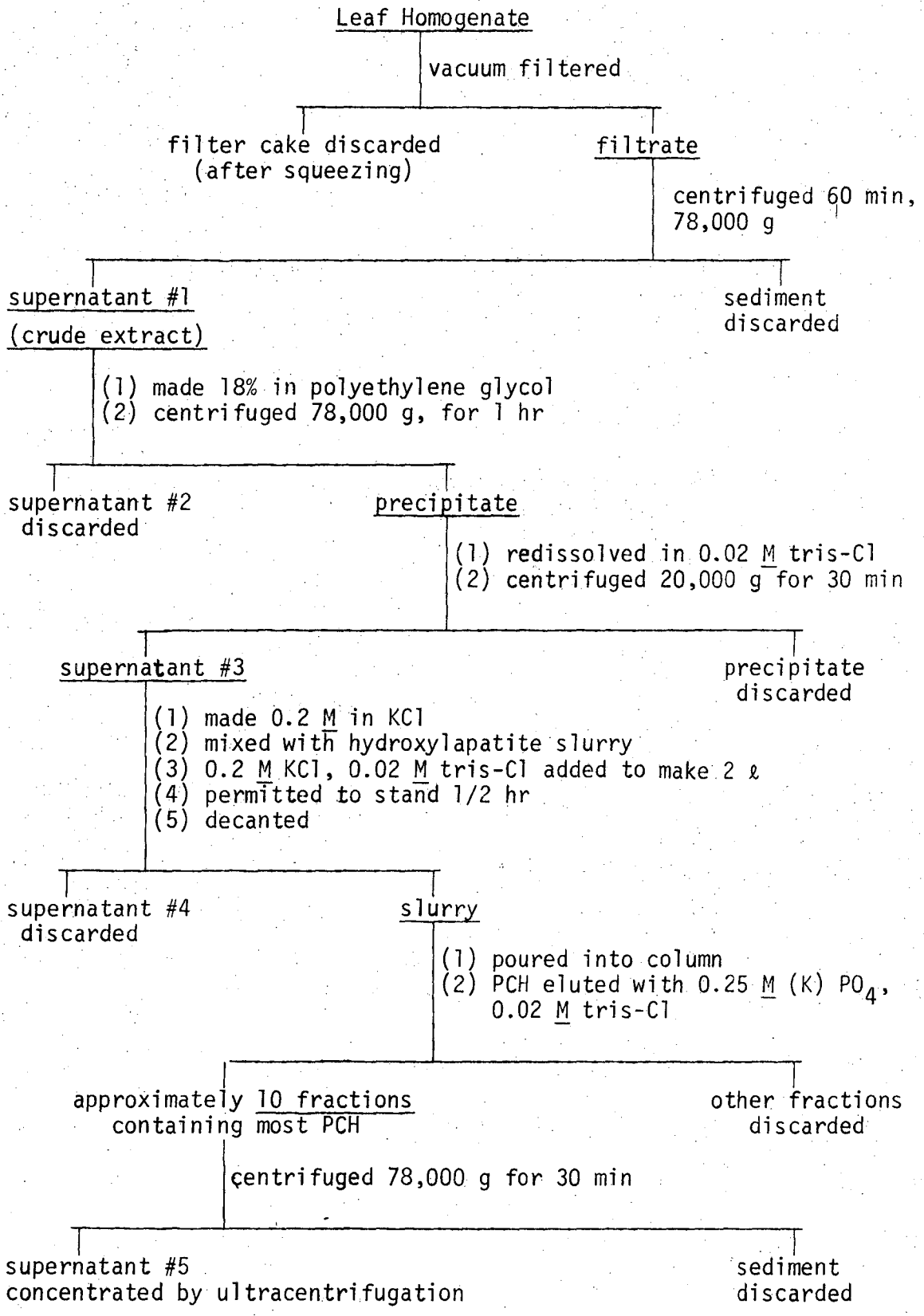

Fig. III-1. Flow chart for the preparation of PCH(suc)

Later the crude extract was thawed and enough 50% (w/w) polyethylene glycol (Polyethylene Glycol 6000, J. T. Baker Chemical Co.; dissolved in 0.02 M tris-Cl) added to make the extract 18% (w/w) polyethylene glycol. After five min of stirring, the solution stood for 30 min and was then centrifuged one hour at 78,000 g as above. The precipitate was redissolved in cold 0.02 M tris-Cl buffer. The redissolved precipitate was again centrifuged, this time at 20,000 g for 30 min, to clarify it. The precipitate of this centrifugation was discarded. Its supernatant was made 0.2 M in KCl, then stirred into 500 ml of settled hydroxylapatite suspension in 0.2 M KCl, 0.02 M tris-Cl. The hydroxylapatite was made by the method of Siegelman et al.[74] It was washed free of fines shortly before use. The slurry with PCH adsorbed was washed by decantation with about 1.2 ℓ of the above buffer. After the washing, it was poured into a 7 cm (d) x 16 cm (ℓ) column. Then the adsorbed protochlorophyllide holochrome was eluted with 0.25 M (K) PO_4, 0.02 M tris-Cl (Fig. III-2). The flow rate was maintained at about 20 ml/min with a Buchler Polystaltic pump, and 17 ml fractions were collected using a Gilson drop counter and fraction collector. Most of the holochrome was contained in about 10 fractions.

These were pooled, centrifuged at 78,000 g for 30 min, and concentrated more than tenfold in 12 hours by vacuum ultrafiltration against powdered sugar (Fig. III-3).[73] The powdered sugar speeds the concentration and preserves the PCH solution by dissolving in it.

The yields of this preparation are given in Table III-1. They are typical for this preparation method. The PCH(suc) solutions can

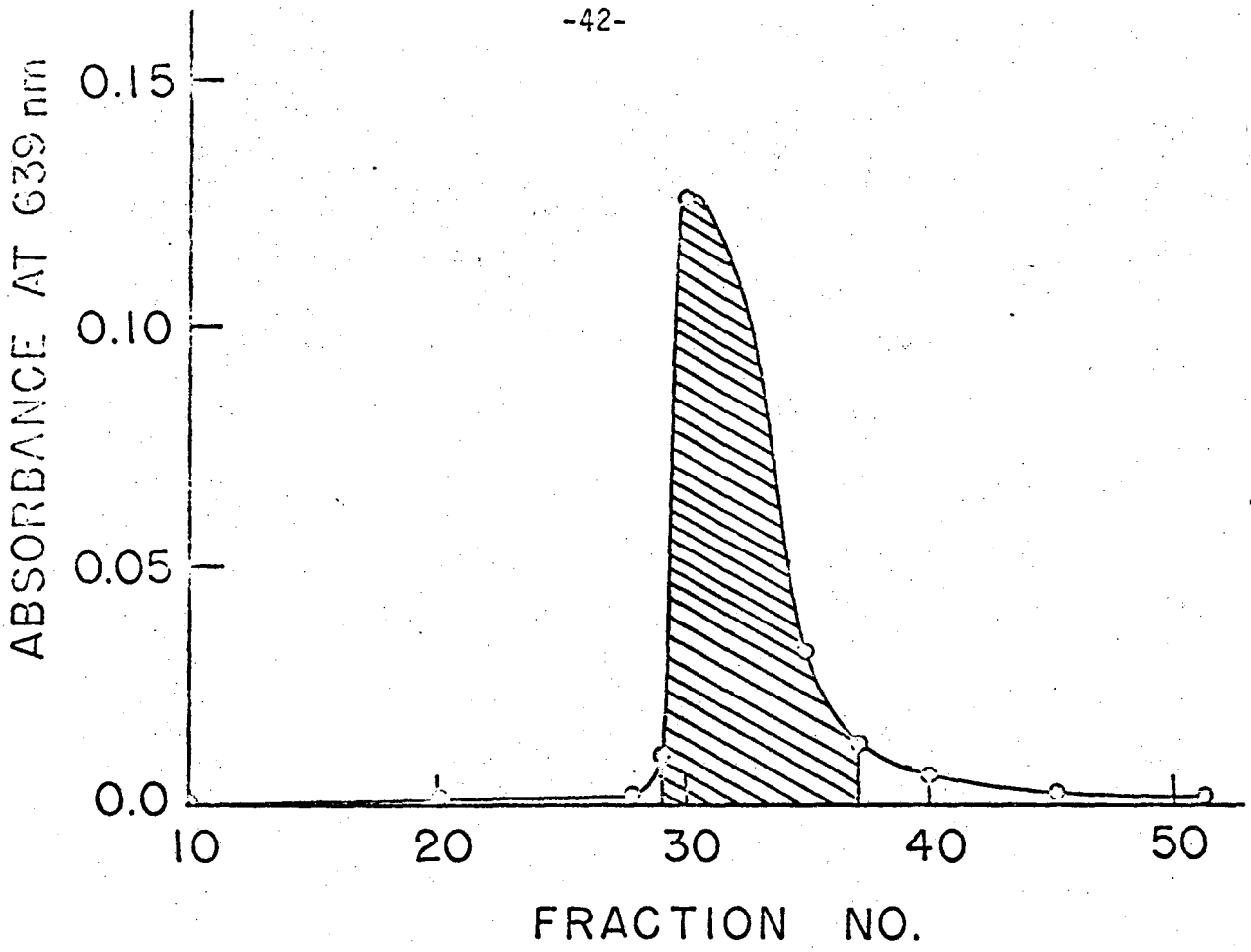

Fig. III-2. Elution of PCH from a hydroxylapatite column. The portion retained is cross-hatched.

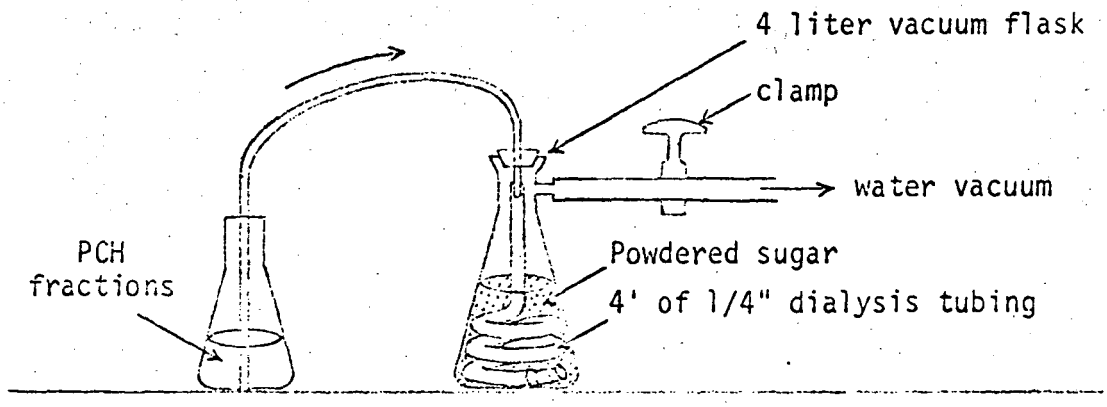

Fig. III-3. Vacuum ultrafiltration apparatus.

Table III-1. Purification of PCH(suc). All optical paths were 1.00 cm. #PCH units = (A-T)·(volume)

Material	A-T (Absorbance -turbidity)	$\frac{T}{A-T}$ (Clarification)	PCH units	Yield (%)	Phototrans-formability (%)
Crude extract	0.175	1.34	29.8	100	
Frozen-thawed crude extract	0.160	1.75	27.2	91	
Supernatant #3 (20,000 g)	0.170	1.29	20.2	68	
Hydroxyapatite eluate retained	0.073	0.59	11	37	
Supernatant #5 (78,000 g)	0.073	0.30	11	37	
Concentrate after ultra-filtration	1.05	0.305	7-8	25	51

be stored frozen at -15°C for at least a month with no loss of activity or concentration. Note that this cannot be done with CH(suc), i.e., once phototransformation has taken place. In general, the chlorophyllide form of the holochrome is less stable than the protochlorophyllide form.

PCH solutions free of the concentrated sucrose found in the above solution [PCH(suc)] were prepared (Fig. III-4), as I have reported above with the following differences. The extraction buffers contained 1.0 M sucrose instead of 0.5 M sucrose. This was simply an attempt to increase the phototransformability of PCH. It did not. To save time and increase the final yield, the polyethylene glycol precipitation was deleted. The hydroxylapatite column was 10 cm (d) by 7 cm (ℓ), in order to facilitate the flow. The absorbance of the eluate at 254 nm was measured with an Isco Model 222 ultraviolet analyzer. The absorbance at 254 nm was a good indicator of the PCH content in this instance. The fractions containing PCH were centrifuged at 361,300 g with a Spinco L_2-65B ultracentrifuge, #60 Ti rotor, for 240 min at 1°C. After standing covered with a thin layer of 0.02 M tris-Cl for several hours, the sediment was very gently resuspended in it, then centrifuged at 20,000 g with a Spinco L_2 ultracentrifuge (#30 rotor) for 30 min at 0°C. The supernatant was used immediately for measurements; the sediment was discarded. Typical yields for this type of preparation are given in Table III-2.

Chlorophyllide a holochrome [CH or CH(suc)] was produced by exposing protochlorophyllide a holochrome [PCH or PCH(suc)] in a

cuvette immersed in a 150 ml beaker of water-ice mixture to illumination from a 150 W flood lamp about 12 cm away for 5 to 10 sec. Since the photoreduction of PChlide to Chlide was usually about 50%, what is called CH or CH(suc) actually contains both Chlide and PChlide.

These preparation procedures produce PCH [or PCH(suc)] solutions of high activity, high concentration, and low turbidity. They are also relatively simple and rapid to prepare.

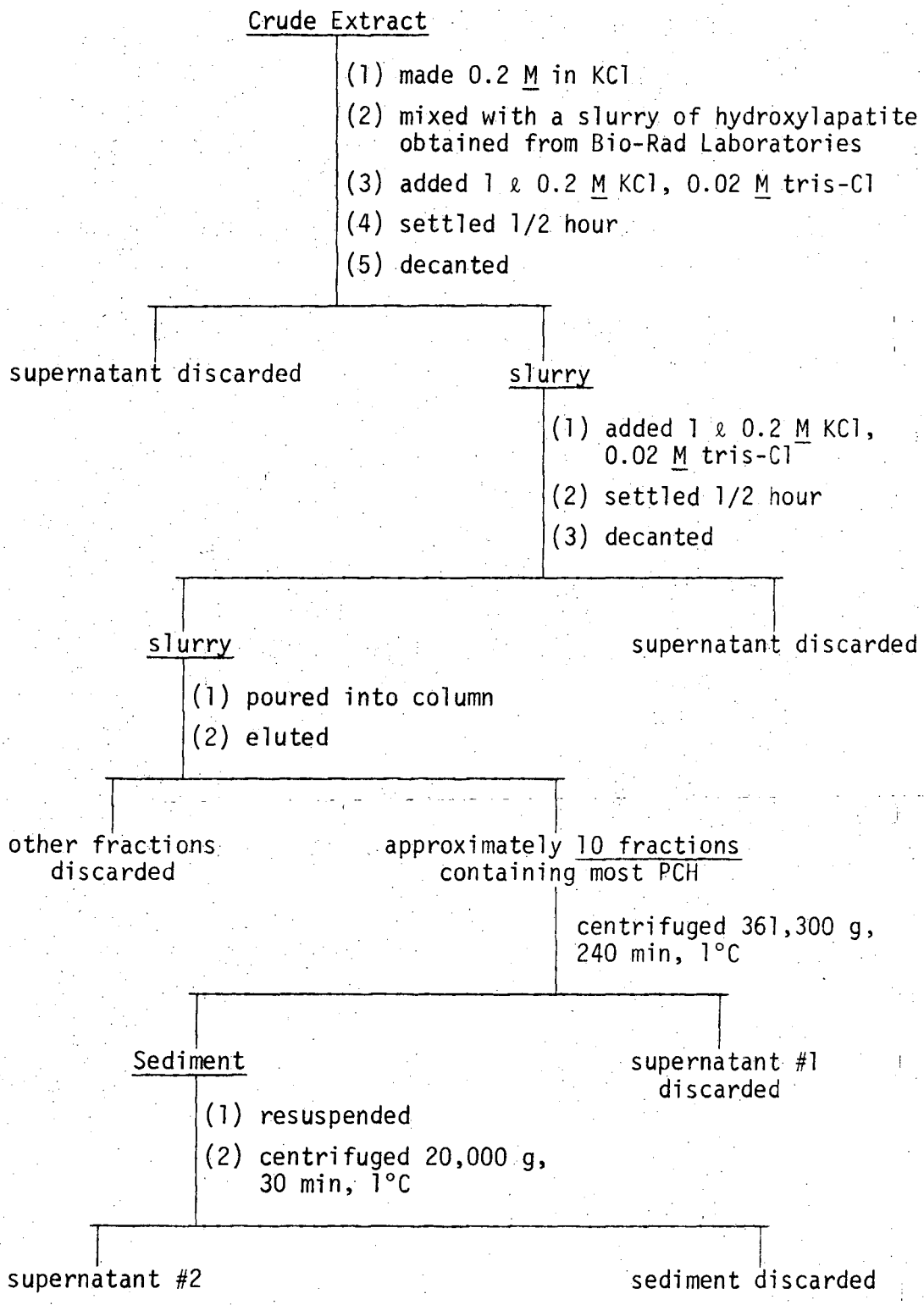

Fig. III-4. Flow chart for the preparation of PCH.

Table III-2. Purification of PCH. All optical paths were 1.00 cm. #PCH units = (A-T)·(volume)

Material	A-T (Absorbance -turbidity)	$\frac{T}{A-T}$ (Clarification)	PCH units	Yield (%)	Phototrans-formability (%)
Crude extract	0.220	0.91	23	100	63
PCH fractions	0.091	0.55	14	61	--
Supernatant #1 (361,300 g)	0.031	1.0	4.6	20	--
Resuspended sediment	0.930	1.2	6.5	28	--
Supernatant #2 (20,000 g)	0.840	0.76	5.9	25	49

IV. THE CD AND MCD OF PROTOCHLOROPHYLLIDE HOLOCHROME AND CHLOROPHYLLIDE HOLOCHROME

A. Introduction

Schopfer and Siegelman[67] found that their PCH solutions had absorption maxima at 639, 440, and 278 nm, with absorbance shoulders at about 615, 418, 382, and 332 nm. Light induced absorption changes in PCH have been reported to occur in less than 10^{-5} sec.[68] Immediately after phototransformation, the CH, which actually contains both Chlide a and nontransformable PChlide a, had maxima at 678 nm, about 630 nm, and 438 nm.[67] They found that the red absorption maximum subsequently shifted in a temperature-dependent manner toward a limit at about 672 nm with a slight decrease in its absorbance. The shift required 30 min at 12°C, and 10 to 15 min at 25°C.[67]

Both Smith and Boardman have reported observing absorption maxima at wavelengths longer than 678 nm immediately after phototransformation.[87,8] Since this represents one of the major differences between conversion of protochlorophyllide inside and outside the leaf, a careful study of the light induced absorption changes of the holochrome should be done.

In the absorption spectrum of an etiolated leaf, the red maximum is usually at 650 nm. The first product of phototransformation, presumably an *in vivo* form of Chlide a, has an absorption maximum at

678 nm.[12,22] In a dark reaction, the 678 nm species is transformed to a 684 nm absorbing form in about 6 to 10 min at 2°C. At 25°C the reaction was complete in 20 to 60 sec. This shift shows no isosbestic point. Next, there is a slower shift from 684 to 673 nm (the Shibata shift). This requires about 15 min at room temperature, longer as the temperature decreases, and finally appears to be completely prevented at 2°C.[12] But the speed also depends upon the age of the plants, occurring more rapidly in younger etiolated plants.[71] The Shibata shift has an isosbestic point at 680 $\pm$ 1 nm.[71] As was described in Chapter II, the red maximum then gradually shifts from 673 to 678 or 679 nm as the plant greens.

A study of optical activity proved useful in the investigations of leaf homogenates. It seemed probable that it would also be helpful in a study of the holochrome, especially since the photoreduction of PChlide creates two asymmetric centers at C-7 and C-8 (see Fig. II-1). In addition, the holochrome should be a good model for the interpretation of pigment-carrier complexes. Unlike many such complexes extracted from leaves, one can be reasonably sure that it is not an artifact, because it retains its biological activity, i.e., the photoreduction of PChlide to Chlide. The holochrome is also simple, having only a few pigment molecules per particle[67] compared to the hundreds present on even the basic unit of an intact chloroplast lamellae.[56]

The CD and MCD of chlorophyll and protochlorophyll pigments in organic solvents have been studied extensively by Houssier and Sauer.[38,37] Those studies will be used as a basis for an interpretation of the results obtained here.

The phytyl group of chlorophyll and protochlorophyll does not absorb in the visible region of the spectrum. Although it might indirectly affect the visible spectra of chlorophyll and protochlorophyll by influencing the type of aggregation permitted and the type of environment preferred by the pigments.

Magnetic circular dichroism is the CD measured while the sample is in a magnetic field. All substances have MCD spectra associated with their absorption bands, whether they are asymmetric or not. MCD originates from the interaction of an electron with the external magnetic field during an electronic transition. The MCD will be used here primarily for determining the position of transitions.

B. Experimental

Absorption spectra were measured with a Cary Model 14 spectrophotometer. CD and MCD spectra were measured with the Dratz circular dichroism (CD) and magnetic circular dichroism (MCD) instrument.[18] The CD instrument's calibration was checked with d-camphorsulfonic acid. The magnet strength during the MCD measurements was 11 kilogauss. However, the MCD of the holochrome preparations were measured while the sample was inside a dewar. This may have reduced the magnetic field strength during those measurements. The sample CD was subtracted from the MCD during the measurements. One can do this easily because, although the sign of the MCD is reversed when the direction of the magnetic field is reversed, the sign of the CD remains the same. Spectra of holochrome were recorded at 0 to 5°C.

No CD or MCD spectra of whole leaves were attempted, because of the high light scattering of these samples. Such measurements might, however, be possible by placing the leaf very close to the photomultiplier (light detector) and increasing the instrument's sensitivity.

Sucrose, which was present in 2 M concentrations in the PCH(suc) and CH(suc) samples, has an optically active transition in the far ultraviolet, making ORD measurements difficult even when it is present in very small concentrations. Still, high concentrations of sucrose (1 to 2 M) do not alter the CD spectra of chloroplast lamellar fragments suspended in it. In addition, the CD spectra of PCH and PCH(suc) are virtually identical. Thus the presence of high concentrations of sucrose does not appear to alter visible CD spectra. This is not unreasonable since, like absorption, CD extends over rather small wavelength ranges; ORD, like index of refraction, extends over the entire wavelength range.

Chlorophyll a (Chl a) was prepared by the method of Anderson and Calvin.[2,3] Protochlorophyll a (PChl a) was prepared by Houssier.[36] Both procedures employ chromatography on a polyethylene column, then a powdered sugar column.

Water was removed from the carbon tetrachloride (Matheson, Coleman and Bell, spectroquality reagent, maximum water content 0.02%) used as a spectral solvent by passing it through a 15 cm aluminum oxide column (Woelm basic), then a 15 cm column of molecular sieve (Linde, type 4A). The molecular sieve was previously washed with water, dried, washed with CCl_4, and activated at 200°C for 1 day.

Finally, the spectral CCl_4 was dried over molecular sieve for at least 1 day. The absence of water was checked for in the near infrared spectrum. The absorbance at 1.89 μ was 0.00 ± 0.01; and at 2.6 μ, it was 0.05 ± 0.02 (10.0 cm pathlength). An absorbance at 1.89 μ of 0.01 corresponds to a water content of 3×10^{-4} M [$\varepsilon_{H_2O}^{1.89\ \mu} = 3.73 \frac{\ell}{\text{mole-cm}}$, (88)]. The Chl a and PChl a were stored under vacuum at room temperature for at least one week prior to the experiment. Cuvettes and volumetric flasks were dried in a vacuum drying oven overnight before the dilutions were carried out in a box flushed with dry nitrogen.

