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PROTOCHLOROPHYLLIDE AGGREGATION IN SOLUTION AND ASSOCIATED SPECTRAL CHANGES*

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SUMMARY

When protochlorophyllide was dissolved in solvents of low dielectric constants (*e.g.*, benzene, chloroform, diethyl ether, *etc.*), its absorption spectrum showed a red shift with time. For instance, in 5 h, the original absorption bands in benzene at 634, 581 and 539 nm were shifted to 651, 587 and 546 nm. In addition, the blue band at 443 nm was split into two bands at 442 and 472 nm. The red shift was reversed upon addition of a trace amount of a more polar solvent such as methanol and upon standing. Also, in more polar solvents, no red shift in the absorption spectrum occurred.

The time-dependent spectrum shift was accompanied by a loss of fluorescence intensity at 640 nm and by an increase in scattering of the 440 nm excitation light. Again, these changes could be reversed by a trace amount of a more polar solvent.

The red shift in the absorption spectrum, the decrease in fluorescence intensity and the increase in light scattering were interpreted as due to an aggregation of the pigment molecules.

INTRODUCTION

Recent spectroscopic studies have provided evidence suggesting that chlorophyll *in vivo* is present in a variety of forms representing different states of aggregation (see ref. 1 for a review). The different forms of chlorophyll are generally considered to play some important roles in the energy transfer process of photosynthesis. In this connection, the aggregation phenomenon of chlorophylls *in vitro* has become of increasing interest, because the spectral properties of the chlorophyll aggregates, rather than the free pigment in solution, bear a closer resemblance to the pigments *in vivo*. Thus, studies of pigment aggregation may lead to information regarding the function of these pigments in photosynthesis. Chlorophyll aggregation usually results in a displacement of the absorption bands, hence visible absorption spectra are most often used for such studies. More recently, infrared absorption spectra and nuclear magnetic resonance have also been applied to pigment aggregation studies[2].

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In the course of a study of pigment transformations in etiolated bean leaves[3], an anomaly was found in the absorption spectrum in solution of the photoreceptor pigment, protochlorophyllide, depending on the nature of the solvent medium used. This note presents some experimental evidence from visible absorption spectra, fluorescence, and light scattering, indicating that the spectral shifts are probably associated with protochlorophyllide aggregation in solvents of low dielectric constants.

EXPERIMENTAL

Larger amounts of protochlorophyllide were obtained by biosynthesis[4] by incubating 10–14-day-old etiolated bean leaves in $5 \cdot 10^{-3}$ M δ-aminolevulinic acid by vacuum infiltration[3]. After 16 h of incubation in the dark, the leaves were blotted dry with tissue paper and immediately ground in cold acetone containing a trace of $MgCO_3$. The acetone extract was centrifuged free of particulate matter and the supernate placed in a separatory funnel. To the acetone extract was added an additional half volume of benzene until two phases were formed. Complete partition of the pigments into the benzene phase was achieved by washing with an excess of 10% aqueous NaCl solution. The benzene solution was removed and dried over anhydrous Na_2SO_4. It was then reduced to a small volume by vacuum evaporation prior to chromatographic separation.

The pigments were purified by thin-layer chromatography[5] on 500 μ thick layers of silica gel H and developed with a solvent mixture containing benzene–ethyl acetate–ethanol (80:20:20, by vol.). Untreated etiolated leaves yielded 2 bands, whose absorption spectra resembled that of protochlorophyllide[5], and having R_F values of 0.1 (band 1) and 0.8 (band 2), respectively. The carotenoids were also separated into distinct bands. In methanol, the major red absorption band of both pigments was located near 630 nm. The δ-aminolevulinic acid-incubated leaves yielded about 10 times as much band-1 pigment.

The band-1 pigment contained a trace amount of protochlorophyll. Phytol analysis by thin-layer chromatography followed by permanganate staining according to the method of SHIMIZU, FUKUSHIMA AND TAMAKI[6] showed the once chromatographed band-1 pigment had a phytol content which corresponded to approx. 5% protochlorophyll. Pigments used in this study were rechromatographed at least 4 times, and thus contained a negligible amount of protochlorophyll.

The absorption spectra were measured with a Cary Model 14 R spectrophotometer, and fluorescence emission and excitation spectra were taken with an Aminco-Bowman spectrophotofluorimeter.

RESULTS

Absorption spectra of protochlorophyllide in different solvents

A solution of the rechromatographed band-1 pigment was collected by eluting with "spectro-grade" methanol and the solid pigment was obtained by drying such solutions. The absorption spectra of protochlorophyllide in acetone, methanol or ethanol are practically identical. The absorption spectrum of protochlorophyllide in acetone is shown in Fig. 1. This spectrum is similar to that reported by KOSKI AND

SMITH[7] for protochlorophyll in methanol with respect to both the positions and heights of the absorption bands.