C. Results and Conclusions

1. Protochlorophyllide Holochrome in 2 M Sucrose

The CD of PCH(suc) (see Fig. IV-1) is a complex having minima at 647 and 613 nm, and possibly a maximum at 637 nm. In PChl(ide) a spectra, the Q_y (0,0) and Q_x (0,0) bands are quite close to each other. The transition dipole moments for Q_y and Q_x are, respectively, oriented approximately along the y and x direction, as shown in Fig. II-1. The MCD of PCH(suc) (Fig. IV-1) indicates that Q_y (0,0) is at about 643 nm and Q_x (0,0) is at about 615 nm for holochrome PChlide. For solutions of PChl a,[38] it has been demonstrated that Q_y (0,0), the lowest energy electronic transition, has positive rotational strength and Q_x (0,0), the next band, has negative rotational strength. By comparison, PChlide in the holochrome is probably not a monomer, because its lowest energy CD band is negative (Fig. IV-1).

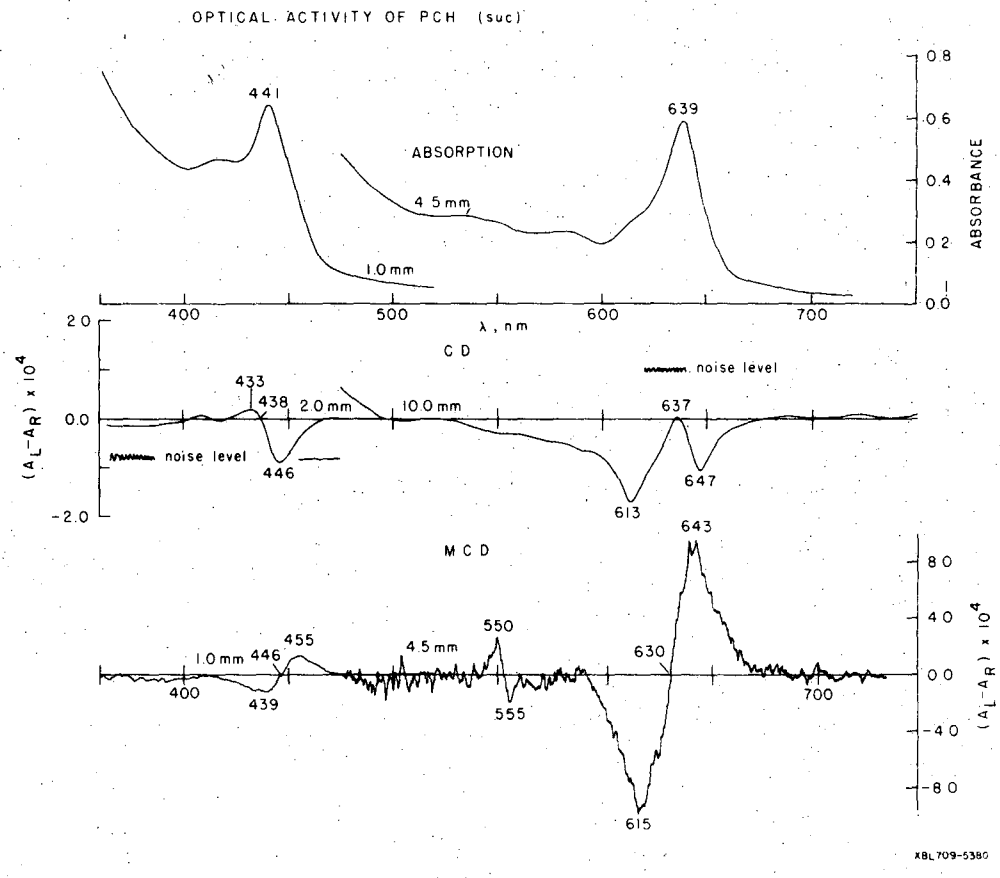

Fig. IV-1. Optical activity of PCH(suc). Optical pathlengths are indicated on the figure.

The next step in complexity would be a pigment dimer, in which case one or both the Q_y (0,0) and Q_x (0,0) bands might be split into positive and negative CD components whose magnitudes and splittings would depend upon the dimer's geometry (see Eqns. II-1 to II-4). Qualitatively, the PCH(suc) CD resembles that of PChl a in very dry CCl_4 where the PChl a is completely aggregated and may well be a dimer.[38] However, in the absence of supporting evidence, one can only conclude that the PChlide in the holochrome is aggregated.

Monomers as well as dimers are present in the solution whose spectra are shown in Fig. IV-3. The CD spectrum of a solution of dimers in CCl_4 has a sharp minimum at 628 nm, a sharp maximum at 622 nm and a broad minimum at 603 nm about equal in amplitude to that at 628 nm.[38] The lowest energy of the solution monomer CD, as mentioned above, is positive. When the spectra shown in Fig. IV-3 were measured, some H_2O was apparently present in the solution. H_2O will complex with the pigment molecules, preventing the formation of a solution of pure dimers.[38,95,43]

2. Chlorophyllide Holochrome in 2 M Sucrose

CH(suc) was the PCH(suc) solution used in recording the spectra in Fig. IV-1 after a 10 sec flood lamp illumination. The CD spectrum of CH(suc) (Fig. IV-2) is also complex, suggesting the presence of at least a Chlide a dimer. The CD spectrum shows that the absorption band at 678 nm [Q_y (0,0)] is clearly split, with a strong positive component at 678 nm and a weaker negative component at 687 nm.

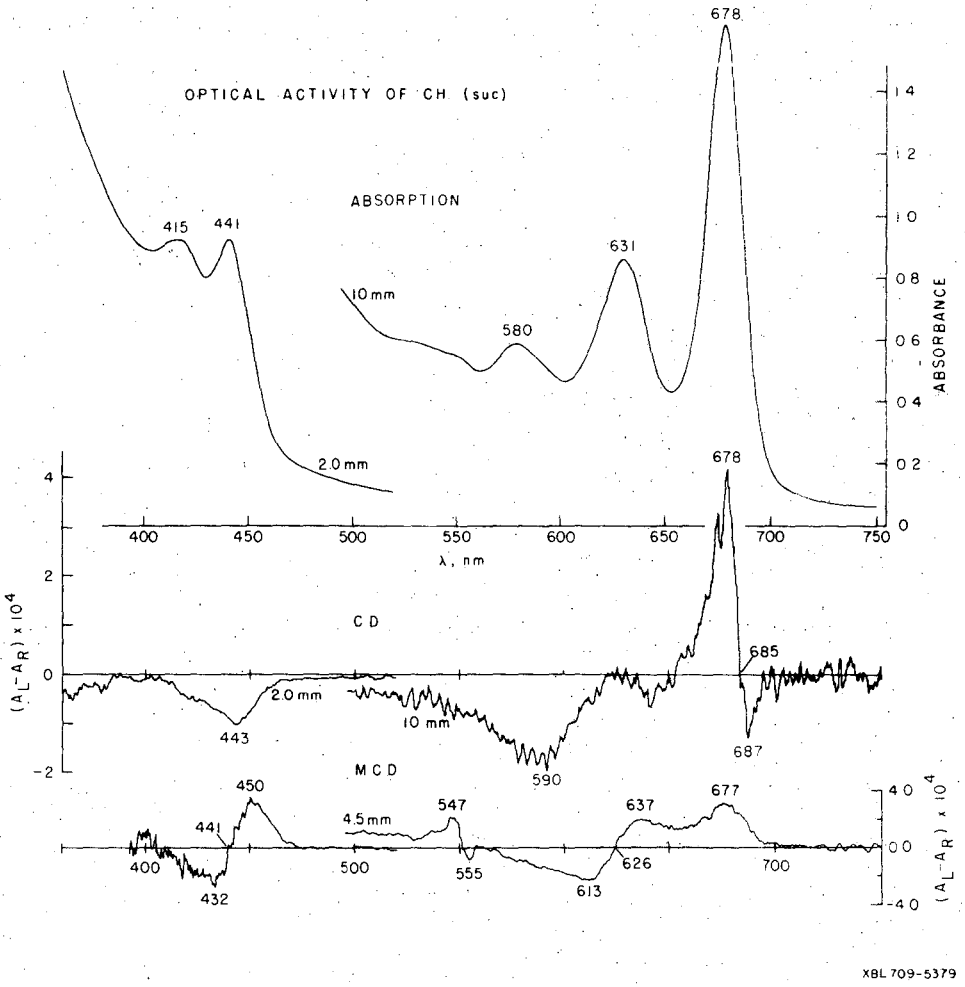

Fig. IV-2. Optical activity of CH(suc). Optical pathlengths are indicated on the figure.

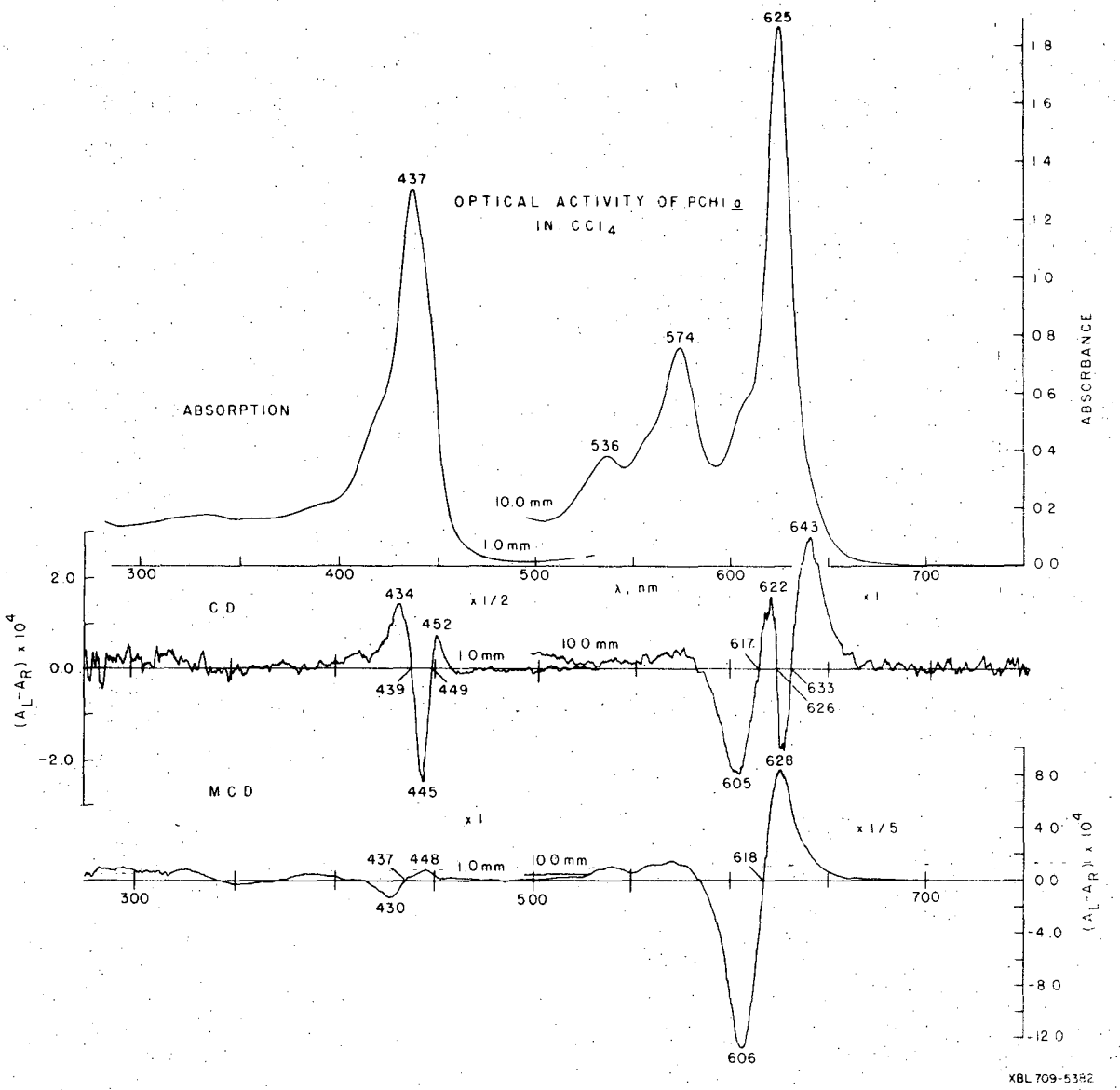

Fig. IV-3. Optical activity of PChl a in CCl_4. Optical pathlengths were as shown.

The CD in the wavelength region around 590 nm where the Q_y (0,0) transition is located is much more intense than it is either in solution dimers or mature chloroplast fragments. It does not appear to be split.

3. Protochlorophyllide a which Cannot be Photoreduced

Inactive PChlide a appears in the absorption spectrum of CH(suc) (Fig. IV-2) as a maximum at 631 nm. Inactive PChlide a optical activity can be seen in the CD spectrum of CH(suc) (see Fig. IV-5 or Fig. IV-2) as the small negative band at about 640 nm. Inactive PChlide a can be seen in the MCD spectrum (Fig. IV-2) as a positive peak at 637 nm and a negative trough at 613 nm. The CD spectrum of CH(suc) from 650 nm to 600 nm is compatible with inactive PChlide having a CD similar to that of active PChlide (Fig. IV-5), but blue shifted about 6 to 7 nm. Thus, inactive PChlide might also be aggregated. In addition, comparing the CD of PCH(suc) and CH(suc) in this wavelength region, one can see that if inactive PChlide's CD were subtracted from the CD of PCH(suc), there would be a true maximum at 634 nm in the CD of PCH(suc). Thus, the CD of active PChlide a would resemble that of the solution dimer even more closely than the CD of PCH.

4. Effect of Sucrose on Dark Shift from 678 to 674 nm

Spectra were taken of the holochrome both with and without added sucrose. Although the holochrome is more active and more stable in the former, there is apparently no dark shift of the red absorption

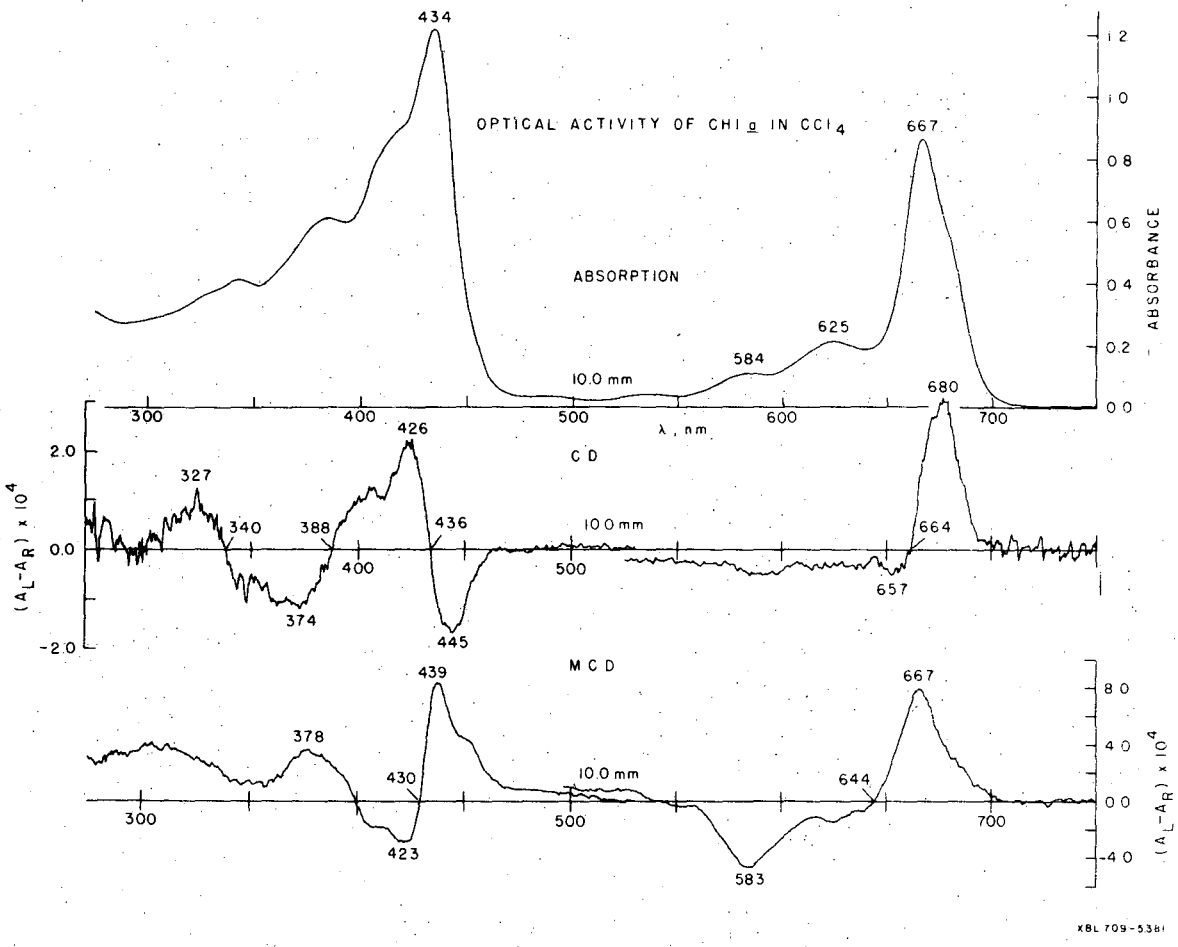

Fig. IV-4. Optical activity of Chl a in CCl_4. Optical pathlength was 10.0 mm.

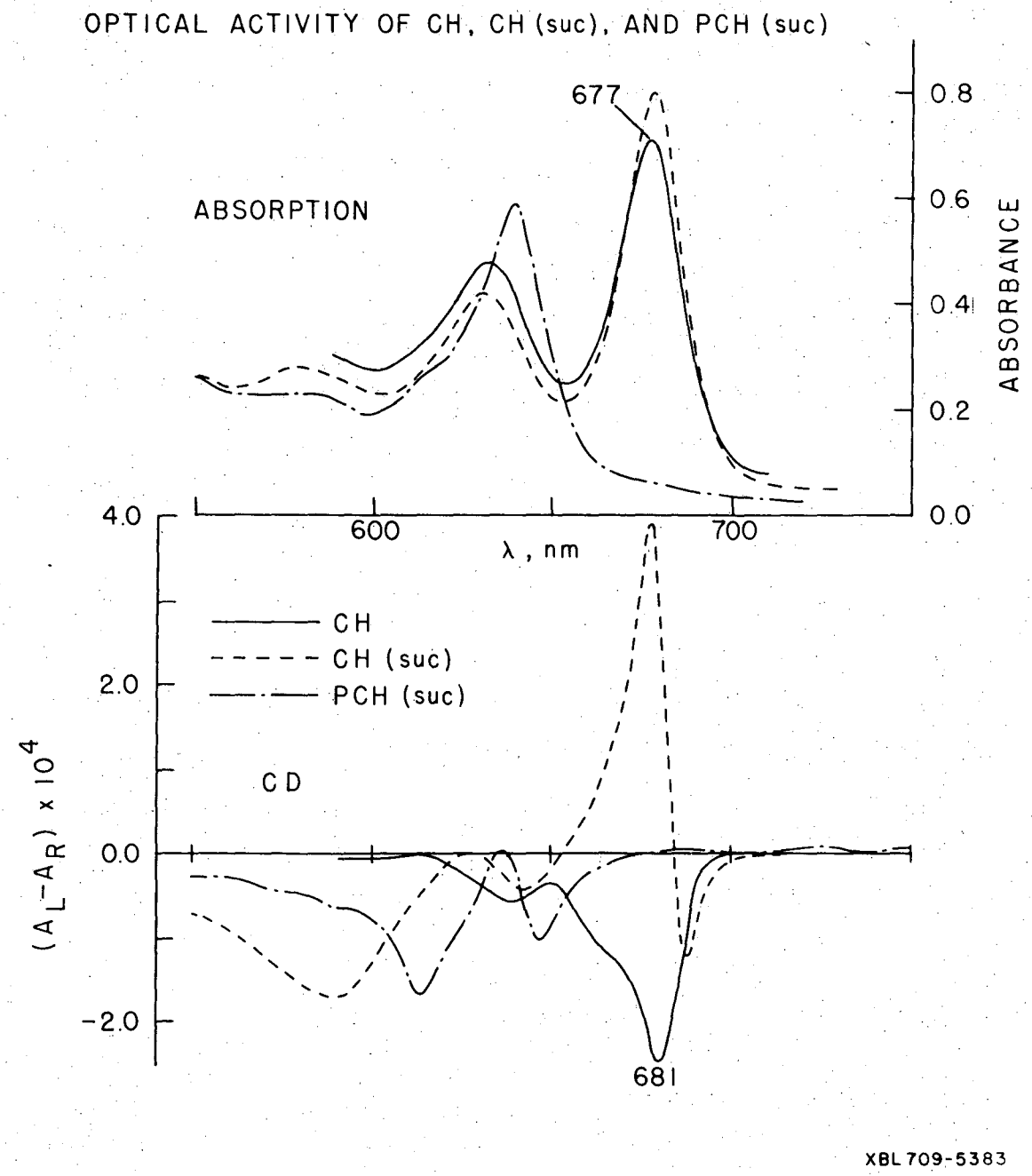

Fig. IV-5. Optical activity of CH: (————), CH(suc): (--------), and PCH(suc): (————·————). Optical pathlengths: CH(suc) and PCH(suc) absorption, 4.5 mm; CH absorption and all CD, 10.0 mm. CH spectra were recorded at the very beginning of the dark shift.

maximum of the newly formed CH(suc) to shorter wavelengths in the presence of 2 M sucrose. When PCH(suc) was transformed into CH(suc) by a 5 sec illumination, its absorption maximum was at 678 nm (Fig. IV-2), remaining at this wavelength for at least an hour at 0 to 5°C. On the other hand, the absorption maximum of CH shifts toward shorter wavelengths, as it does in intact leaves. Sucrose in high concentrations (1 to 2 M) has been shown to be an inhibitor of many enzymatic reactions.[34] Phytylation of the chlorophyllide a, an enzymatic reaction, might be responsible for the dark shift.[76] However, in this instance the dark shift has been completely blocked by 2 M sucrose.

5. Protochlorophyllide a Holochrome

To be able to measure the spectrum of a sample capable of the dark shift, PCH was prepared as described in the experimental section. The CD spectrum of PCH was the same as that of PCH(suc).

6. Chlorophyllide a Holochrome

After PCH was transformed to CH, the red maximum of the first absorption spectrum recorded was at 677 nm (see Fig. IV-5: ———), and after the first CD spectrum was completed it was at 676 nm. I was unable to stop the shift toward higher energies, even though the CH sample was at 0 to 5°C. If the PCH sample was first made 2 M in sucrose, the initial absorption peak after transformation was at 678 nm, and it did not shift. When the absorption maximum of CH was at 676 to 677 nm, the CD spectrum had a minimum at 681 nm (Fig. IV-5: ———).

After 1 hr at 5°C and 15 min at 22°C, the absorption maximum had shifted to 674 nm and the CD minimum was at 679 nm. The absorbance and the magnitude of CD had both decreased about 11%. The CD band also appeared to have broadened slightly. The CD spectra obtained for CH resemble those of Chl *a* monomers.[38] As will be seen later, the fluorescence depolarization spectra of CH support the idea that its Chlide *a* is monomeric.

The qualitative agreement between the CD spectra of CH and those of homogenates prepared from leaves first illuminated about 1 hr (Chapter II) was very good (see Fig. II-4 and Fig. IV-5). The chromophore of CH is the acidic form of the pigment, *i.e.*, Chlide *a*.[81] On the other hand, the chromophore of homogenates prepared from leaves after 1 hr of illumination is probably Chl *a*, *i.e.*, phytylation has probably been completed.[104,76,100] Thus the addition of the phytyl esterifying group has altered the CD spectrum very little qualitatively. The magnitude of the CD of CH per unit absorbance at the wavelength of the absorption maximum, however, was about 40% greater than that of the leaf homogenate (Fig. II-4, 1 hr; Fig. IV-5, CH).

Because Chlide *a* is apparently monomeric during most of the dark shift, some cause other than a change in the state of aggregation must be sought for most of the dark shift. Although a change in aggregation appears to initiate the dark shift. Perhaps the cause is a change in the Chlide *a* environment, for example, some rearrangement of the carrier that the Chlide is bound to and possibly surrounds it.

The failure to find CH CD corresponding to those of CH(suc) has three possible explanations. First, CH may have passed through the 678 nm form too rapidly to be measured. This is the most plausible explanation. The very first step after phototransformation may be the breakup of the aggregate, after which the dark shift occurs. Apparently, high concentrations of sucrose preserve the aggregate. It seems probable that an aggregate of PChlide a would persist for at least a short period after its conversion to Chlide a. The phototransformation is completed within 4 msec after the beginning of a 1 msec illumination with an electronic flash.[54] Schopfer and Siegelman[68] reported light induced absorption changes occurring in less than 10^{-5} sec. In addition, phototransformation can take place in the frozen state both in the leaf and in homochrome preparations. Conversion is fairly rapid at -70°C, but has stopped after the temperature is lowered to -195°C.[84,7]

The second possible explanation is that CH(suc) is an artifact. This seems unlikely because, in addition to the reasons given above, the phototransformation of PChlide into Chlide has occurred.

The third possible explanation is that CH is an artifact. However, the transformation of PChlide into Chlide has also worked in this preparation, the Chlide a red maximum goes through a dark shift in dark grown leaves, and a similar form is found in preparations made from newly illuminated etiolated leaves (Chapter II). The dark shift which occurs in CH still might, however, differ from the Shibata shift which occurs in leaves.[71] Shibata found that as the absorption maximum shifts from 684 nm to 673 nm, the successively

recorded absorption spectra form an isosbestic point at 680 ± 1 nm.[71] The isosbestic point indicates that the dark shift is the disappearance of the 683 nm band and the appearance of a 673 nm band, and that the process is the transformation from a single compound to a single compound.[71,39] The absorbance of the 673 nm band was greater than that at 684 nm.[71] Schopfer and Siegelman[67] found a slight decrease in absorbance as the 678 nm peak of newly formed CH shifted toward shorter wavelengths. I found that in CH the absorbance at the maximum when it was at 674 nm was about 11% less than the absorbance when it was at 677 nm. In addition, no isosbestic point could be demonstrated for the shift. Both the decrease in absorbance and the lack of an isosbestic point might be due to decomposition of some of the Chlide *a* of CH. After CH(suc) was dialyzed overnight in the dark at 5°C against 0.02 M tris-Cl, pH 8.0, the absorption band at 678 nm had almost disappeared. The absorbance at the 630 nm band was now about twice as great as at the band in the 670 nm region.

7. Similarity of Pigment Monomer and Aggregate MCD

Qualitatively, the MCD of PCH(suc) (Fig. IV-1), PChl *a* in CCl_4 (Fig. IV-3) and PChl *a* in ether[38] are very similar. This also appears to be true of the MCD of CH(suc) (Fig. IV-2), Chl *a* in CCl_4 (Fig. IV-4), and Chl *a* in ether.[18,19,38] The similarity of these MCD spectra implies that MCD probably is no more sensitive to intermolecular electronic interactions than the absorption spectrum, since Chl *a* or PChl *a* dissolved in CCl_4 forms dimers,[63,65,19] PCH(suc) and CH(suc) probably contain pigment aggregates, and finally Chl *a* in ether and

PChl a in ether are monomeric.[38] Nevertheless, the absorption bands of the aggregates should be split. This is certainly so in the case of Chl a in CCl_4.[65] However, if the split components of an electronic transition (see Fig. IV-6) have the same sign MCD, they might not be resolved. This is apparently the case for Chl(ide) a and PChl(ide) a aggregates. However, the aggregate MCD bands should be broader than those of the monomers. A careful study of the MCD band widths in the MCD of the pigments in the aggregated and unaggregated states might support this hypothesis.

8. MCD Show Presence of Cytochrome

There is an additional feature worth noting in the holochrome MCD's; the sharp double bands crossing the baseline at about 552 nm (Fig. IV-1, IV-2). The MCD of reduced cytochrome c shows this feature, that of oxidized cytochrome c does not.[18,1] Apparently, there was a reduced cytochrome present in these holochrome solutions. This is not at all obvious from the absorption spectra. The cytochrome might be cytochrome b_6, which was shown by Boardman[10] to be present in etioplasts in its reduced form. Its α band is located at 554 nm. However, the cytochrome may not be associated with the holochrome.

9. Summary

An aggregate of PChlide molecules in, or on the carrier molecule, is phototransformed into an aggregate of Chlide chromophores which rapidly disintegrates into Chlide monomers. After the breakup of

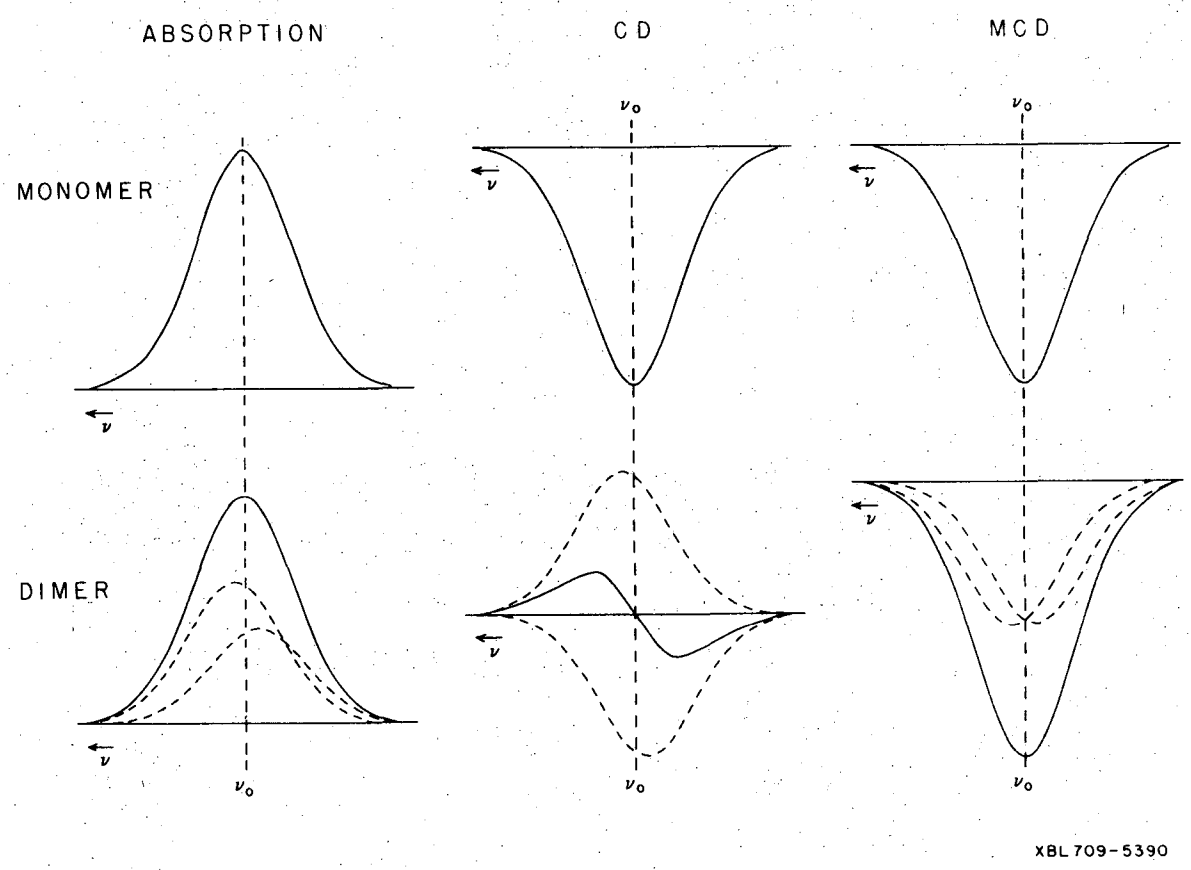

Fig. IV-6. A decomposition of the degenerate components of the absorption, CD, and MCD of the split transition of a hypothetical dimer showing how the MCD bands might fail to be resolved. Observed band (————), proposed decomposition (--------).

the aggregate, the dark shift results from a change in the environment of the Chlide chromophores. In all cases the pigments are bound to carrier molecules.

V. FLUORESCENCE SPECTRA OF THE HOLOCHROMES

A. Introduction

Light energy captured by a molecule can often be transferred to a nearby molecule which may emit the energy as fluorescence. CD measurements indicated that in PCH, PCH(suc), and CH(suc) the chromophores were aggregated. But in CH the CD spectra indicated that the chromophores were monomeric. Fluorescence measurements might demonstrate energy transfer in the former cases, but not in the latter case.

B. Materials and Methods

PCH and CH were prepared as was described in the preceding chapter. Samples designated PCH(suc), unlike those in the preceding chapter, were PCH made 2 M in sucrose by adding a sufficient amount of 4 M sucrose; 0.02 M tris-Cl. CH(suc) was made by maximal transformation of these PCH(suc) samples with a brief flood lamp illumination in the same manner as was described in Chapter III. Absorption spectra were recorded with a Cary Model 14 spectrophotometer. The sample temperature while the fluorescence spectra were being measured was about 7°C.

A modified Aminco-Bowman spectrophotofluorometer (American Instrument Co.) (Fig. V-1) was used for all fluorescence measurements. The emission monochromator grating was manufactured by Bausch and Lomb to perform optimally at 700 nm. Excitation light was chopped at 200 cps (chopper, type Cal-5A, American Time Products). The following electronic components manufactured by Princeton Applied Research Corp. were utilized to amplify the 200 cps signal: high voltage power supply (Model #221), selective amplifier (Model #210), and lock-in amplifier (Model #220). A cooled (dry ice snow), shielded photomultiplier (RCA #7102) with S-1 response detected emitted light. Spectra were plotted on a Moseley Autograf Model 2D-2A x-y recorder. The emission spectra were not corrected either for the wavelength dependence of the photomultiplier sensitivity or for wavelength dependent variations in the monochromator efficiency.

The bandwidths given were determined from the widths of bands of scattered exciting light at 1/2 their height. The bands were measured with the same combination of slits as was used when the fluorescence spectra were recorded.

Four-sided cuvettes with 10.0 mm optical paths were used in all the experiments. Excitation spectra were measured with a Corning sharp cut yellow filter (C.S. #3-73) in the emission beam to prevent the transmission of overtones from the excitation grating. An orange filter (C.S. #3-68 or C.S. #3-66) was used for the same purpose during the measurement of the fluorescence polarization spectra.

Polarization data are given as $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$, where $I_{\perp}$ and $I_{\parallel}$ are the intensities of light emitted at 90°C to the activating

MODIFIED AMINCO-BOWMAN SPECTROPHOTOFLUOROMETER

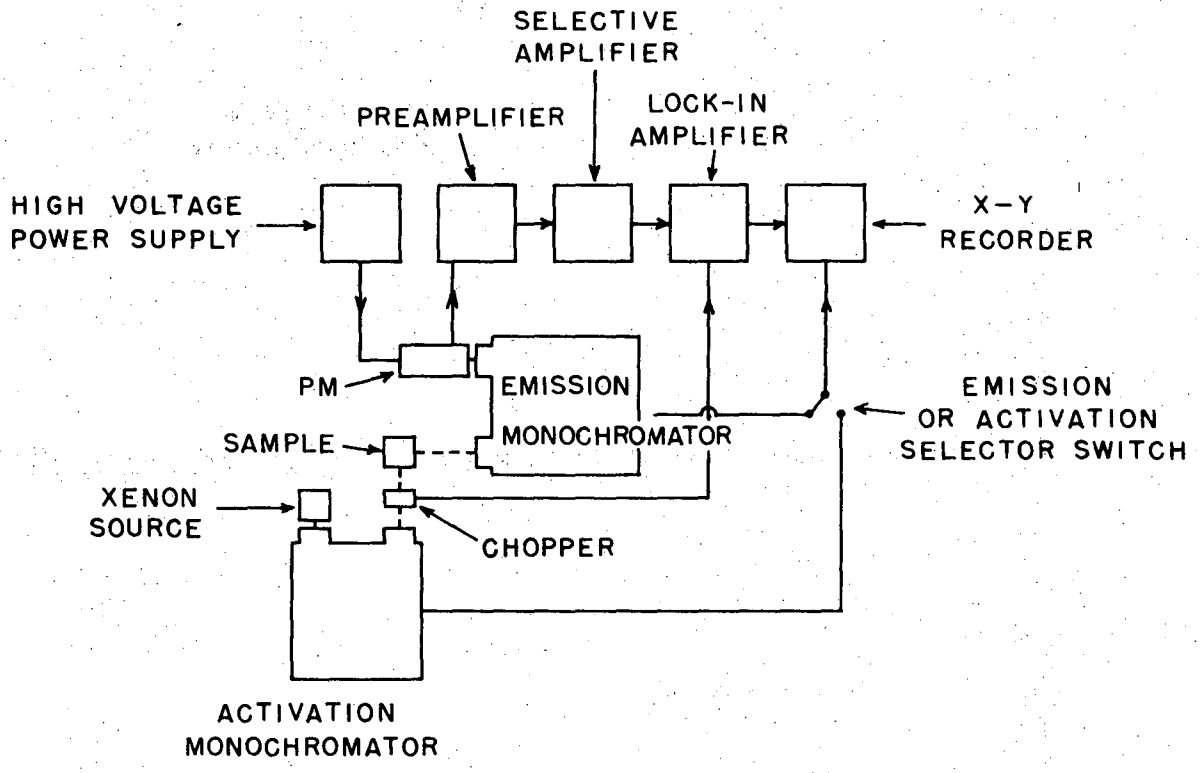

Fig. V-1. Modified Aminco-Bowman spectrophotofluorometer.

light beam and respectively perpendicular and parallel to its plane of polarization. E and B will designate polarization directions perpendicular to and in the plane intersecting the incident and emitted light beams (Fig. V-2). Thus I(EB) will represent the fluorescence intensity when the polarization of the incident light is perpendicular and that of the emitted light is parallel to the plane containing them. One can see that both I(BE) and I(BB) correspond to $I_{\perp}$. Actually, the emission monochromator transmits light polarized in the E and B directions with different efficiencies, producing an instrumental polarization characterized by a ratio $N = I(BB)/(I(BE)$, which depends upon the wavelength setting of the emission monochromator. The correct polarization will then be given by $p = [N \cdot I(EE)-I(EB)]/[N \cdot I(EE)+I(EB)]$.[38]

C. Fluorescence Emission

In practice, it is difficult to measure the fluorescence spectrum of PCH(suc) without the exciting light rapidly transforming active PChlide a into Chlide a. Active PChlide is defined as the PChlide which can be phototransformed into Chlide by a 5 to 10 sec flash lamp illumination. However, by attenuating the exciting light with two neutral density filters of 0.9 and 1.2 optical density (%T = 0.8), spectra could be measured without transforming more than about 12% of the active PChlide. Fig. IV-3 shows the spectrum of PCH(suc) compared with that after complete transformation. The respective

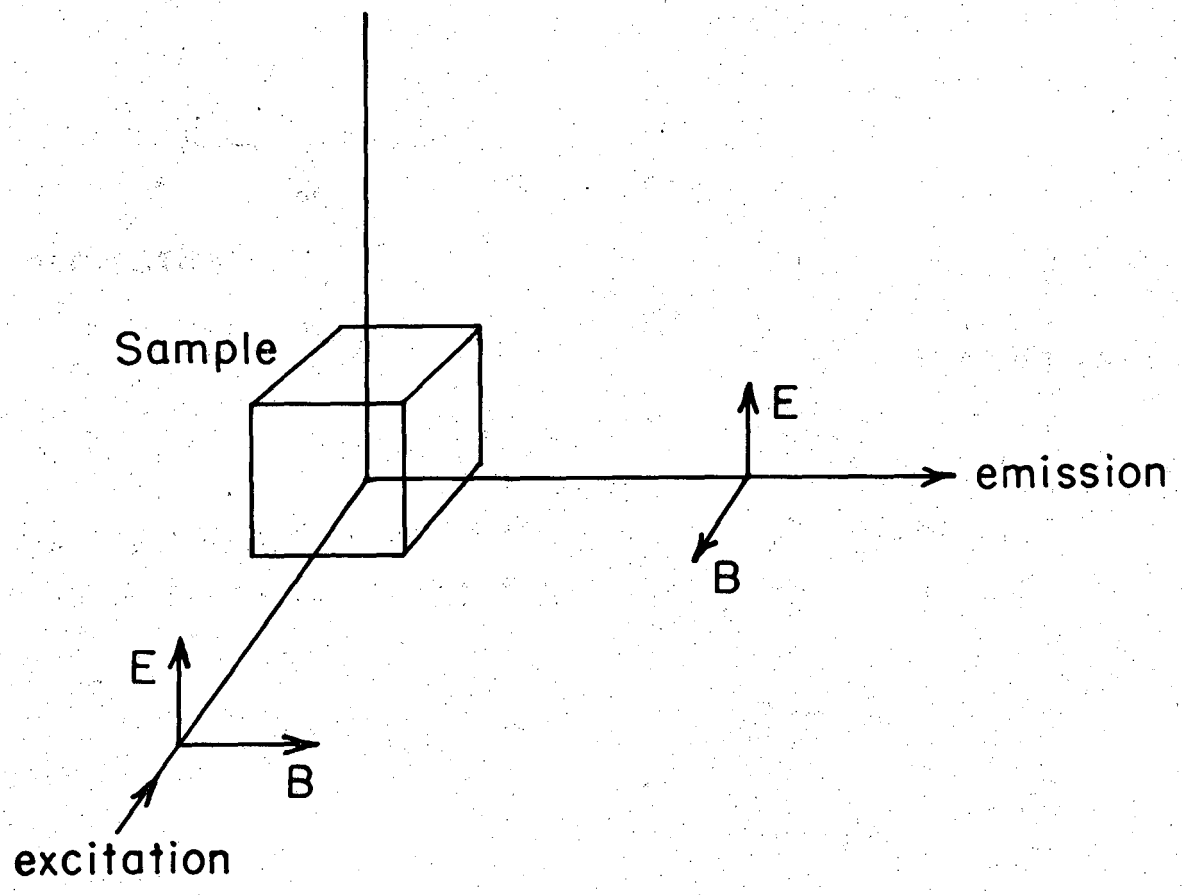

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Fig. V-2. Geometry of polarized fluorescence excitation and emission beams during fluorescence [C. Houssier and K. Sauer, Biochim. Biophys. Acta 172, 492 (1969)].

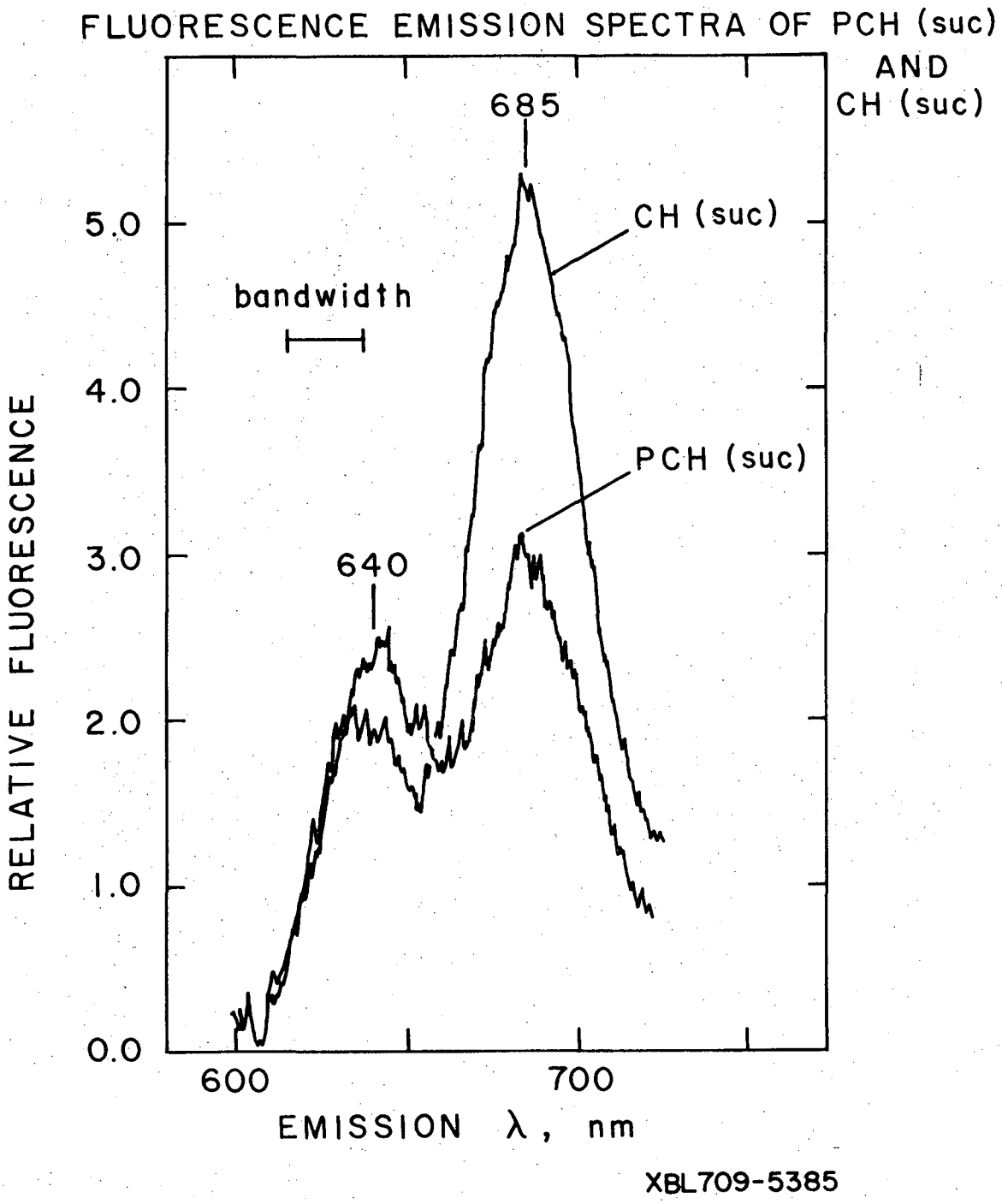

Fig. V-3. Fluorescence emission spectra of PCH(suc) and CH(suc). Excitation is at 440 nm. Optical pathlength, 1.00 cm.

absorption spectra* can be seen in Fig. V-4.

Active PChlide a appears to emit little fluorescence. Very roughly its fluorescence per unit active PChlide a absorbance at 640 nm is $\Delta I_{642}/\Delta A_{640} = 10$, in arbitrary units. The fluorescence maximum of active PChlide is about at 642 nm. Based on the 7 nm Stokes shift found for inactive PChlide, the fluorescence maximum of active PChlide should be located at about 647 nm; however, 5 nm is probably within the uncertainty of the measurement. The fluorescence maximum of inactive PChlide is at 637 nm. Its fluorescence per unit absorbance at 630 nm is approximately $I_{637}/A_{630} = 33$, in the same units as above. Thus, the fluorescence per unit absorbance of active PChlide is less than 1/3 that of inactive PChlide.

There also appears to be a difference between the fluorescence per unit absorbance of the initially formed Chlide and the fluorescence per unit absorbance of the Chlide of the totally transformed sample. The fluorescence per unit absorbance of the first formed Chlide, correcting for Chlide formed during the measurement of the emission spectrum (dashed and dotted line, Fig. V-4), is approximately $I_{685}/A_{678} = 1.6/.01 = 160$. For the final form, it is $I_{685}/A_{678} = 5.2/.1 = 52$, a three-fold decrease.

The most reasonable explanation of the decrease would appear to be concentration quenching of Chlide fluorescence, such as one finds for Chl solutions in solvents favoring its aggregation.[103] Because these holochrome solutions are dilute, there probably is more than one chromophore per holochrome. As the number of PChlide chromophores

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*Absorption spectra were measured at 22°C.

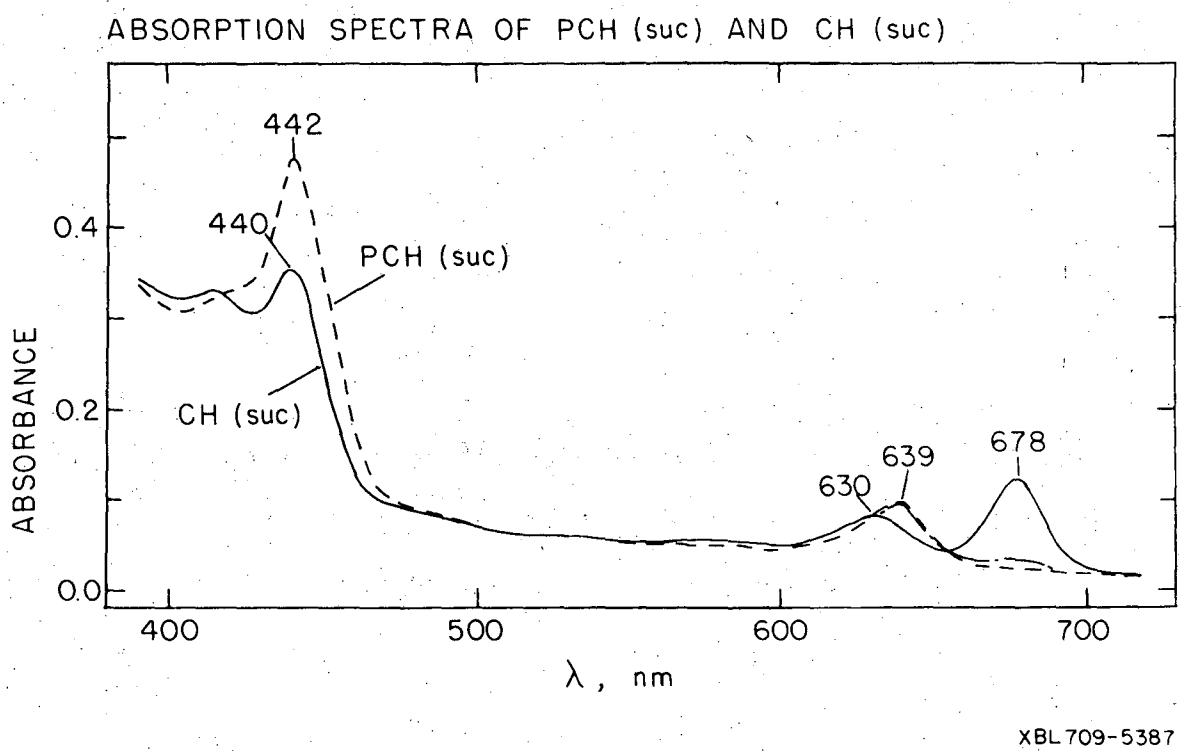

Fig. V-4. Absorption spectra of PCH(suc) before fluorescence emission spectrum (--------), PCH(suc) after fluorescence emission spectrum (———-———-) and CH(suc) (————). Optical pathlength: 1.00 cm.

transformed to Chlide increases, the probability of finding more than one Chlide per holochrome increases, and the fluorescence efficiency decreases.

One cannot, however, exclude the possibility that the holochrome molecules are aggregated. Then, even if there is only one chromophore per holochrome molecule, if the chromophores on separate holochrome molecules in the aggregate are close enough, quenching of Chlide fluorescence could be observed as the conversion proceeds.

Kahn et al.[41] have a different explanation for a similar decrease they observed in the fluorescence from Chlide a as the phototransformation proceeds, although their conclusion is similar. They attribute it to energy transfer from PChlide. Excitation in both instances was at 440 nm where PChlide absorbs more than twice as strongly as Chlide a. Thus they reason, as transformation proceeds, the effective absorption decreases. Kahn et al.'s spectra, however, were recorded at 77°K, where there is no phototransformation of PChlide a. Thus, active PChlide must utilize the energy it receives in some other manner than phototransformation. On the other hand, my spectra were recorded at 7°C. If it is assumed that at 7°C almost all the energy received by active PChl was used for phototransformation, Kahn et al.'s[41] explanation is unlikely for my results. Kahn et al.[41] have additional evidence, that at low temperatures there is energy transfer from active PChlide to Chlide. They measured the fluorescence emission and excitation spectra of a slightly transformed sample of PCH and a mixture of equal volumes of unconverted and maximally converted PCH. For slightly transformed PCH samples,

both the emission spectrum and the excitation spectrum showed evidence of energy transfer, from active PChlide to Chlide, although they are compatible with the presence of concentration quenching also. In the case of the mixture, there was no evidence of energy transfer. They also concluded that there is no transfer of energy between molecules of holochrome. However, if relatively stable aggregates of holochrome molecules were present, there could be energy transfer between molecules of holochrome.

An alternative explanation is that as the phototransformation proceeds, the binding of the chromophore to carrier molecule changes in such a way that the fluorescence efficiency decreases.

The emission spectrum of the same sample of CH(suc), recorded with unattenuated exciting light, is shown in Fig. V-5. The fluorescence maximas are at 637 nm and 684 nm with a shoulder at 745 nm. The tentative assignments of these bands are 637 nm: inactive PChlide (0→0) transition; 684 nm: Chlide (0→0) transition; and 745 nm: Chlide (0→1) transition. The Stokes shift (shift of fluorescence to lower energies) for both inactive PChl and Chl appears to be 7 nm. The fluorescence maximum for CH(suc) is at 684 nm. The absorption maximum is at 678 nm. Thus, the maxima are the same as they are for chloroplast fragments.[29] In mature plants, however, most of the fluorescence supposedly comes from a form of chlorophyll _a_ with absorption at 673 nm.[29] When the absorption maximum of CH shifts from 678 nm to 674 nm, the fluorescence maximum shifts from 684 nm to 682 nm (see Fig. V-7).

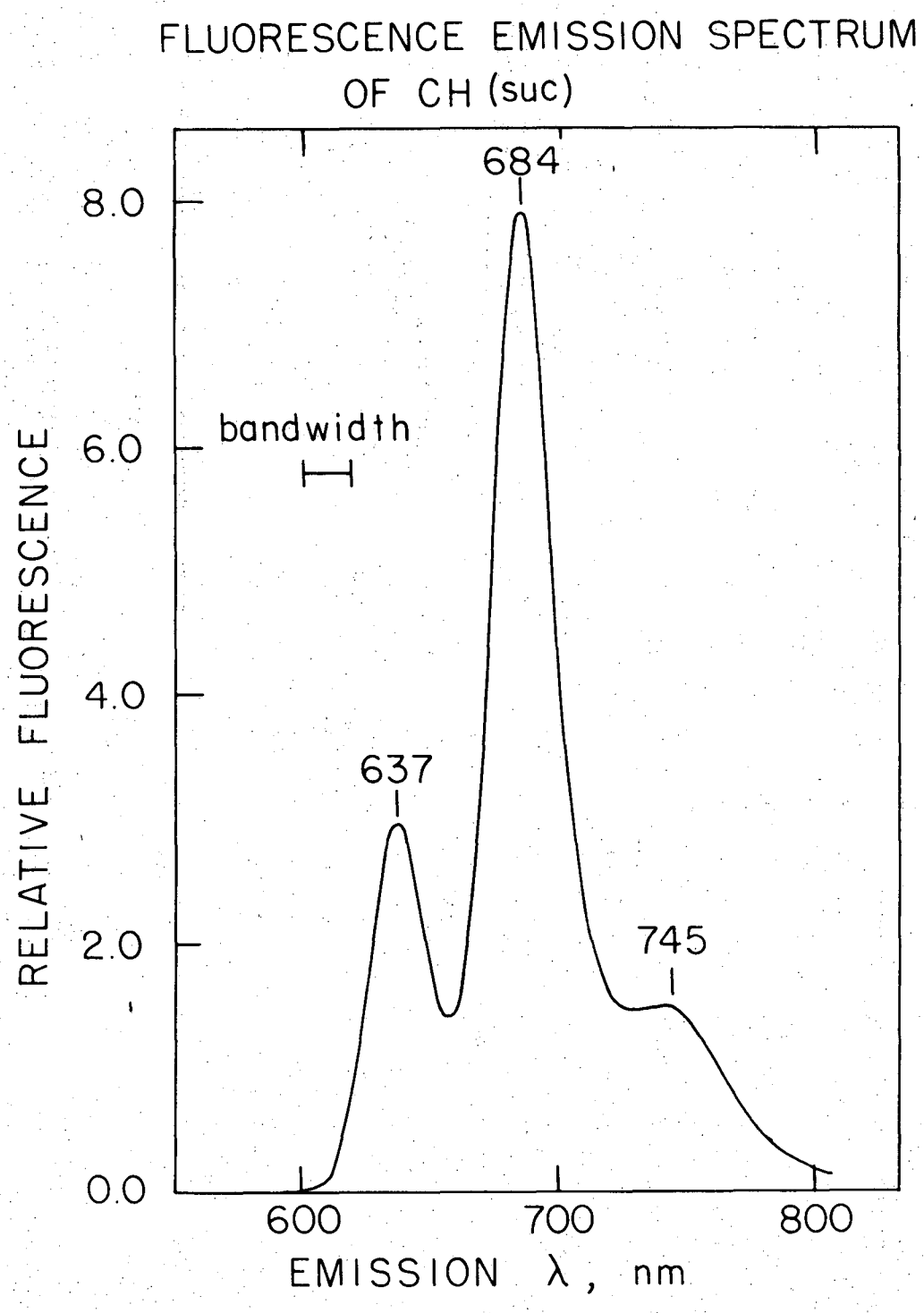

Fig. V-5. Fluorescence emission spectrum of CH(suc). Excitation is at 440 nm. Optical pathlength: 1.00 cm.

D. Fluorescence Excitation

CH(suc) fluorescence excitation spectra (Fig. V-6) of the above sample were measured at observational wavelengths of 637, 684, and 745 nm. The latter two observational wavelengths give qualitatively identical spectra, confirming that both emission bands are from Chlide a.

Both the 685 nm and 745 nm excitation spectra have prominent shoulders at 630 nm. The prominance of the shoulder at 630 nm might indicate transfer of energy absorbed by inactive PChlide a to Chlide a. In the spectrum of chloroplast fragments of the barley mutant lacking Chl b (Chlorina 2) (Fig. II-2: ————) developed by Highkin,[32,33] the absorption at 630 nm is 0.22 that at 678 nm. If the absorbance of the mutant spectrum at 678 nm were the same as CH(suc) at 678 nm, 0.100, then the percent absorption of the mutant at 678 nm would be 20.5% and the percent absorption at 630 would be 4.9%. Using the ratio of the percent absorptions, 0.239, only 58% of the fluorescence excited by 630 nm light can be from Chlide a excited by direct absorption of a 630 nm photon [$.239\ I^{679} / I^{630} = .58$]. The remainder must be from inactive PChlide a. However, the emission of inactive Pchlide a is probably negligible at 745 nm. Therefore, it is probably transferring energy to Chlide a. Because 2/3 of the 630 nm absorption is by inactive PChlide (see Fig. V-4) (calculated using the barley mutant spectrum again), the efficiency of energy transfer from inactive PChlide a to Chlide a is very approximately $.42/.67 = 0.63$.

The spectrum observed with the emission monochromator at 637 nm is that of inactive PChlide emission alone, since Chlide does not emit at this wavelength.

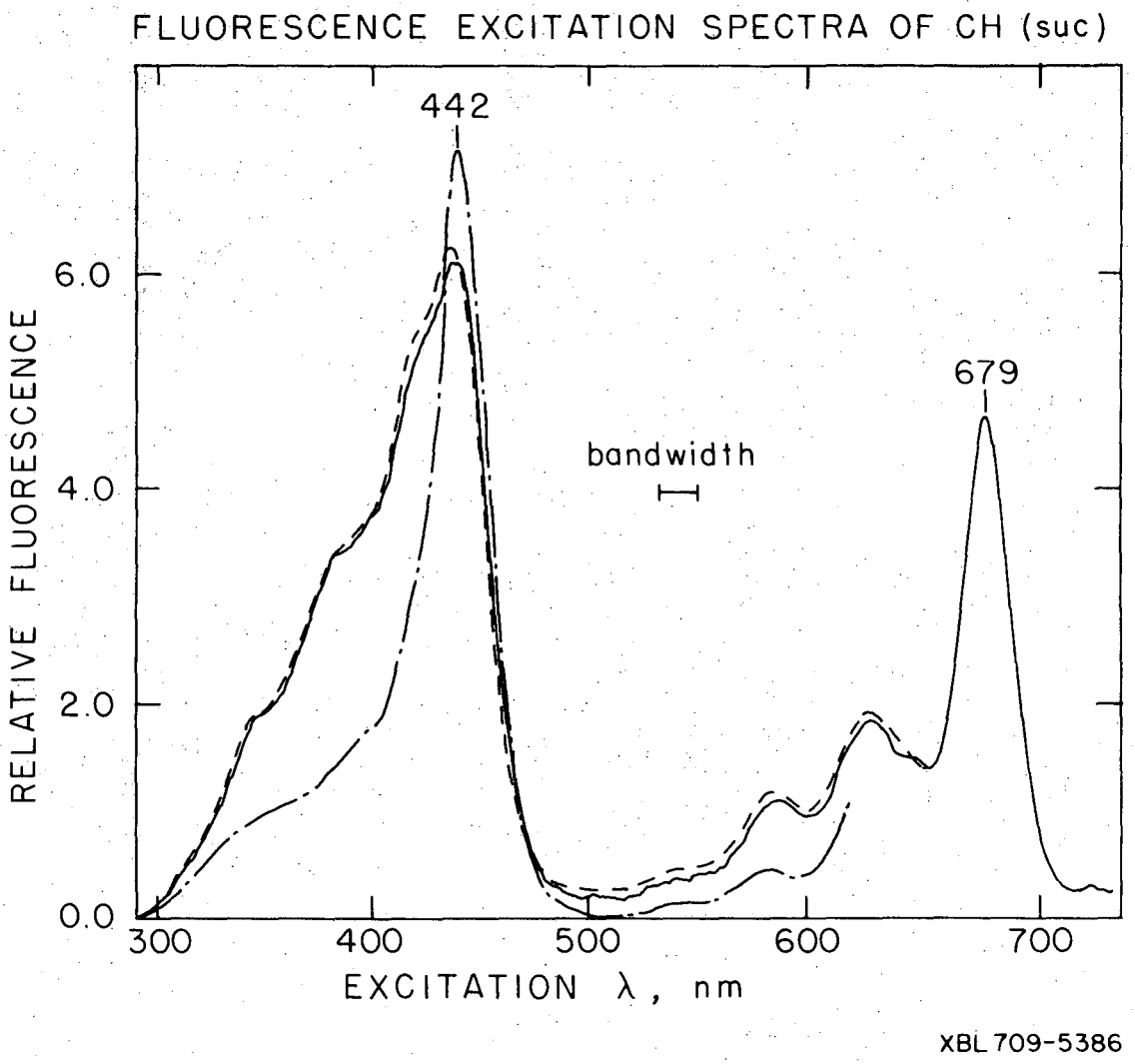

Fig. V-6. Fluorescence excitation spectra of CH(suc) at the emission wavelengths 637 nm (——·——·——), 685 nm (----------), and 745 nm (——————). The instrument gain has been adjusted to facilitate comparison of the spectra. Optical pathlength: 1.00 cm.

Although a weak shoulder in the absorption spectrum at 480 to 490 nm indicates the presence of carotenoids in the sample, there is no corresponding shoulder in any of the activation spectra, demonstrating that there is no energy transfer from carotenoid to either inactive PChlide or Chlide. This is not surprising since the carotenoids can be removed entirely without altering the activity of the sample.[67]

Weber[102] has formulated a method of enumerating the components having distinct absorption and fluorescence spectra in complex systems by fluorescence spectroscopy. The enumeration is possible because of the constancy of the spectral distribution of the fluorescence for a given substance excited with light of different wavelengths. One determines the number of components in a system from the rank of a matrix of the fluorescence intensities of the system at different excitation wavelengths (the matrix columns) and at different emission wavelengths (the matrix rows).

Applying Weber's technique to these spectra, I found that there were two, but not three components. These are surely inactive PChlide and Chlide.

E. Fluorescence Yield during the Dark Shift

Aggregation of molecules often quenches their fluorescence;[58,103] thus a change in fluorescence yield might be an indication of a change in aggregation.

The relative fluorescence yields of Chlide absorbing at 678 nm and Chlide absorbing at 674 nm in CH (Fig. V-7), as well as their respective inactive PChlides, were 36 for Chlide absorbing at 678 nm, 27 for the associated inactive Pchlide, 33 for Chlide absorbing at 674 nm, and 23 for its inactive PChlide.* So the fluorescence efficiency of Chlide absorbing at 674 nm decreased 8% relative to that of Chlide absorbing at 678 nm. A change this small is probably not significant, especially since the fluorescence efficiency of inactive PChlide decreased also (by about 15%). Thus, neither CD nor fluorescence efficiency reveals any great change in Chlide during the dark shift.

As was mentioned above, the fluorescence maximum also shifted from 684 to 682 nm. Because the dark shift could not be halted during the measurement of the spectra, the order in which they were recorded is important. The absorption spectrum with a maximum at 678 nm was made first after photoconversion, then the fluorescence with a maximum at 684 nm, next the absorption spectrum with a maximum at 674 nm, and finally the fluorescence spectrum with a maximum at 682 nm.

F. Fluorescence Polarization

Polarized light absorbed by an isolated molecule which does not rotate during the lifetime of its excited state will emit polarized light. If, however, the molecule tumbles during the lifetime of the

*Absorption spectra were measured at 5°C.

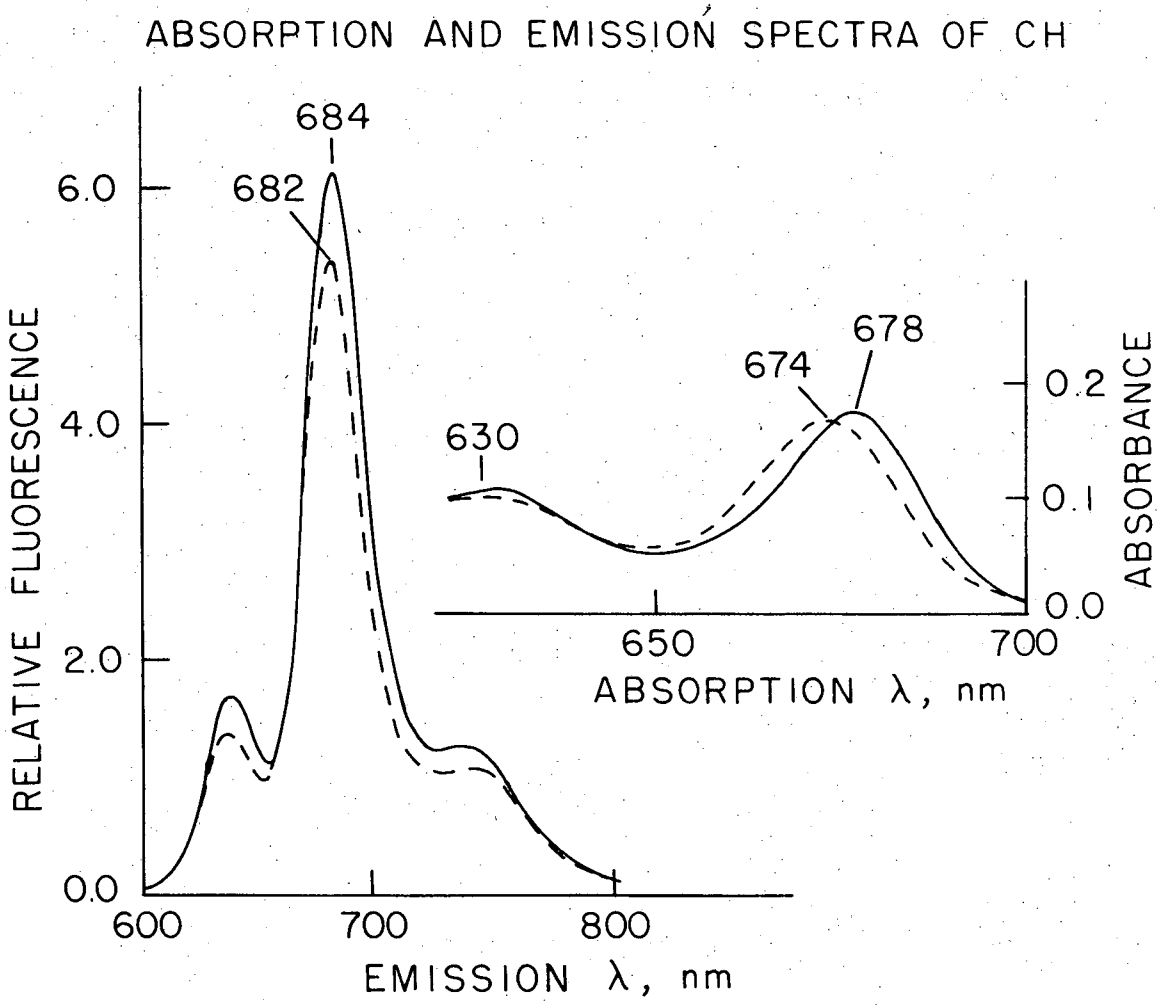

Fig. V-7. Absorption and emission spectra of CH at the beginning (————) and the end (--------) of the dark shift. Excitation was at 440 nm. Optical pathlength: 1.00 cm.

excited state or transfers its energy to another molecule having a different orientation, the emitted light will be depolarized. The extent of depolarization will depend upon the tumbling of the molecule or the orientation of the molecule to which it transfers its energy.

The fluorescence polarization experiment began as an attempt to demonstrate a difference in CH before and after the dark shift. The emission at 680 nm was monitored. The correction factor, N, was 1.07. The CH absorbance at the red maximum was about 0.14 to 0.16*. The fluorescence depolarization spectrum was the same for the Chlide 677 nm form and the dark shifted form of Chlide (Fig. V-8a). These spectra are very much like those obtained by others for nonaggregated Chl a in viscous solvents, such as castor oil, to prevent rotational depolarization of the fluorescence.[27] The similarity between the spectra indicates that Chlide a was probably in a monomeric form in these samples so that there was no energy transfer. The Chlide is also apparently bound tightly enough to something - probably a large protein - to prevent any rotational depolarization. Again, these results tend to support the ideas that Chlide a in CH does not change much during the dark shift, or at least it doesn't after the shift has begun, and that Chlide a in CH is monomeric.

Next, the fluorescence polarization spectrum for CH(suc) (Fig. V-8b), the sample which gave such striking evidence of aggregation

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*Absorption spectra were measured at 5°C.

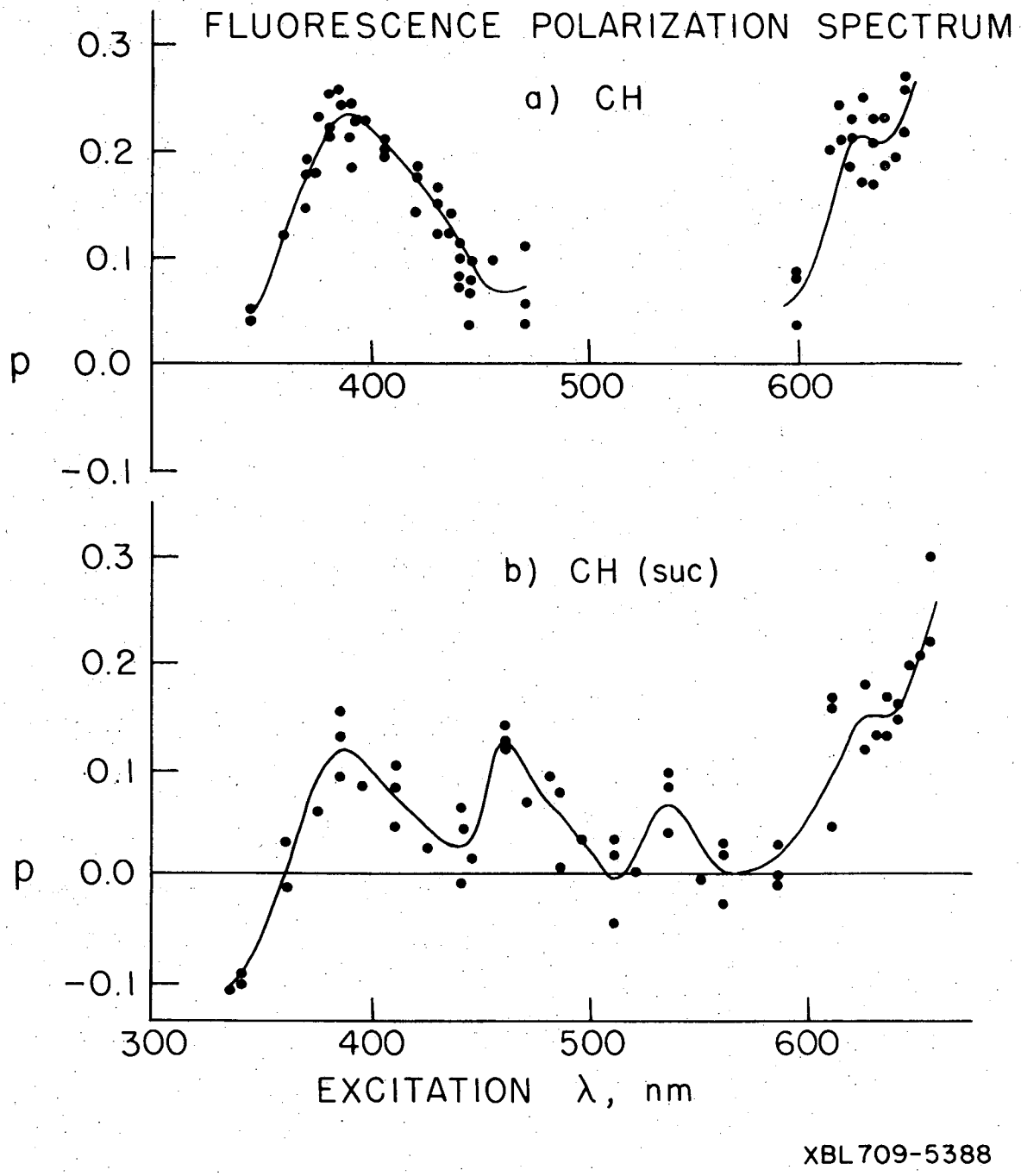

Fig. V-8. Fluorescence polarization spectra. a) CH (emission wavelength = 680 nm); b) CH(suc) (emission wavelength = 682 nm). Optical pathlength: 1.00 cm. Band widths at half-height, approximately 25 nm.

in the CD, was measured.* The emission monochromator monitored at 682 nm. N, the correction factor, was 1.2. The fluorescence polarization spectrum of CH(suc) (Fig. V-8b) is considerably different from that of CH (Fig. V-8a). At most wavelengths the p values for CH(suc) are less than those of CH. The decreased polarization has three possible causes. First, rotation of the chromophore during the lifetime of the excited state might depolarize the fluorescence. But the holochrome is in a fairly viscous medium, 2 M sucrose (at 7°C, η for 2 M $\sim$ 50 to 60 cp; at 20°C, η for castor oil = 986 cp). Also, the chromophore is probably bound to a carrier with a molecular weight of approximately 500,000.[67] Therefore, it seems unlikely that the decreased polarization is being caused by rotation during the lifetime of the excited state. Second, collisions between chromophores on separate holochrome molecules might allow energy transfer between the chromophores, decreasing the polarization. This is an unlikely cause, however, because the solution is viscous, and the concentration of Chl a (absorbance = 0.09 at 678 nm) is low, even lower than it was in the CH solution. The third, and most probable, cause

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*Sucrose solutions do rotate plane polarized light, but the rotation in this case was so small that it had a negligible effect upon the results. For plane polarized light traveling through a 2 M solution of sucrose having an optical path of 5.0 mm, the rotation was calculated from the specific rotations of sucrose given by Lowry[52] to be 1.73° for light having a wavelength of 670.8 nm, and 5.95° for light having a wavelength of 382.6 nm.

is energy transfer between chromophores on the same carrier molecule. This conclusion is consistent with that of Chapter IV, i.e., Chlide a of CH(suc) is aggregated. It is also consistent with the other conclusions of this chapter, i.e., the apparent concentration quenching of Chlide a fluorescence as the photoreduction of active PChlide a proceeds, and the transfer of excitation energy from inactive PChlide a to Chlide a.

Finally, I measured the fluorescence polarization of a sample of PCH(suc). Its absorbance at 639 nm was 0.10. The exciting light beam was attenuated with two neutral density filters (O.D. 0.9 and 1.2) to slow the transformation of PChlide to Chlide. Activation was at 440 nm and emission was measured at 642 nm. The correction factor N was 1.2. The value of p found was +0.06 ± 0.30. The value for PChl a in mineral oil at its blue peak (432 nm) was found by Houssier[37] to be +0.23. Thus the fluorescence from PChlide in the holochrome also appears to be depolarized. And again the cause is probably energy transfer between chromophores on the same holochrome molecule for the same reasons given above for CH(suc).

Goedheer and Smith[83] obtained a polarization value of 0.15 for a glycerine extract of PCH and 0.18 for a glycine buffer extract. In both cases excitation was at 644 nm. The emission was apparently not monochromatized. The PCH sample was transformed by the exciting light; consequently, most of the fluorescence is from Chlide. Because the emission was not monochromatized, there must have been a substantial fraction from inactive PChlide a Sixty percent (v/v) glycerine will halt the dark shift just as sucrose will.[47] Thus

it may be appropriate to compare the polarization value of the glycerine extract of PCH with the polarization value for CH(suc) at 644 nm which was about 0.15, although Goedheer and Smith[83] did not give the positions of any absorption maxima for their experiment. Since the polarization of the glycine extract was measured immediately upon transformation, the dark shift has probably not begun yet, although the polarization of this sample was slightly greater, 0.18. The polarization of my CH sample, which was phototransformed outside the spectrometer, was 0.21 $\pm$ .02, still greater. On the other hand, these values are all less than the 0.286 given by Goedheer and Smith for Chl _a_ in castor oil at this excitation wavelength. It may, however, be deceptive to use only one activation wavelength to compare the fluorescence polarization of chlorophyll in plant materials and organic solvents, because p can change rapidly with wavelength, and the transitions in plant materials are usually shifted relative to those in organic solvents. In any case, Goedheer and Smith[83] concluded that because the fluorescence from the holochrome was more depolarized than that from chlorophyll in castor oil, and because they believed that there was only one pigment molecule per holochrome, the most plausible explanation was that the chromophore was free to rotate within the molecule. In view of the more recent work of Schopfer and Siegelman,[67] and others,[41,76] as well as the results reported in this chapter and Chapter IV, it seems likely that there can be more than one chromophore per holochrome. Consequently, energy transfer between Chlide molecules seems to be the most likely explanation for the lowered polarization.

Goedheer and Smith[30] also measured the fluorescence polarization of holochrome extracted from leaves at different stages of greening with glycine buffer containing 20% glycerol, pH 9.5. The polarization decreased as the greening proceeded. They then extrapolated their values to zero Chl(ide) concentration, finding a p of 0.154. The Chl(ide) concentration was measured by the absorbance of the homogenate at 670 nm at the chlorophyll peak. This value of 0.15 is considerably lower than the value 0.21 which I found for CH. Their illumination periods were 0.0 (this sample was photoreduced in the spectrometer), 0.08, 1.00, 5.86, and 17.33 hrs. The p values of even the earliest samples showed a downward trend. If the chlorophyll absorption maximum was really at 670 nm after 17.33 hrs of illumination, it is in substantial disagreement with my finding in Chapter II that the absorption maximum had shifted to 678 nm after only 13 hrs of illumination.

More recently, Losev and Gurinovich[51] reported polarization spectra measured at -100°C and 20°C using excitation wavelengths from 400 to 600 nm. They examined homogenates prepared from etiolated barley seedlings which had been illuminated immediately before homogenation. The polarization spectrum was qualitatively similar to the spectrum of Chl _a_ in castor oil. The spectra of the homogenates were identical at -100°C and 20°C, leading them to the conclusion that there is no rotational depolarization of the fluorescence. The polarization values of the homogenates were only 20% less than those of Chl _a_ in castor oil. From this result, Losev and Gurinovich[51] concluded that at the start of the lag phase in chlorophyll synthesis the molecules of Chl _a_ are uncoupled.

In conclusion, my fluorescence polarization spectrum for CH (Fig. V-8a) agrees with those of Losev and Gurinovich.[51] And the spectrum for CH(suc) (Fig. V-8b) agrees at 644 nm with the p value obtained by Goedheer and Smith[83] for a glycerine extract of PCH which is phototransformed in the spectrometer during the measurement. However, all the p values obtained by Goedheer and Smith[83,30] are less than those found by me for CH and those found by Losev and Gurinovich[51] for homogenates prepared from leaves immediately after illumination.

G. Summary

The following results indicate that CH(suc) and PCH(suc) contain chromophores close enough to interact. First, in CH(suc) energy absorbed by inactive PChlide excites Chlide fluorescence. Second, the polarizations of CH(suc) and PCH(suc) fluorescence are lower than they are for Chl a and PChl a in viscous solvent. Third, the fluorescence yield of Chlide a decreases as the phototransformation of PChlide a proceeds.

The fluorescence polarization spectrum of CH resembles that of Chl a in viscous solvents. The similarity of these spectra indicates that the Chlide a of CH cannot rotate on its carrier. It also indicates that the Chlide a of CH is monomeric.

The CH polarization spectrum is the same whether measured toward the beginning or the end of the dark shift. Similarly, the fluorescence yield apparently changes little during the dark shift.

In all, the above findings support those obtained from the CD measurements reported in Chapter IV.

The fluorescence yield of active PChlide is low relative to that of inactive PChlide. This probably means that energy received by active PChlide is efficiently utilized in phototransformation.

Energy transfer from carotenoid to either inactive PChlide a or Chlide a could not be detected from the excitation spectra of CH(suc).

VI. THE QUANTUM REQUIREMENTS FOR THE TRANSFORMATION OF PCH(suc) TO CH(suc)

A. Introduction

The quantum requirement for the photoreduction of PChlide to PCH would be very helpful in formulating a mechanism for the reaction and a model for PCH. It was determined by Smith[86] more than a decade ago. The absorption maxima of his holochrome preparations were positioned at 636 nm before transformation, and 674 nm after transformation. He obtained a quantum requirement of 1.7 quanta of light absorbed by active PChlide per molecule of Chlide formed, assuming no energy transfer from Chlide or inactive PChlide to active PChlide. This value was an average of four ranging from 1.43 to 2.09. Smith considered it likely that the transformation is a one-quantum process with an over-all efficiency of 0.6.[82]

The initial rate of photoconversion of PChlide *a* to Chlide *a* is directly proportional to the incident light intensity.[84]

Smith and Benitez,[84] and Boardman[7] found that the photoreduction of PChlide *a* to Chlide *a* does not follow simple first order kinetics in an etiolated leaf and in holochrome preparations. However, Sironval *et al.*[75] found that the photoreduction follows first order kinetics in etiolated bean leaves when the illumination is with 647 nm, or longer wavelength photons, and non-first order

kinetics when the illumination is with 630 nm, or shorter wavelength photons.

Smith and Benitez[84] reported no phototransformation of etiolated barley leaves at -195°C, but fairly rapid conversion at -70°C. The rate of conversion continues to increase up to 40°C. Five minutes at 55°C will almost completely destroy phototransformation activity.

Boardman[7] obtained essentially the same results for the temperature dependence of photoreduction of PChlide a to Chlide a in a holochrome preparation.

B. Materials and Methods

PCH(suc), except where noted, was made by sedimentation in the same manner as that in IV-B. All absorption spectra were measured using a Cary Model 14 spectrophotometer having a modified Model 1462 scattered transmission accessory (photomultiplier tube RCA #6217) as described by Sauer and Biggins[64] at ambient temperature (21°C). Side (actinic) illumination was provided by a Bausch and Lomb grating monochromator (500 mm model, dispersion 33A/mm) with at 750 nm blaze wavelength grating and a 1200 (General Electric DHT projection lamp) source operated at 100 $\pm$ 2 volts. The entrance and exit slits of the monochromator were 1.03 mm and 1.00 mm, respectively. In addition, the actinic light passed through a Corning sharp cut filter (C.S. #3-66; transmission $\geq$ 80% from 608 nm to 750 nm and $<$0.5% at wavelengths shorter than 547 nm). Light intensity

measurements were made with a photovoltaic cell (Silicon Cell-120 CG) 3 days before the experiments, which took two days, and again the day after them. The two light intensity measurements were in excellent agreement. The experiments were performed 2/9/70 and 2/10/70. The photovoltaic cell was calibrated on 3/8/66 against a thermopile which was calibrated with an incandescent electric lamp standardized by the National Bureau of Standards.

A typical experiment (see Fig. VI-2) consisted of: (1) measuring the absorption spectrum from 720 nm to 600 nm of an unilluminated PCH sample (pathlength 10.0 mm); (2) illuminating with actinic light for 10 sec (pathlength 5.00 mm); (3) measuring the absorption spectrum again; (4) illuminating 10.0 sec more; (5) remeasuring the absorption spectrum, etc. The illumination periods were lengthened as the transformation of PChlide to Chlide proceeded. A typical set of illumination periods was 10.2 sec, 10.0 sec, 15.0 sec, 29.7 sec, and 60.2 sec. These periods were measured by operating a stop watch with one hand and the monochromator light source switch with the other. The total duration of each experiment was about 1 hour. The duration might be significant, since the sample was not refrigerated during this period, and its activity may have declined. However, 51% of one sample was transformable following weak illumination for about 1 hour at ambient temperature, compared to 49% of another sample illuminated immediately with a strong light.

To convert all of the active PChlide to Chlide, the PCH preparation was illuminated with a floodlamp at about 0°C for 7 sec, as was related earlier (section III-B).

The pigment content of each sample was determined by mixing 1.00 ml of it with 4.00 ml of acetone (Baker Analyzed Reagent), then filtering the mixture through filter paper (No. 1 Whatman). The spectrum of each acetone extract was then recorded, and the pigment concentrations determined from the following equations:

$$\text{Chlide } \underline{a}\ \left(\frac{\mu\text{moles}}{\ell}\right) = 14.0\ A_{664} - 0.150\ A_{627} \qquad \text{(VI-1)},$$

$$\text{PChlide } \underline{a}\ \left(\frac{\mu\text{moles}}{\ell}\right) = -8.85\ A_{664} + 48.8\ A_{627} \qquad \text{(VI-2)}.$$

The pathlengths are 1.00 cm. The millimolar extinction coefficients were determined from spectra of Chl _a_ and PChl _a_ (the compounds were isolated by C. Houssier[36] in 80% aqueous acetone. The concentrations of pigments were determined by first measuring their absorbances in ether, then calculating the concentrations using molar extinction coefficients of $\varepsilon_{622} = 22 \times 10^3\ \left(\frac{\ell}{\text{mole-cm}}\right)$ for PChl _a_ in ether,[37] and $\varepsilon_{661} = 86.3 \times 10^3\ \left(\frac{\ell}{\text{mole-cm}}\right)$ for Chl _a_ in ether.[89] Next, the ether was carefully evaporated, after which the pigments were again dissolved in volumes of 80% aqueous acetone equal to the ether evaporated. Finally, the absorbances in 80% aqueous acetone were measured, and the extinction coefficients in this solvent calculated. These values are compared in Table V-1 with those given by Anderson and Boardman[4] for PChlide _a_ in 80% aqueous acetone and those of Mackinney[53] for Chl _a_ in 80% aqueous acetone. It was assumed that Anderson and Boardman[4] obtained their values with PChlide _a_. The molar extinction coefficients for PChl _a_, obtained here, were used in Eqns. (VI-1) and

Table VI-1. Maxima and molar extinction coefficients for the absorption spectra of PChl(ide) a and Chl(ide) a in 80% acetone/20% water.

				PChl(ide) a				
Wavelength (nm)	664	627*	575*	535*	436*	431	412.5	This work
$\varepsilon \times 10^{-3}$ ($\frac{\ell}{\text{mole-cm}}$)	0.21	20.5	7.79	---	141	123	47.1	
Wavelength (nm)	663	626*	---	---	---	---	---	Anderson &
$\varepsilon \times 10^{-3}$ ($\frac{\ell}{\text{mole-cm}}$)	0.12	20.8	---	---	---	---	---	Boardman[3] **
				Chl(ide) a				
Wavelength (nm)	664*	627	619*	575	436	431*	412.5*	This work
$\varepsilon \times 10^{-3}$ ($\frac{\ell}{\text{mole-cm}}$)	68.4	12.4	---	7.6	69.3	77.1	67.1	
Wavelength (nm)	663*	626	---	---	---	---	---	Mackinney[53]
$\varepsilon \times 10^{-3}$ ($\frac{\ell}{\text{mole-cm}}$)	73.3	13.3	---	---	---	---	---	

* Signifies a maximum.

**It was assumed that Anderson and Boardman's measurements were made using PChlide a.

(VI-2). But for Chl a, averages of my values and Mackinney's values[53] were used, i.e., 70.8×10^3 ($\frac{\ell}{\text{mole-cm}}$) at 664 nm and 12.9×10^3 ($\frac{\ell}{\text{mole-cm}}$) at 627 nm.

C. Results and Conclusions

On the basis of analysis of three different holochrome samples,* 1 mole of PChlide a was found to disappear for 0.91 ± 0.21, 0.73 ± 0.07, and 0.46 ± 0.12 moles of Chlide a appearing (Fig. VI-1). The source of the wide variation between samples is not known. However, the pigment concentrations were greatest in the sample where 0.91 ± 0.21 moles of Chlide a appeared for each mole of PChlide a which disappeared (see Fig. VI-1). It will be assumed here that 1 molecule of PChlide is phototransformed to 1 molecule of Chlide. Using etiolated barley leaves, Smith[77] reported that 0.8 to 0.9 mole of Chlide a appears for each mole of PChlide which disappears. Changing Smith's values to conform to my assumption (Chapter III-A) that he actually determined the specific absorption coefficient of PChlide a rather than PChl a, one obtains 0.54 to 0.60 moles of Chlide a appearing for each mole of PChlide a disappearing. More recently, Granick and Gassman[25] reported stoichiometric photoconversion of PChlide a into Chlide a in whole leaves. These authors used a molar extinction coefficient of $\varepsilon_{623} = 35.6 \times 10^3$ ($\frac{\ell}{\text{mole-cm}}$) in ether. Using the value

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*PCH(suc) prepared by ultrafiltration (slope = 0.91 ± 0.21), PCH crude extract (slope = 0.73 ± 0.07), PCH(suc) prepared by sedimentation (slope = 0.46 ± 0.12).

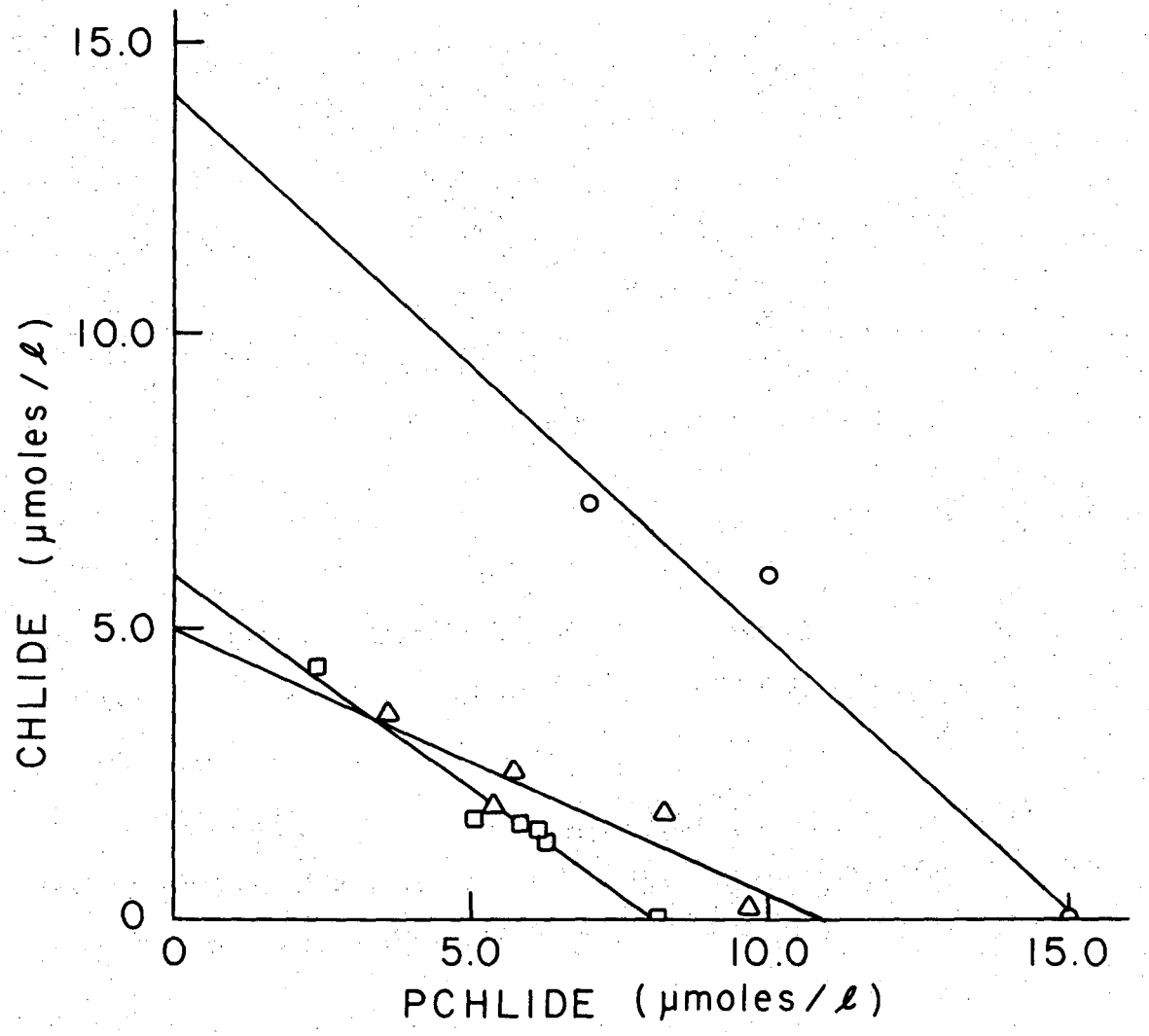

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Fig. VI-1. Pigment concentrations in three different samples of PCH at different stages of transformation. Each point represents a separate determination by 80% aqueous acetone extraction. □: PCH crude extract; slope = 0.73 ± 0.07 $\frac{\mu\text{mole Chlide}}{\mu\text{mole PChlide}}$; △: PCH(suc) prepared by sedimentation; slope = 0.46 ± 0.12 $\frac{\mu\text{mole Chlide}}{\mu\text{mole PChlide}}$; ○: PCH(suc) prepared by ultrafiltration; slope = 0.91 ± 0.21 $\frac{\mu\text{mole Chlide}}{\mu\text{mole PChlide}}$.

I have assumed to be correct, 22×10^3 ($\frac{\ell}{\text{mole-cm}}$), one obtains 0.64 moles of Chlide a appearing for each mole of PChlide a disappearing. If the extinction coefficients used for PChl(ide) a are incorrect, the number of moles of Chlide a forming for each mole of PChlide a disappearing would be increased by about 35%.

The spectra taken at intervals during the progressive transformation of PCH(suc) to CH(suc) by the actinic wavelengths 630.0 nm, 640.0 nm or 650.0 nm or a floodlamp are shown in Fig. VI-2. They are very similar for each of the three actinic wavelengths. Each set appears to have isosbestic points at 654 nm, 630.0 nm and 615 nm.

Other authors have also found an isosbestic point at about 650 nm. Boardman's spectra[7] for the transformation of PChlide in PCH apparently have an isosbestic point at about 650 to 655 nm. Spectra for the transformation in old etiolated leaves published by Shibata[71] also appear to have an isosbestic point at a wavelength slightly greater than 650 nm.

The presence of an isosbestic point at 654 nm indicates that there is only one form of active PChlide and one Chlide product (transformation of a single compound to a single compound).[71,39] The invariance of the isosbestic points for the 3 actinic illumination wavelengths and white light appears to be further evidence supporting this contention.

The presence of an isosbestic point seems to exclude the conclusion of Kahn et al.,[41] that there are two active forms of PChlide having absorption maximum at 637 nm and 650 nm, respectively.

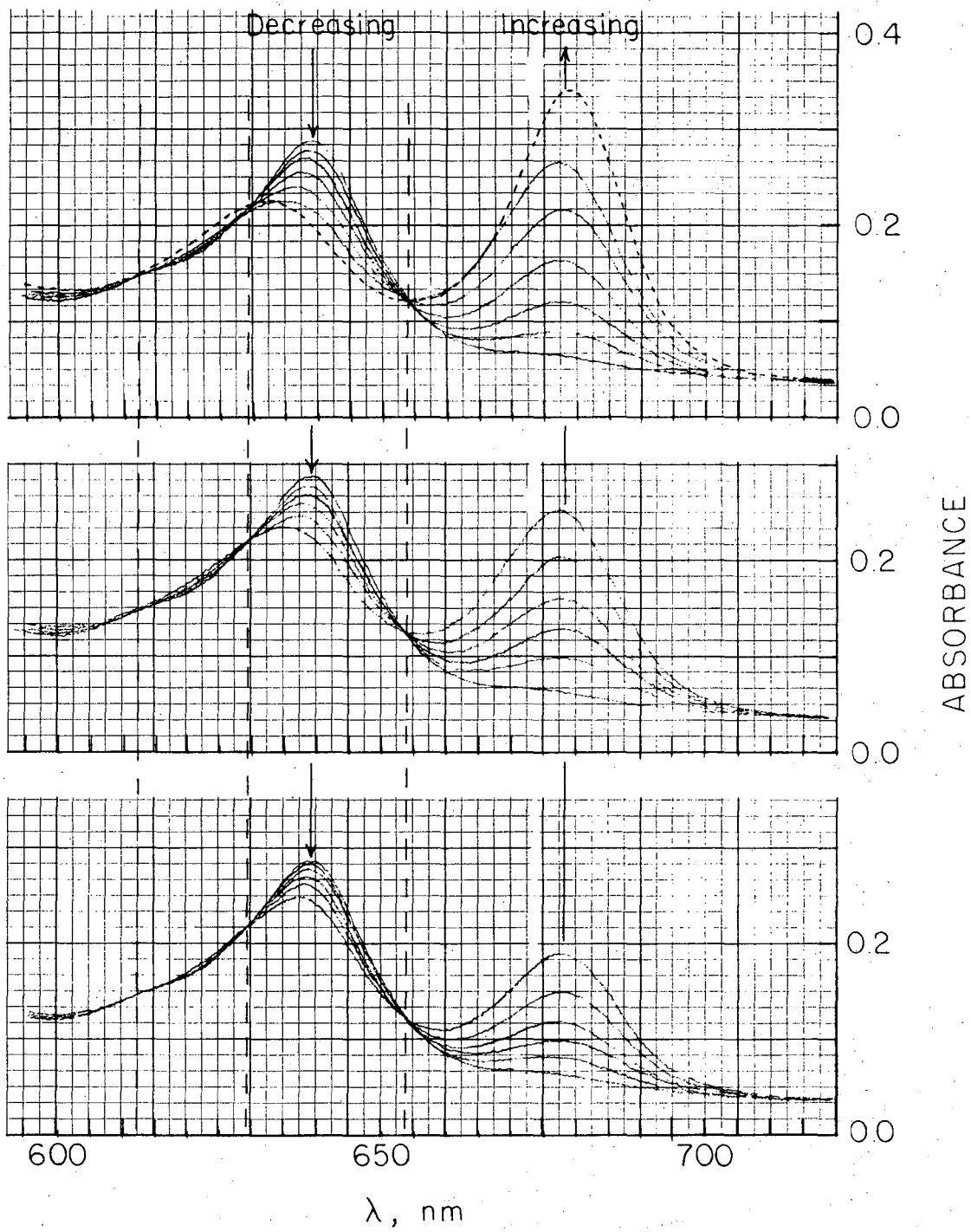

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Fig. VI-2. Absorption spectra taken at intervals during the progressive transformation of PCH(suc) to CH(suc). Actinic wavelength bottom set of spectra: 630.0 nm; middle set: 640.0 nm; upper set: 650.0 nm, with exception of (-----) which was total floodlamp conversion of separate portion of sample. Dashed vertical lines indicate isosbestic points.

Figure VI-3 is a semi-logarithmic plot of the total light energy incident on the sample vs the % of active PChlide remaining. The method in which % active PChlide was determined is explained in Appendix I. The plots begin at 96% active PChlide remaining instead of 100% active PChlide remaining, because 4% of the active PChlide was transformed to Chlide during the preparation of the PCH(suc) sample. Sironval et al.[75] found that the kinetics of the phototransformation in the whole leaves were first order, when they illuminated with 647 nm photons, and the sum of two first order processes when the actinic illumination was at 630 nm. They proposed a model for interpreting the data which postulates energy transfer from inactive to active PChlide.

Quantum requirements were calculated by several different methods of increasing sophistication and accuracy.

Preliminary quantum requirements were determined using the following expression:

$$\text{Quantum requirement} = \frac{\bar{F}\, Q\, \Delta t}{\Delta C_a} \qquad \text{(VI-3)},$$

where: Q = density of actinic photons incident on the sample in $\frac{\text{Einsteins}}{\text{cm}^3\text{-sec}}$,

Δt = illumination period in sec,

ΔC_a = Chlide *a* concentration in $(\frac{\text{moles}}{\text{ml}})$ formed in Δt,

and $\bar{F}$ = the mean of the fractions of actinic light absorbed at the beginning and at the end of an illumination period Δt.

$$\bar{F} = \frac{1}{2}\left\{2 - \frac{1}{\text{antilog}\,(\frac{\text{initial absorbance}}{2})} - \frac{1}{\text{antilog}\,(\frac{\text{final absorbance}}{2})}\right\}.$$

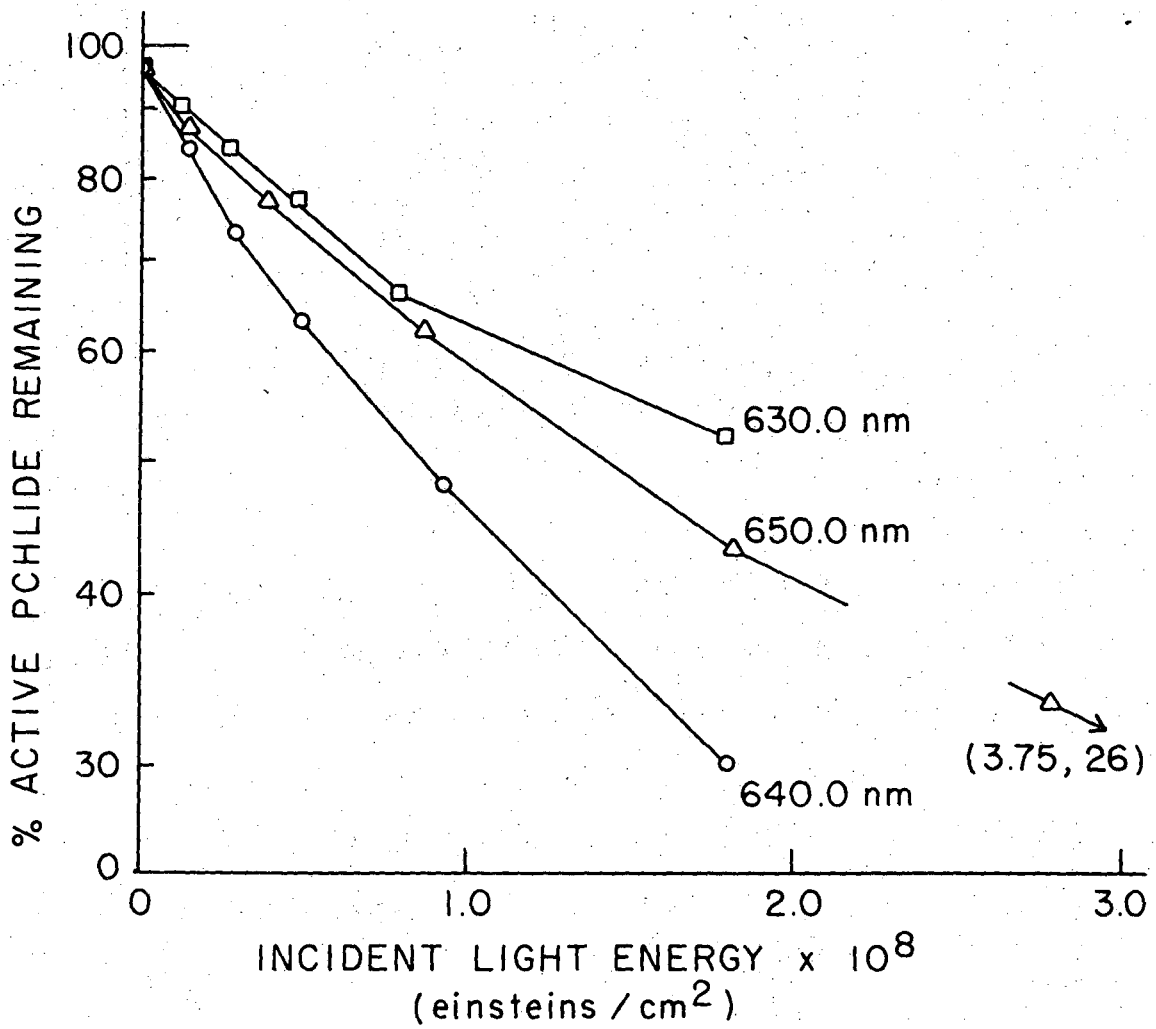

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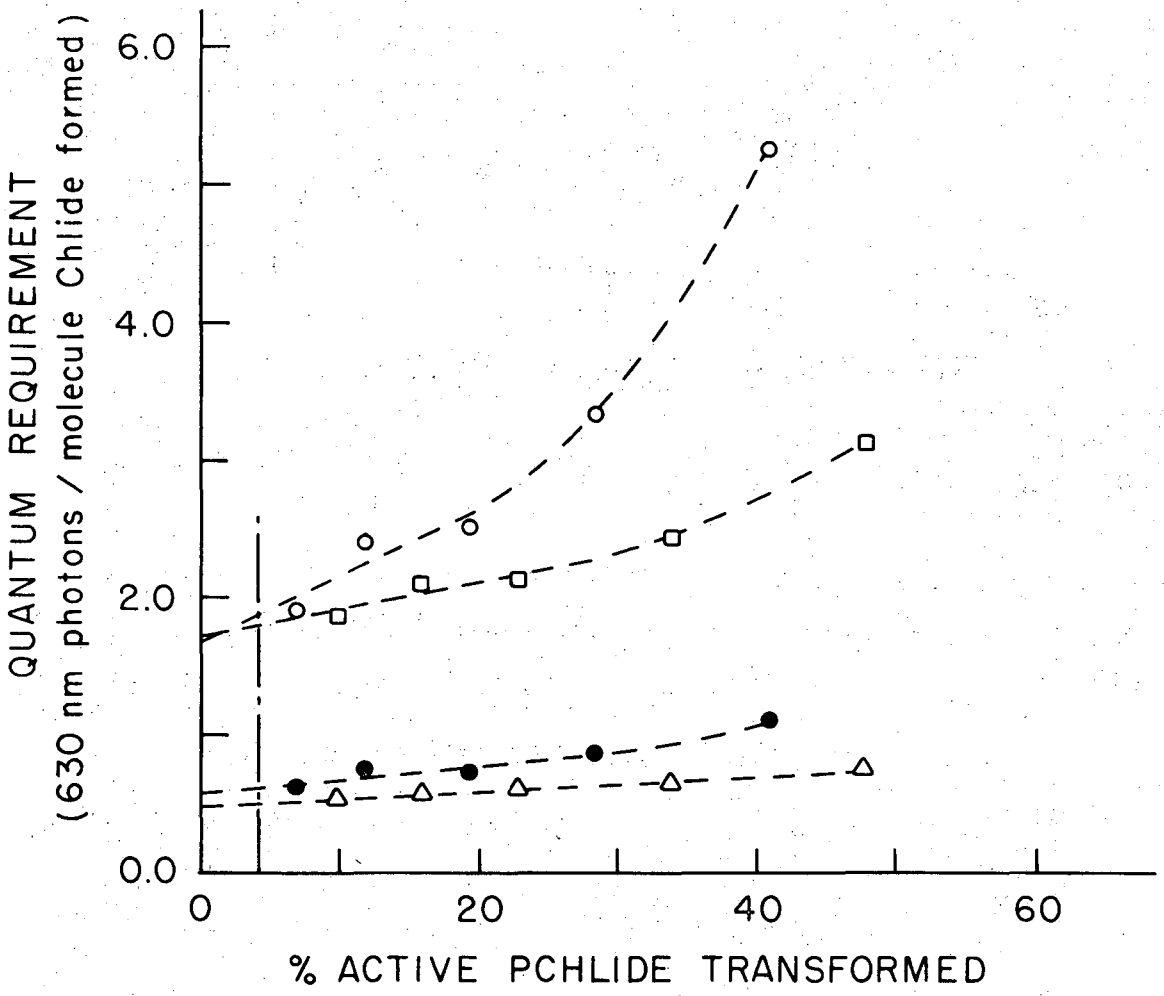
Fig. VI-3. Semi-logarithmic plot of total light energy incident on 3 portions of one sample vs the % active PChlide a remaining. The method of determining % active PChlide remaining is explained in Appendix I. Incident light was 630.0 nm (□), 640.0 nm (○), and 650.0 nm (△). 4% of the active PChlide was transformed during the preparation of the sample.

ΔC_a was determined from the millimolar extinction coefficient of Chlide *a* in CH(suc), which in our case was 66.0 at 678 nm. The detailed procedure for the determination of this value is given in Appendix I. There are no corrections in Eqn. (VI-3) for the proportion of the actinic light absorbed by Chlide *a* or inactive PChlide *a*, *i.e.*, this light is assumed to be 100% effective for phototransformation. The quantum requirements calculated from Eqn. (VI-3) are plotted for actinic wavelengths 630.0 nm, 640.0 nm, and 650.0 nm in Figs. VI-4 (o), VI-5 (o), and VI-6 (o). Extrapolated to 0% active PChlide transformed, they are 1.66 (quanta per molecule of Chlide *a* formed) for 630.0 nm illumination, 1.22 for 640.0 nm illumination, and 0.91 for 650.0 nm illumination.

A correction was applied to Eqn. (VI-3) for the case where the light absorbed by Chlide is not photochemically active. The effect upon the quantum requirements during the early stages of transformation was negligible. The maximum correction was on the last point for 650.0 nm light, where the calculated quantum requirement was decreased approximately 30%. In any case, the quantum requirements continued to increase with increasing percent transformation.

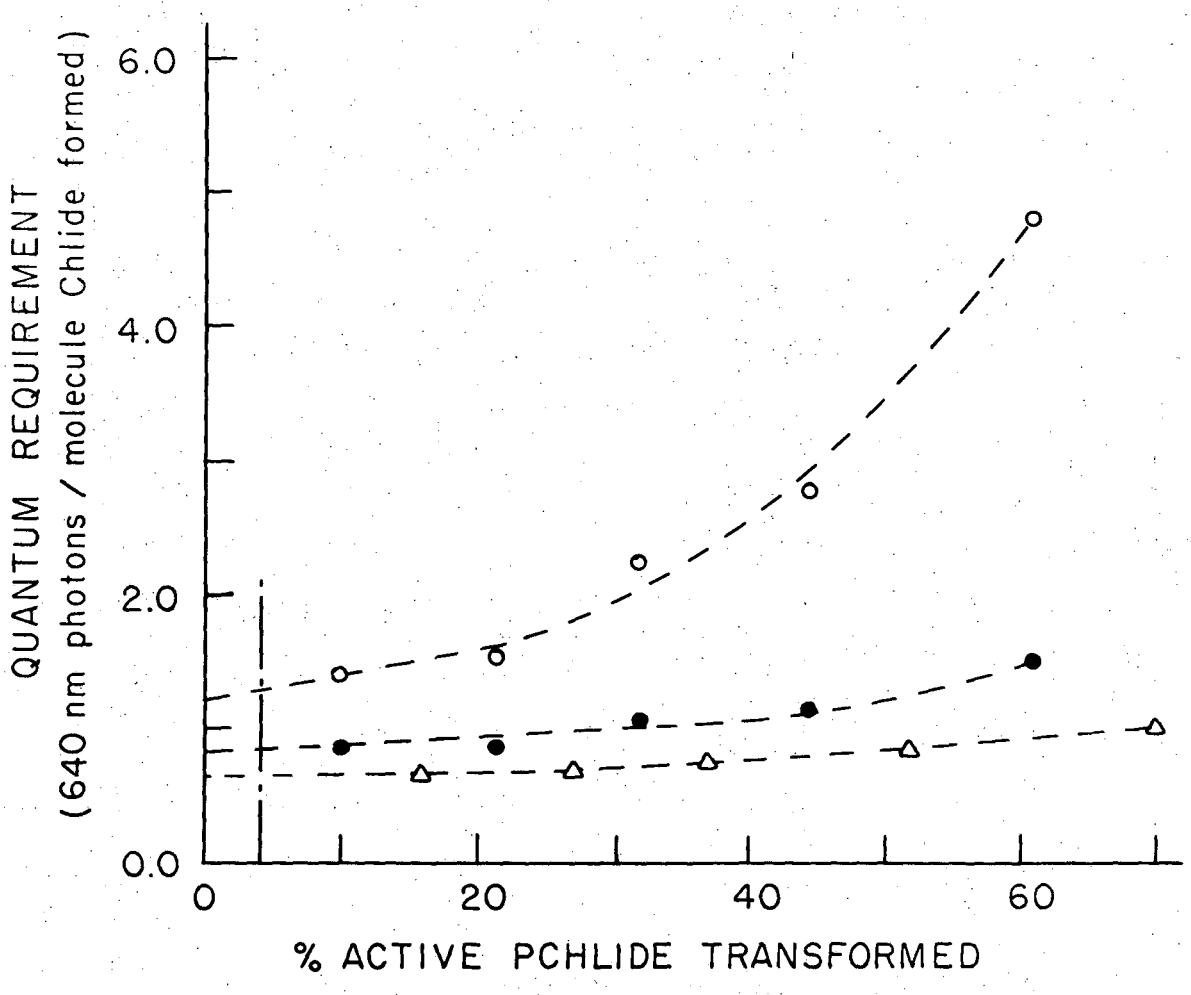
Eqn. (VI-3) was then corrected for both the absorption of Chlide *a* and the absorption of inactive PChlide *a* at the actinic wavelength. This is equivalent to assuming that both inactive PChlide *a* and Chlide *a* are photochemically inactive. The relation for this determination of the quantum requirement is

$$\text{Quantum requirement} = \frac{\overline{F}'Q\ \Delta t}{\Delta C_a} , \qquad \text{(VI-4)}$$



XBL 709-5397

Fig. VI-4. Quantum requirements for 630.0 nm photons calculated from Eqn. (VI-3): (○), Eqn. (VI-5): (□), Eqn. (VI-4): (●), and Eqn. (VI-6): (△). Vertical dash-dot line indicates % active PChlide a transformed during the preparation of sample.



XBL 709-5399

Fig. VI-5. Quantum requirements for 640.0 nm photons calculated from Eqn. (VI-3): (○), Eqn. (VI-4): (●), and Eqn. (VI-7): (△). Vertical dash-dot line indicates % active PChlide a transformed during the preparation of the PCH(suc) sample.

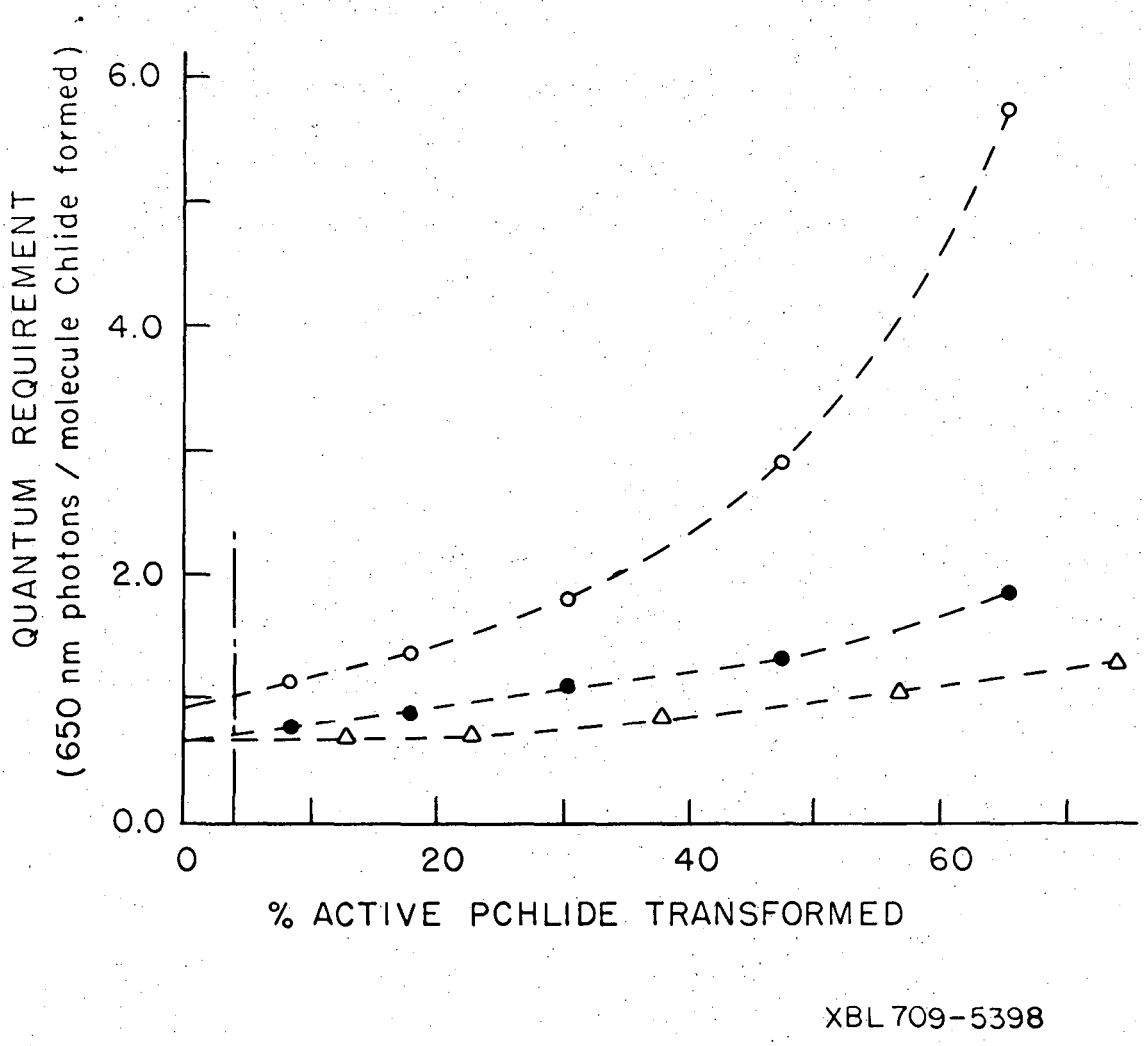


Fig. VI-6. Quantum requirements for 650.0 nm photons calculated from Eqn. (VI-3) (○), Eqn. (VI-4): (●), and Eqn. (VI-7): (△). Vertical dash-dot line indicates % active PChlide a transformed during the preparation of the PCH(suc) sample.

where: Q, Δt, ΔC_a are as defined for Eqn. (VI-3), and

$\bar{F}'$ = the mean of the fractions of actinic light absorbed by active PChlide *a* at the beginning and at the end of an illumination period Δt

$$= \frac{1}{2}\left\{2 - \frac{1}{\text{antilog}\left(\frac{A_i}{2}\right)} - \frac{1}{\text{antilog}\left(\frac{A_f}{2}\right)}\right\} ,$$

where: A_i = the absorbance of active PChlide *a* at the actinic wavelength at the beginning of Δt

$$= a - \frac{B}{B_{678}}(C_{678}) - \left[D - \frac{B}{B_{678}}(E_{678})\right] ,$$

and A_f = the absorbance at the actinic wavelength at the end of Δt

$$= a' - \frac{B}{B_{678}}(C'_{678}) - \left[D - \frac{B}{B_{678}}(E_{678})\right] .$$

where: a = total absorbance at the beginning of Δt at the actinic wavelength (= initial absorbance in Eqn. (VI-3)),

a' = total absorbance at the end of Δt at the actinic wavelength (= final absorbance in Eqn. (VI-3)),

B = absorbance of Chl *b* minus barley mutant at the actinic wavelength,

B_{678} = absorbance of Chl *b* minus barley mutant at its red maximum, 678 nm,

C_{678} = absorbance of the sample at 678 nm at the beginning of Δt,

C'_{678} = absorbance of the sample at 678 nm at the end of Δt,

D = absorbance of the completely transformed sample at the actinic wavelength, and

E_{678} = absorbance of the completely transformed sample at 678 nm.

Quantum requirements calculated with Eqn. (VI-4) are plotted, as the photoreaction proceeded, in Fig. VI-4 (•) for 630.0 nm actinic photons, in Fig. VI-5 (•) for 640.0 nm actinic photons, and in Fig. VI-6 (•) for 650.0 nm actinic photons. The curves extrapolate to approximately 0.58 quanta/molecule, 0.85 quanta/molecule, and 0.66 quanta/molecule at the beginning of the photoreaction for 630.0 nm, 640.0 nm, and 650.0 nm illumination, respectively. The increase in the quantum requirements, as the photoreaction proceeds, is less than it is using Eqn. (VI-3). Because the quantum requirement should remain constant, Eqn. (VI-4) is probably a better description of the photoreaction than Eqn. (VI-3).

Another method was used to determine the quantum requirement. It is essentially an exact treatment instead of the approximate treatment used in Eqn. (VI-3) and Eqn. (VI-4). It follows quite closely the method described by Kling et al.,[45] for a first order photoreaction where both reactant and product absorb photochemically effective wavelengths and there is incomplete absorption in the reaction vessel.

Deriving an equation (Appendix II) for the case of 630.0 nm actinic illumination, the wavelength where an isosbestic point occurred in the spectra at successive stages of transformation (Fig. VI-2), one obtains, assuming complete transfer of energy from inactive PChlide to active PChlide, but none from Chlide to active PChlide:

$$\text{Quantum requirement} = \frac{\bar{Q}_o \, t \, x_A^\lambda \left(1 - e^{-[x_A^\lambda a_o + x_P^\lambda p_o]d}\right)}{(x_A^\lambda a_o + x_P^\lambda p_o) \log\left(\frac{x_A^\lambda a_o + x_P^\lambda p_o}{x_A^\lambda a + x_P^\lambda p_o}\right)} , \quad \text{(VI-5)}$$

and for no energy transfer from either inactive PChlide or Chlide a to

active PChlide:

$$\text{Quantum requirement} = \frac{\overline{Q}_o \; t \; \chi_A^\lambda \; (1-e^{-[\chi_A^\lambda a_o + \chi_P^\lambda p_o]d})}{(\chi_A^\lambda a_o + \chi_P^\lambda p_o) \log\left(\frac{a_o}{a}\right)} \quad . \quad \text{(VI-6)}$$

The expression (derived in Appendix II) for the case at wavelengths other than at the isosbestic points, and assuming no energy transfer from either inactive PChlide or Chlide to active PChlide, is:

$$\text{Quantum requirement} \cong \overline{Q}_o \; d \; \chi_A^\lambda \; t \left\{ \log \frac{(1-A)(n)}{(A)(1-n)} - \frac{1}{2(1-m)} \right.$$

$$\cdot \log \frac{a^2 \left[-C^2 a_o^2 + C(1-2m)a_o + m(1-m)\right]}{a_o^2 \left[-C^2 a^2 + C(1-2m)a + m(1-m)\right]}$$

$$\left. + \frac{(1-2m)}{2(1-m)} \cdot \log \frac{[Ca+m][1-Ca_o-m]}{[1-Ca-m][Ca_o+m]} \right\}^{-1} \quad . \quad \text{(VI-7)}$$

where in Eqns. (VI-5), (VI-6), and (VI-7) $\overline{Q}_o$ represents the time average incident quantum density in $\frac{\text{mole}}{\ell \cdot \text{sec}}$; χ_A^λ , χ_B^λ and χ_P^λ are the natural extinction coefficients respectively for active PChlide, Chlide and inactive PChlide at the actinic wavelength in $\frac{\text{mole}}{\ell \cdot \text{cm}}$, ($\varepsilon = 0.4343\chi$); d is the pathlength for the actinic light in the reaction vessel (5.00 mm); and a, b, and p_o are respectively the concentrations of active PChlide, Chlide, and inactive PChlide at time t in mole/ℓ ; a_o is the concentration of active PChlide at time t = 0; $A = [(\chi_A^\lambda - \chi_B^\lambda)a + \chi_B^\lambda a_o + \chi_P^\lambda p_o]d$; $n = (\chi_A^\lambda a_o + \chi_P^\lambda p_o)d$; $m = (\chi_B^\lambda a_o + \chi_P^\lambda p_o)d$; and $C = (\chi_A^\lambda - \chi_B^\lambda)d$.

The following experimental quantities, obtained directly from the holochrome solution spectra, were substituted into Eqns. (VI-5), (VI-6) and (VI-7):

A = measured absorbance of the sample at time t at the actinic wavelength · $[2(0.4343)]^{-1}$,

n = measured absorbance of the unilluminated sample at the actinic wavelength · $[2(0.4343)]^{-1}$, and

m = measured absorbance of the fully transformed sample at the actinic wavelength · $[2(0.4343)]^{-1}$.

Quantities involving a (a and a_o were obtained as described in Appendix I) were:

$$C = \frac{A-m}{a} \quad \text{and}$$

$$x_A^{\lambda} = \frac{\text{Abs. Chl } b^- \text{ mutant at actinic } \lambda}{\text{Abs. Chl } \underline{b}^- \text{ mutant at 678 nm}} \cdot \frac{\varepsilon_B^{678}}{(0.4343)} + \frac{C}{d} \quad .$$

Finally,

$$x_A^{\lambda} a + x_P^{\lambda} p_o = 2A - \frac{\text{Abs. Chl } b^- \text{ mutant at actinic } \lambda}{\text{Abs. Chl } \underline{b}^- \text{ mutant at 678 nm}} \left[\frac{\text{in vivo abs. of sample}}{\text{at time, } t \cdot (0.4343)^{-1}}\right]$$

The quantum requirements at 630.0 nm determined using Eqn. (VI-5) [Fig. VI-4 (□)] extrapolate at 0% transformation to 1.70, about the same value at 0% active PChlide transformed as the quantum requirements calculated using Eqn. (VI-3) for 630.0 nm actinic photons.

The quantum requirements determined from Eqn. (VI-5), however, increase much less rapidly as the photoreaction proceeds. Because the quantum requirement should remain constant during the reaction, Eqn. (VI-5) probably describes the photoreaction more accurately than Eqn. (VI-3). Eqn. (VI-3) assumes both Chlide a and inactive PChlide, as well as active PChlide, are photochemically active, but Eqn. (VI-5) assumes that only inactive and active PChlide are photochemically

active. In addition, in terms of % increase in quantum requirements as the reaction proceeds, the quantum requirements from Eqn. (VI-5) also increase less rapidly than those from Eqn. (VI-4), which was the preliminary treatment assuming both inactive PChlide and Chlide to be ineffective photochemically.

Finally, of all the quantum requirements, at 630.0 nm, those obtained through Eqn. (VI-6) [Fig. VI-4: (Δ)] increase least rapidly. They extrapolate to a value of 0.48 photons per molecule Chlide formed.

Eqn. (VI-7) extrapolates (at 0% transformation) to a quantum requirement for PChlide to Chlide transformation of 0.60 for 640.0 nm illumination and 0.66 for 650.0 nm illumination. The quantum requirements extrapolated to 0% transformation are summarized in Table VI-2.

Table VI-2. Quantum requirements for PChlide to Chlide transformation (extrapolated to 0% transformation).

Actinic (nm)	Eqn. (VI-3)	Eqn. (VI-4)	Eqn. (VI-5)	Eqn. (VI-6)	Eqn. (VI-7)
630.0	1.66	0.58	1.70	0.48	---
640.0	1.22	0.85	---	---	0.60
650.0	0.91	0.66	---	---	0.66

Eqns. (VI-6) and (VI-7) give the most meaningful analysis of the kinetics of the photoreaction of the equations considered, i.e., the

quantum requirement is most nearly a constant during the course of phototransformation.

Even if the molar extinction coefficients for PChlide a assumed here are incorrect, the quantum requirements would be increased by only 18% (see Appendix II), through alteration of ΔCa in Eqns. (VI-3) and (VI-4) or χ_A^λ in Eqns. (VI-5), (VI-6) and (VI-7).

Eqn. (VI-7) and Eqn. (VI-6) seem to give the lowest quantum requirements of those considered. Eqn. (VI-4) gives only slightly higher quantum requirements. This is not surprising, since Eqns. (VI-4), (VI-6) and (VI-7) all neglect the possibility of energy transfer from inactive PChlide and Chlide.

If there is no energy transfer from inactive PChlide to active PChlide, then there should be little difference between the quantum requirements calculated, assuming no energy transfer from inactive to active PChlide (Eqns. (VI-4), (VI-6) and (VI-7)) at the different wavelengths. In fact, these quantum requirements are least at 630 nm (see Table VI-2) where inactive PChlide absorption is greatest. Therefore, energy transfer from inactive PChlide to active PChlide, where it is used for the transformation of active PChlide to Chlide, probably exists.

By the same reasoning, any appreciable transfer of energy from Chlide to active PChlide should have the effect of decreasing the corrected quantum requirements as the transformation proceeds and the amount of Chlide increases. Such a decrease in quantum requirement is not seen for any of three actinic wavelengths used.

The efficiency of energy transfer from inactive PChlide is difficult to determine with confidence. As was mentioned previously,

Eqn. (VI-6), which assumes no energy transfer from inactive PChlide, is a better description of the reaction than Eqn. (VI-5), which assumes complete energy transfer.

In addition, the quantum requirements, calculated using Eqn. (VI-3) and extrapolated to 0% transformation (Table VI-2), increase 82% when one goes from 650 nm illumination where the absorption of inactive PChlide is least, to 630 nm illumination where the absorption of inactive PChlide is greatest. On the other hand, the quantum requirements,calculated using Eqn. (VI-7) (Table VI-2), decrease by only 27% when one goes from 650 nm photons to 630 nm photons. Thus the assumption of no energy transfer would seem to be closer to reality than assuming complete energy transfer.

If one assumes that the absorption of inactive PChlide is negligible at 650 nm (the ratios of the absorbances of PChl a in 80% aqueous acetone at 627 nm (the maximum), 637 nm, and 647 nm are 1.00:0.586:0.163), then Eqn. (AII-9, see Appendix II) should equal Eqn. (AII-13), and Eqn. (AII-9) divided by Eqn. (AII-11) should be equal to the quantum requirement calculated from Eqn. (AII-11) at time, t, divided by the quantum requirement calculated from Eqn. (AII-13) at time, t.

For t = 9.6 sec, we have

$$\frac{0.52}{0.69} = \frac{\log\left(\frac{\chi_A^\lambda a_o + f\chi_P^\lambda p_o}{\chi_A^\lambda a + f\chi_P^\lambda p_o}\right)}{\log\left(\frac{a_o}{a}\right)} \qquad \text{(VI-8)}$$

solving for f: f = 0.11 = energy absorbed by inactive PChlide transferred to active PChlide.

Thus, by this calculation approximately 11% of the energy absorbed by inactive PChlide is transferred to active PChlide.

D. Summary

In conclusion, the following mechanism seems to be the most plausible for the transformation of PChlide to Chlide on the basis of the results obtained here

$$2\ \text{PChlide}\ \underline{a} \xrightarrow{h\nu} 2\ \text{Chlide}\ \underline{a} \quad . \qquad \text{(VI-9)}$$

A quantum requirement of less than one is unusual. However, the CD spectra indicate that the PChlide molecules of PCH form an aggregate. The proximity of the molecules in the aggregate could permit utilization of one quantum for the reduction of two molecules.

No reason for the discrepancy between the quantum requirements found in this work and those reported by Smith could be determined.[81,86]

VII. SUMMARY AND MODELS

A. PCH

The results of CD, fluorescence, and quantum requirements all support the hypothesis that there are two or more interacting PChlide chromophores per carrier unit. In brief, the most plausible explanation of the complex CD found for both PCH and PCH(suc) is that it is the result of an exciton interaction between PChlides. The fluorescence depolarization of PCH(suc) at the Soret maximum is much less than that of PChl *a* in a viscous solvent, indicating transfer of the absorbed energy between PChlide *a* molecules during the lifetime of the excited state. Finally, the quantum requirements for photoconversion of PChlide are 0.48 at 630.0 nm, 0.60 at 640.0 nm, and 0.66 at 650.0 nm, strongly supporting the idea of cooperative action between two PChlide chromophores.

This result has been visualized in Fig. VII-1. On the basis of the results of Schopfer and Siegelman,[67] two PChlide chromophores have been placed upon each protein subunit and four per each protein unit.

There is some evidence that, when PCH is prepared in the absence of the detergent Triton X-100, larger protein aggregates exist. Boardman[6] found an indication of such aggregates in sedimentation patterns of PCH. I have found that when PCH is prepared without

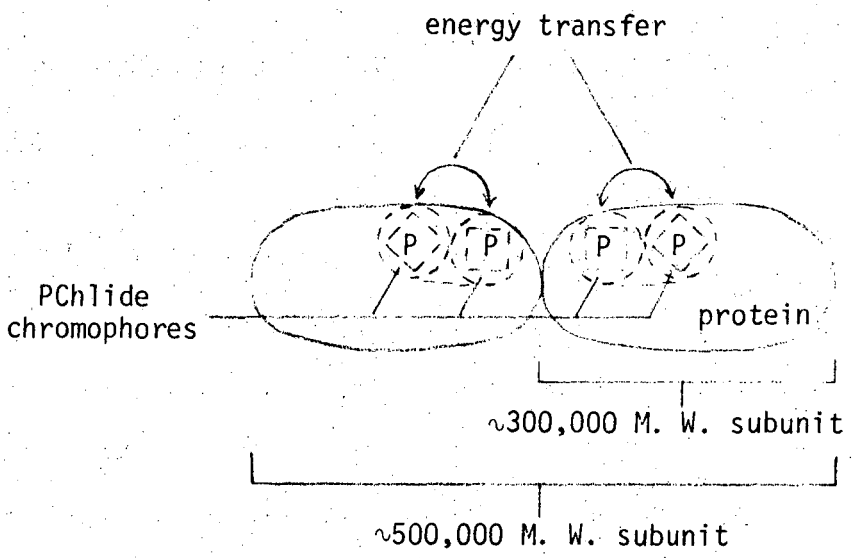


Fig. VII-1. Proposed model for active PCH.

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using Triton X-100, little of it enters polyacrylamide gels during electrophoresis. On the other hand, Schopfer and Siegelman[67] reported that their PCH preparations would enter polyacrylamide gels during electrophoresis.

The chromophores have been placed inside the carrier somewhat arbitrarily. However, Chlide a in CH was found not to pheophytinize measurably at pH 4.16. In solution, Chl a pheophytinizes at pH 5.00. The stability of Chlide a in CH at pH 4.16 suggests that the Chlide a is protected by its carrier.

When one attempts to include inactive PChlide in this model, one should recall that the ratio of inactive to active PChlide varies. In PCH(suc), it was approximately 1:1. The ratio depends on the age

of the leaf.[71] It also depends upon the physical or chemical treatments the leaf has undergone, such as freezing and thawing,[17] or supplying δ-aminolevulinic acid.[25,90] Thus, inactive PChlide will be shown as part of a partially denatured or incomplete holochrome subunit (Fig. VII-2).

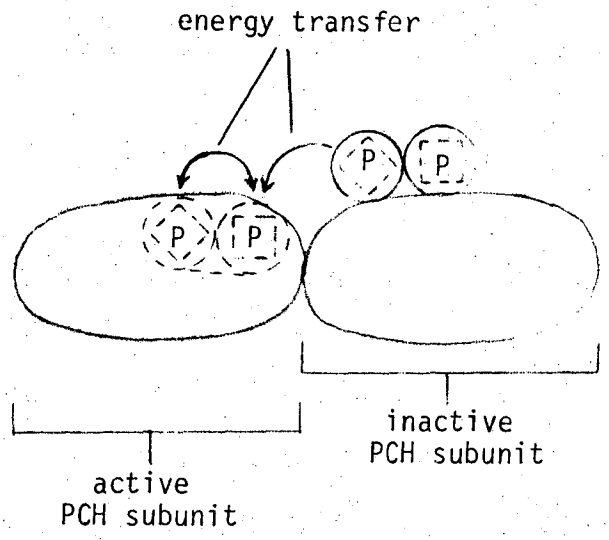

Fig. VII-2. Proposed model for PCH including inactive PChlide.

B. Phototransformation

The quantum yield for the phototransformation of PCH (Fig. VII-3) appears to be about 2. Energy absorbed by Chlide is photochemically ineffective, but that absorbed by inactive PChlide may be partially effective (Fig. VII-2). Isosbestic points in the absorption spectra

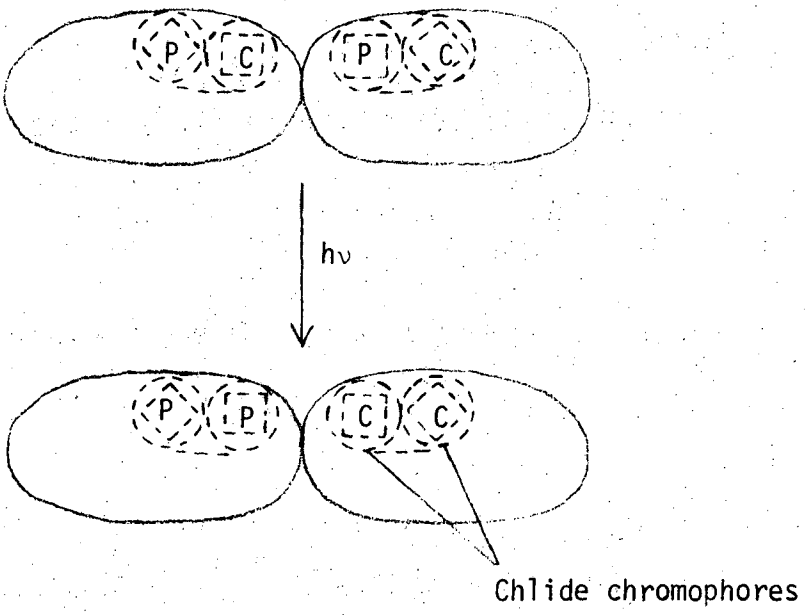

Fig. VII-3. Proposed model for phototransformation.

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recorded sequentially during the phototransformation indicate that there is only one form of active PChlide present and a single product.

The source of hydrogen to reduce ring IV of PChlide is still unknown. Phototransforming activity can not be dialyzed away. And phototransformation will proceed in the frozen or even freeze-dried state.[82] The hydrogen donor is apparently bound to the holochrome. The phototransformation of PCH is not significantly affected by being in D_2O.[7] H_2O is thought not to be the hydrogen donor. In addition, Smith[79] determined the quantity of oxygen released during the phototransformation of PChlide in the leaves of etiolated barley

by measuring the degree of quenching of phosphorescence of trypaflavine. The oxygen actually released was only 2.5% of that expected if H_2O had been the source of the hydrogen used to reduce PChlide. Röbbelen[62] found a Mendelian inherited mutation of Arabidopsis thaliana which prevented the phototransformation of PChlide to Chlide. But, if an aqueous extract of normal plants was added to a homogenate of the mutant, the reduction was reported to take place. Rudolph and Bukatsch[106] have reported that PChl(ide) reduction corresponds to the photooxidation of ascorbic acid.

Perhaps PCH contains a strongly bound cofactor which acts as a hydrogen donor, such as is found in the flavoproteins. The flavin components of flavoproteins have an equilibrium constant for dissociation of 10^{-8} or 10^{-9}. It is difficult, however, to account for Röbbelen's experiments with this proposal.

Now that PCH can be isolated in a pure, concentrated, stable form, the question of the hydrogen source should be ripe for answer.

The arrangement of Chlide a in CH immediately after transformation is pictured in Fig. VII-3 as similar to the arrangement of PChlide a from which it was formed. The CD of phototransformed PCH(suc) and its fluorescence indicate that its Chlide chromophores are aggregated. Phototransformation occurs so rapidly and at such low temperatures that any significant rearrangement of the chromophores on the holochrome during phototransformation would appear to be unlikely. Fluorescence excitation spectra of CH(suc) indicate the existence of energy transfer from inactive PChlide to Chlide.

C. The Dark Shift

CD and fluorescence polarization spectra imply that the shift in the absorption maximum of newly formed Chlide a from 678 nm to 674 nm is preceded by a disaggregation of the pigment (Fig. VII-4). The disaggregation can be prevented by high concentrations of

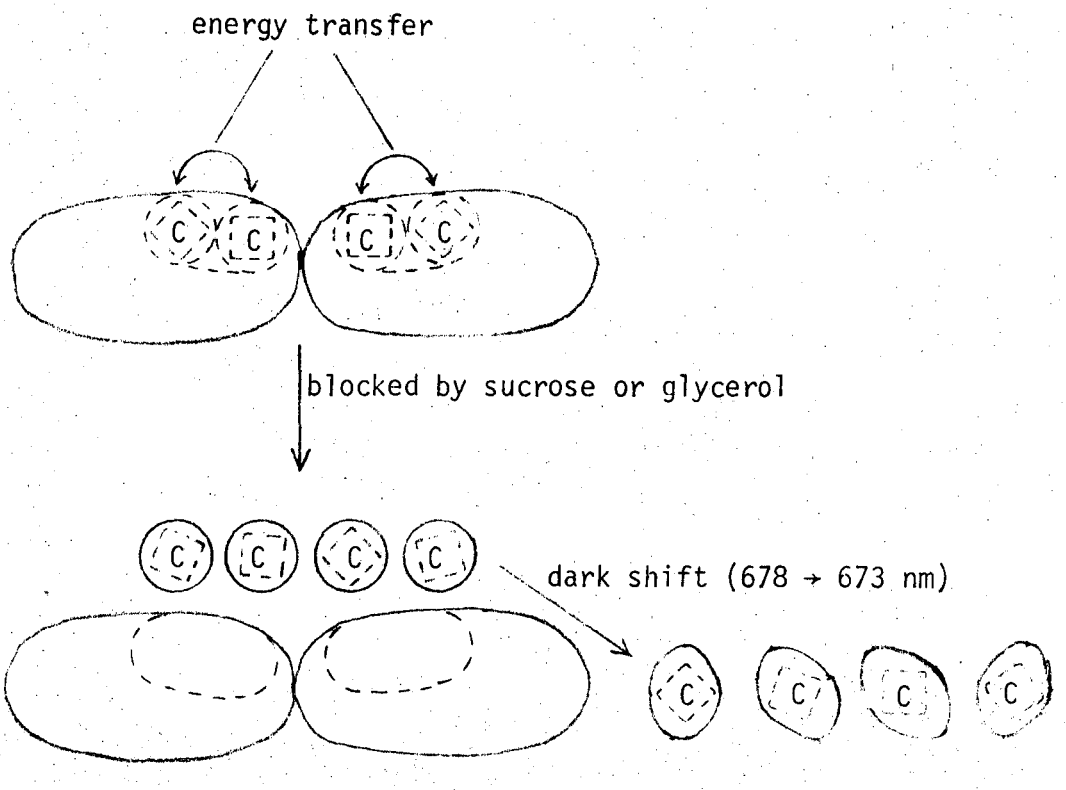

Fig. VII-4. Proposed model for the dark shift from 678 nm to 673 nm.

sucrose or glycerol. Both the CD spectra and fluorescence polarization spectra of CH measured during the shift of the absorption maximum from 678 to 674 nm indicated that the Chlide a chromophores were monomeric. In view of this, the most plausible cause of the shift is a change in the environment of the Chlide a.

Sundqvist,[90] as well as Granick and Gassman,[25] have reported experiments indicating that after phototransformation, the pigment leaves the active site of the holochrome and is replaced by new PChlide. Using plants fed δ-aminolevulic acid,* so that there is a great increase in the amount of an inactive type of PChlide present, Sundqvist[90] found that following photoconversion, the pool of active PChlide is rapidly refilled in the dark from the pool of inactive PChlide. The half-rise time to refill the pool of active PChlide was about 50 sec. This time is about 10-fold more rapid than the dark shift to shorter wavelengths.

The regeneration of active PChlide may be related to the disintegration of Chlide aggregates. The holochrome in the proposed model (Fig. VII-4) is reusable.

Sundqvist's results[90] differed from those of Granick and Gassman[25] in that he found the refilling to be relatively independent of temperature following the first flash, but dependent after the second. On the other hand, Granick and Gassman[25] found that the regeneration was very temperature-dependent even after the first flash.

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*δ-aminolevulinic acid is a precursor of PChlide.

The Chl(ide) a carrier units may be released as is shown in Fig. VII-4, or they may simply move away from the active site to some other place on or in the holochrome. The released Chlide a carriers are pictured (Fig. VII-4) as undergoing a change in conformation which causes the dark shift.

Holochrome has been shown to behave differently upon sucrose gradient centrifugation before and after phototransformation.[11,9] Both Bogorad et al.[11] and Boardman[9] found CH extracted from leaves after light transformation to be denser than PCH. In addition, Bogorad found a transitory form of CH which was less dense than PCH. Phototransformation was carried out both in the leaf and in holochrome solutions.

The dark shift in the leaf may be concurrent with the esterification of the phytol chain to Chlide.[104,100,76] However, this could not be the primary cause of the dark shift for a number of reasons. First, there is very little difference between the visible spectra of Chl a and Chlide a in organic solvents. Second, a dark shift occurs in holochrome preparations where the Chlide is not phytolated. Third, physical disruption, such as freezing and thawing, can effect a dark shift.

D. Greening after the Initial Events

The CD spectra of homogenates of greening leaves indicate that after the disintegration of the holochrome aggregate, other aggregates, apparently differing from the holochrome aggregate, are

built. At least two different types exist, one containing Chl b, and one containing Chl a. Each of these has at least two molecules per unit cell, and both form after about two hours of illumination. Although the extent of the aggregates may increase as greening proceeds after two hours of illumination, the geometries within the unit cells do not appear to change.

Energy transfer from Chl b to Chl a in homogenates of greening leaves appears within 4 hours after the beginning of illumination.[51] Energy transfer from carotenoids to Chl a begins within 6 hours after the beginning of illumination.[51,14,28] On the other hand, photosynthesis does not appear to begin until about 15 hours after the beginning of illumination.[4,13]

E. Conclusion and Future Experiments

The picture of the development and organization of photosynthetic pigment systems found in my thesis has a protein bound aggregate of PChlide a being photoreduced to a protein bound aggregate of Chlide a. The aggregate of Chlide a then disperses into monomers of Chlide a which are still protein bound. Then the environment of the protein bound Chlide a monomers changes. The Chlide a is phytolated at about this time. Finally, a new aggregate of Chl a of different structure from the aggregate of Chlide a is formed from the monomers. This aggregate apparently has at least 2 molecules per unit cell. An aggregate of Chl b also forms. Its structure differs from the aggregate of Chl a, but Chl b also

apparently has at least two molecules per unit cell.

The development and organization of photosynthetic pigment systems should continue to be an area of fruitful research in the future. A number of experiments seem to me to be worthwhile.

Another determination of the extinction coefficient of PChlide a or PChl a is very badly needed. In conjunction with this an analysis of the groups esterified to PChlide a in etiolated leaves might be useful.

The molecular weight of PCH should be determined both before and after phototransformation to determine if the molecular weight of the holochrome changes. To ascertain whether the chromophore leaves the holochrome after phototransformation, radioactive tracers might be used. One would have to be certain, however, that only the holochrome was labeled, but not other molecules or even a piece of the holochrome protein which might come off with the chromophore.

In order to rule out H_2O as the source of hydrogen, PCH could be phototransformed in D_2O. After the pigment was extracted with organic solvents, the C-7 and C-8 positions of it could be checked for deuterium substitution by nuclear magnetic resonance spectrometry. Alternately, the transformation could be carried out in tritiated water, and the organic solvent extracted pigment checked for the presence of radioactivity.

A detailed biochemical analysis of the PCH, in particular, the cofactors present in it, would be useful in determining the hydrogen donor.

Further investigations into the optical activity of developing leaves seem likely to be useful. Measurements at liquid nitrogen temperatures, where resolution of the spectral features should be improved, may be especially informative. The optical activity measurements might enable one ultimately to determine the geometry of the pigment arrays in photosynthetic organisms. To begin the determination of the geometry, it might be helpful to measure the CD spectrum of a well characterized protein crystal such as hemoglobin. Hemoglobin contains four pigment (heme) chromophores. The geometry of the chromophore arrangement predicted by the theoretical analysis of the CD spectrum could then be checked against the structure of hemoglobin known from the analysis of X-ray diffraction patterns.

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APPENDIX I

The PChlide *a* and Chlide *a* concentrations used in Figs. VI-3 through VI-6 were determined in the following manner. Only one sample of PCH(suc) was used in all the above measurements. The sample was divided into 5 portions. One portion received no illumination. Another was maximally transformed with a 7 sec floodlamp illumination. The third received 630.0 nm illuminations. The fourth received 640.0 nm illuminations. And the fifth received 650.0 nm illuminations. Then the PChlide *a* and Chlide *a* concentrations of each of the 5 portions were determined through 80% aqueous-acetone extraction and Eqns. (VI-1) and (VI-2) as was described in Section VI-B.

Next, the % of PChlide *a* transformed in the portion receiving floodlamp illumination was determined from

$$\%\, T_{(max)} = \frac{\text{Chlide } a_{(max)} \cdot 100\%}{\text{PChlide } a_{(max)} + \text{Chlide } a_{(max)}} , \qquad \text{(AI-1)}$$

where Chlide $a_{(max)}$ and PChlide $a_{(max)}$ are respectively the Chlide *a* and PChlide *a* concentrations in the maximally transformed portion in $\frac{\text{moles}}{\ell}$.

Similarly, the % PChlide *a* transformed during the preparation of the sample, *i.e.*, in the unilluminated portion, was

$$\%\ T_{(min)} = \frac{\text{Chlide } \underline{a}_{(min)} \cdot 100\%}{\text{PChlide } \underline{a}_{(min)} + \text{Chlide } \underline{a}_{(min)}} , \qquad \text{(AI-2)}$$

where Chlide $\underline{a}_{(min)}$ and PChlide $\underline{a}_{(min)}$ are respectively the Chlide $\underline{a}$ and PChlide $\underline{a}$ concentrations in the unilluminated portion in $\frac{\text{moles}}{\ell}$.

Next, the mean total pigment concentration $\overline{(\text{PChlide } \underline{a} + \text{Chlide } \underline{a})}$ of the 5 determinations was calculated. From the mean total pigment concentration and the previously determined $\%\ T_{(max)}$ and $\%\ T_{(min)}$ the concentrations of Chlide $\underline{a}$ in the maximally converted portion and unilluminated portion could be determined:

$$\overline{\text{Chlide } \underline{a}_{(max)}} = \overline{(\text{PChlide } \underline{a} + \text{Chlide } \underline{a})} \cdot \frac{\%\ T_{(max)}}{100\%} , \qquad \text{(AI-3)}$$

and

$$\overline{\text{Chlide } \underline{a}_{(min)}} = \overline{(\text{PChlide } \underline{a} + \text{Chlide } \underline{a})} \cdot \frac{\%\ T_{(min)}}{100\%} . \qquad \text{(AI-4)}$$

Now the molar extinction coefficient of Chlide $\underline{a}$ at 678 nm in CH(suc) could be determined from

$$\varepsilon_{\text{in CH(suc)}} = \frac{[A_{678(max)} - A_{678(min)}]}{[\overline{\text{Chlide } \underline{a}_{(max)}} - \overline{\text{Chlide } \underline{a}_{(min)}}][\ell']}$$

$$= 66.0 \times 10^3 \frac{\ell}{\text{mole-cm}} , \qquad \text{(AI-5)}$$

where $A_{678(max)}$ and $A_{678(min)}$ are respectively the absorbances of the maximally transformed portion and the unilluminated portion (see Fig. VI-2), and ℓ' is the optical pathlength, which was 10.0 mm.

The $\overline{\text{Chlide } \underline{a}}$ concentrations at intermediate periods of actinic illumination could now be determined from Fig. VI-2 and the molar extinction coefficient of Chlide $\underline{a}$ in CH(suc).

$$\overline{\text{Chlide } \underline{a}}_{(t)} = \frac{A_{678(t)} - A_{678(min)}}{\ell \cdot \varepsilon_{\text{in CH(suc)}}} + \overline{\text{Chlide } \underline{a}}_{(min)} \quad , \qquad \text{(AI-6)}$$

where $A_{678(t)}$ is the absorbance at 678 nm of the portion after illumination time, t.

The concentrations of active $\overline{\text{PChlide } \underline{a}}$ were determined from the concentrations of $\overline{\text{Chlide } \underline{a}}$,

$$\text{active } \overline{\text{PChlide } \underline{a}}_{(t)} = \overline{\text{Chlide } \underline{a}}_{(max)} - \overline{\text{Chlide } \underline{a}}_{(t)} \quad . \qquad \text{(AI-7)}$$

The maximum concentration of active $\overline{\text{PChlide } \underline{a}}$ was assumed to be equal to the maximum concentration of $\overline{\text{Chlide } \underline{a}}$,

$$\text{active } \overline{\text{PChlide } \underline{a}}_{(max)} = \overline{\text{Chlide } \underline{a}}_{(max)} \quad . \qquad \text{(AI-8)}$$

At the beginning of the illuminations, t = 0, then from Eqns. (AI-6) and (AI-7):

$$\text{active } \overline{\text{PChlide } \underline{a}}_{(0)} = \overline{\text{Chlide } \underline{a}}_{(max)} - \overline{\text{Chlide } \underline{a}}_{(0)} =$$

$$\overline{\text{Chlide } \underline{a}}_{(max)} = \overline{\text{Chlide } \underline{a}}_{(min)} \quad . \qquad \text{(AI-9)}$$

Thus, at t = 0, % active PChlide remaining at t = 0, =

$$\frac{\overline{\text{Chlide } \underline{a}}_{(max)} - \overline{\text{Chlide } \underline{a}}_{(min)}}{\overline{\text{Chlide } \underline{a}}_{(max)}} \cdot 100\% \quad . \qquad \text{(AI-10)}$$

One can see that the molar extinction coefficients assumed for PChlide *a* (see Table VI-1) enter only indirectly into the determination of the pigment concentrations used in Fig. VI-3 and the quantum requirement measurements. The effect of PChlide *a* on Chlide $\underline{a}_{(max)}$, being less than 1% [see Eqn. (VI-1)], can be neglected in the following. Thus, even if the values used for the molar extinction coefficients of PChlide *a* are incorrect, the concentrations of Chlide *a* will be changed at most only by about

$$\text{Chlide } \underline{a}_{(t)} = \overline{\text{Chlide } \underline{a}}_{(t)} \cdot \frac{\text{Chlide } \underline{a}_{(max)}}{\overline{\text{Chlide } \underline{a}}_{(max)}} = \overline{\text{Chlide } \underline{a}}_{(t)}$$

$$\cdot \frac{3.53 \times 10^{-6} \frac{\text{moles}}{\ell}}{4.17 \times 10^{-6} \frac{\text{moles}}{\ell}} = 0.847 \, \overline{\text{Chlide } \underline{a}}_{(t)} \, . \qquad \text{(AI-11)}$$

APPENDIX II

In our case, consider the photochemical reaction

$$A + P \xrightarrow{h\nu} B + P \quad , \qquad \text{(AII-1)}$$

where A represents active PChlide, P represents inactive PChlide, and B represents Chlide.

Now

$$Q_o = Q + Q_D = Q_A + Q_B + Q_P + Q_D \quad , \qquad \text{(AII-2)}$$

where Q_o represents the quantum density of incoming monochromatic light at time t in $(\text{mole}\cdot\ell^{-1}\cdot t^{-1})$, Q represents the quantum density absorbed at time t in $(\text{mole}\cdot\ell^{-1}\cdot t^{-1})$, Q_D represents the quantum density not absorbed at time t in $(\text{mole}\cdot\ell^{-1}\cdot t^{-1})$; and Q_A, Q_B, and Q_P represent the quantum density absorbed at time t by active PChlide, Chlide and inactive PChlide respectively in $(\text{mole}\cdot\ell^{-1}\cdot t^{-1})$. If the Lambert-Beers Law holds, then

$$Q_D = Q_o e^{-[x_A^\lambda a + x_B^\lambda b + x_P^\lambda P_o]d} \quad , \qquad \text{(AII-3)}$$

where x_A^λ, x_B^λ, and x_P^λ are the natural extinction coefficients respectively for active PChlide, Chlide, and inactive PChlide at the actinic wavelength in $(\text{mole}\cdot\ell^{-1}\cdot\text{cm}^{-1})$, $(\varepsilon = 0.4343\chi)$; d is the pathlength for the actinic light in the reaction vessel (5.00 mm); and a, b, and p_o are respectively the concentrations of active PChlide,

Chlide and inactive PChlide at time t in (mole·ℓ^{-1}). a_o is the concentration of active PChlide at t = 0. p_o will be assumed to be constant. From Eqns. (AII-2) and (AII-3), we obtain

$$Q_A + Q_B + Q_P = Q_o \left(1-e^{-[x_A^\lambda a + x_B^\lambda b + x_P^\lambda p_o]d}\right) . \qquad \text{(AII-4)}$$

Assuming first that inactive PChlide transfers some fraction f of its energy to active PChlide, the quantum density received by active PChlide is

$$fQ_P + Q_A = Q_o \left(\frac{x_A^\lambda a + f x_P^\lambda p_o}{x_A^\lambda a + x_B^\lambda b + x_P^\lambda p_o}\right)\left(1-e^{-[x_A^\lambda a + x_B^\lambda b + x_P^\lambda p_o]d}\right) . \qquad \text{(AII-5)}$$

Now

$$-\frac{da}{dt} = \phi (Q_A + fQ_P) , \left(\frac{dp_o}{dt}\right) = 0 , \qquad \text{(AII-6)}$$

where ϕ is the quantum yield (number of transformed molecules divided by the number of quanta received by active PChlide). ϕ is just the inverse of the quantum requirement. Substituting Eqn. (AII-5) and the relation $a + b = a_o$ in Eqn. (AII-6) gives

$$-\frac{da}{dt} = \phi Q_o \left(\frac{x_A^\lambda a + f x_P^\lambda p_o}{(x_A^\lambda - x_B^\lambda)a + x_B^\lambda a_o + x_P^\lambda p_o}\right)\left(1-e^{-[(x_A^\lambda - x_B^\lambda)a + x_B^\lambda a_o + x_P^\lambda p_o]d}\right). \qquad \text{(AII-7)}$$

Because there appears to be an isosbestic point at 630 nm, let $x_A^{630} = x_B^{630} \neq x_P^{630} \neq 0$, then

$$-\frac{da}{dt} = \phi Q_o \left(\frac{x_A^{630} a + f x_P^{630} p_o}{x_A^{630} a_o + x_P^{630} p_o}\right)\left(1-e^{-[x_A^{630} a_o + x_P^{630} p_o]d}\right) . \qquad \text{(AII-8)}$$

Integrating, we obtain

$$\phi = \frac{(\chi_A^{630} a_o + \chi_P^{630} p_o) \log \left(\frac{\chi_A^{630} a_o + f\chi_P^{630} p_o}{\chi_A^{630} a + f\chi_P^{630} p_o} \right)}{\bar{Q}_o \, t \, \chi_A^{630} \, (1-e^{-[\chi_A^{630} a_o + \chi_P^{630} p_o]d})} \quad . \qquad \text{(AII-9)}$$

When f = 1, i.e., inactive PChlide transfers all its energy to active PChlide (considering energy transfers from either inactive PChlide or Chlide only served to increase the curvature of the ϕ plot).

$$\phi = \frac{(\chi_A^{630} a_o + \chi_P^{630} p_o) \log \left(\frac{\chi_A^{630} a_o + \chi_P^{630} p_o}{\chi_A^{630} a + \chi_P^{630} p_o} \right)}{\bar{Q}_o \, t \, \chi_A^{630} \, (1-e^{-[\chi_A^{630} a_o + \chi_P^{630} p_o]d})} \quad . \qquad \text{(AII-10) or (VI-5)}$$

When f = 0, i.e., inactive PChlide transfers none of its energy to active PChlide, then

$$\phi = \frac{(\chi_A^{630} a_o + \chi_P^{630} p_o) \log \left(\frac{a_o}{a} \right)}{\bar{Q}_o \, t \, \chi_A^{\lambda} \, (1-e^{-[\chi_A a_o + \chi_P p_o]d})} \quad . \qquad \text{(AII-11) or (VI-6)}$$

The following experimentally determined quantitites were substituted into Eqns. (AII-10) and (AII-11):

$$\chi_A^{630} a_o + \chi_P^{630} p_o = \frac{\text{Abs.}(t = 0, 630 \text{ nm})}{2d(0.4343)}$$

(1/2 enters because the measuring pathlength was twice the actinic pathlength.)

$$\chi_A^{630} a + \chi_P^{630} p_o = \frac{1}{2d(0.4343)} \left\{ \text{Abs}(t=0, 630 \text{ nm}) - \frac{\text{Abs.Chl. a, 630 nm}}{\text{Abs.Chl. a, 678 nm}} [\text{Abs.}(t=t, 678 \text{ nm})] \right\} .$$

$\frac{\text{Abs.Chl.a, 630 nm}}{\text{Abs.Chl.a, 678 nm}}$ was measured from the spectrum of Chl b minus barley mutant (Chlorina 2) chloroplast fragments (Fig. II-2).

$$\chi_A^\lambda = \chi_B^\lambda = \frac{\text{Abs.Chl a, 630 nm}}{\text{Abs.Chl a, 678 nm}} \chi_B^{678} = \frac{\text{Abs.Chl a, 630 nm}}{\text{Abs.Chl a, 678 nm}} \cdot \frac{\epsilon_B^{678}}{0.4343} .$$

Eqn. (AII-7) may also be integrated for the general case when $\chi_A^\lambda \neq \chi_B^\lambda \neq \chi_P^\lambda \neq 0$. However, let us first attempt to simplify it. Looking at Figure VI-4, the plot of quantum requirements from Eqn. (VI-6) is flatter than that of Eqn. (VI-5), even after they have been normalized. It must be the better description of the kinetics of transformation. Therefore, let us assume no energy transfer from inactive PChlide or Chlide. Then we obtain

$$-\frac{da}{dt} = \phi Q_o \frac{\chi_A^\lambda a}{(\chi_A^\lambda - \chi_B^\lambda)a + \chi_B^\lambda a_o + \chi_P^\lambda p_o} \left(1-e^{-[(\chi_A^\lambda-\chi_B^\lambda)a+\chi_B^\lambda a_o+\chi_P^\lambda p_o]d}\right). \qquad \text{(AII-12)}$$

Substituting the series expansion $e^y = 1 + y + \frac{y^2}{2!} + \ldots$ into Eqn. (AII-12), retaining only the first 3 terms ($y < 0.3$), and integrating, one finds

$$\phi \overset{\sim}{=} \frac{1}{\overline{Q}_o d\, \chi_A^\lambda t} \left\{ \log \frac{(1-A)(n)}{(A)(1-n)} - \frac{1}{2(1-m)} \log \frac{a^2[-C^2a_o^2+C(1-2m)a_o+m(1-m)]}{a_o^2[-C^2a^2+C(1-2m)a+m(1-m)]} \right.$$

$$\left. + \frac{(1-2m)}{2(1-m)} \log \frac{[Ca+m][1-Ca_o-m]}{[1-Ca-m][Ca_o+m]} \right\}, \qquad \text{(AII-13) or (VI-7)}$$

where in addition to the symbols already defined:

$A = [(\chi_A^\lambda - \chi_B^\lambda)a + \chi_B^\lambda a_o + \chi_P^\lambda p_o]d$ = measured absorbance of the

sample at time t at the actinic wavelength under consideration · $[2(0.4343)]^{-1}$,

$n = (\chi_A^\lambda a_o + \chi_P^\lambda p_o)d$ = measured absorbance of the unilluminated sample at the actinic wavelength under consideration · $[2(0.4343)]^{-1}$,

$m = (\chi_B^\lambda a_o + \chi_P^\lambda p_o)d$ = measured absorbance of the fully transformed sample at the actinic wavelength under consideration · $[2(0.4343)]^{-1}$, and

$C = (\chi_A^\lambda - \chi_B^\lambda)d = \frac{A-m}{d}$.

χ_A^λ was determined using the Chl b less barley mutant (Chlorina 2) as before.

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TECHNICAL INFORMATION DIVISION
LAWRENCE RADIATION LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720