The absorption-spectrum data for several different solvents examined are summarized in Table I. Because of the absorption-band shift and change in the magnitude of absorption associated with pigment aggregation (see below) the data presented in Table I for benzene and chloroform were taken at the earliest moment before substantial aggregation occurred. The relative band heights were expressed with reference to the Soret band height taken as unity. Note that the absorption bands in pyridine are generally at a longer wavelength than in most other solvents.

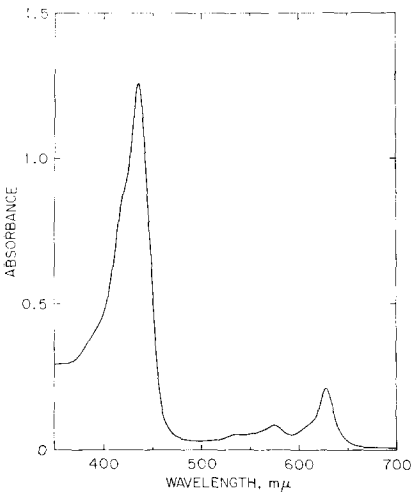

Fig. 1. Absorption spectrum of protochlorophyllide in acetone.

TABLE I

WAVELENGTHS OF MAXIMUM ABSORPTION OF PROTOCHLOROPHYLLIDE IN SEVERAL ORGANIC SOLVENTS

The absorbance values are normalized values, with the blue-band value taken as unity.

Solvent	*Red-1 wavelength (A)*	*Red-2 wavelength (A)*	*Red-3 wavelength (A)*	*Blue wavelength (A)*	*B/R*
Benzene	634 (0.178)	581 (0.068)	539 (0.047)	443 (1.00)	5.62
Chloroform	634	579	537	442	>5.5
Pyridine	634 (0.128)	586 (0.061)	548 (0.036)	452	7.82
2-Propanol	631 (0.165)	578 (0.056)	537 (0.035)	436	6.06
Acetone	628 (0.163)	576 (0.061)	535 (0.037)	436	6.14
Ethanol	630 (0.152)	575 (0.059)	535 (0.035)	434	6.58
Methanol	629 (0.152)	576 (0.062)	537 (0.037)	433	6.58

Photochlorophyllide aggregation and associated changes

When the band-1 pigment was dissolved in benzene, the initial position of the absorption bands was located at slightly longer wavelengths than in acetone or methanol (*cf.* Table I). However, upon standing at ambient temperature, a gradual change in absorption spectrum occurred. A typical set of spectra obtained over a

period of 5 h is presented in Fig. 2A. Upon standing for 1 h, an additional absorption band of longer wavelength developed at 650 nm, resulting in a doublet peak. The original peaks at 539 nm and 581 nm also shifted toward longer wavelengths. At the end of 5 h, the major red band was located at 651 nm and the 2 minor bands at 546

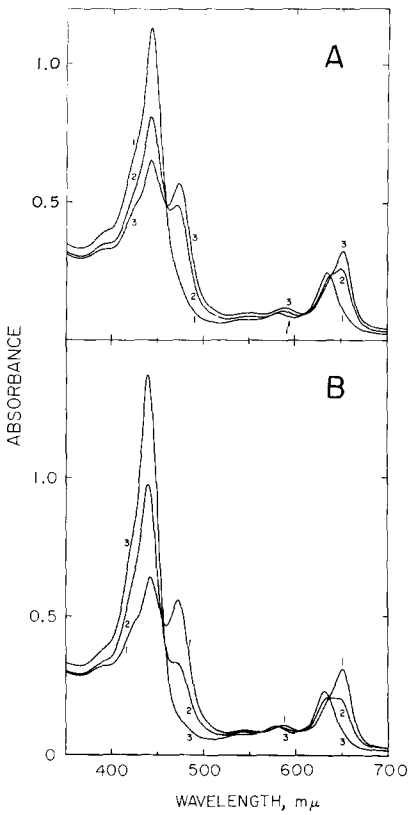

Fig. 2. A. Change in the absorption spectrum of protochlorophyllide in benzene upon standing. Spectra 1, 2 and 3 were taken immediately, 50 min and 289 min after dissolution of the pigment. B. Change in the last absorption spectrum in (A) upon addition of 200 μl methanol and standing. Spectrum 1 is identical with spectrum 3 in A. Spectra 2 and 3 were taken immediately and 16 h after methanol addition.

Curve	*Wavelengths of peaks (nm)*							
A-1	443		539		581		634	
A-2	442, 470		543		584		635, 650	
A-3	442, 472		546		587		(635), 651	
Isosbestic points A		458				609		637
B-1 (= A-3)								
B-2	442, (472)		537		577		632, (650)	
B-3	440		537		577		632, (650)	
Isosbestic points B		450		560		600		635

and 587 nm. The spectral changes in the blue region were even greater. The major blue band at 443 nm decreased rapidly in 1 h and simultaneously a second band developed at 470 nm. In 5 h, the 443 nm band decreased further and the second band became much more prominent and shifted to 472 nm. Similar spectral changes as a function of time were also observed in chloroform. Preliminary experiments with anhydrous ether as the solvent showed the major absorption bands were at 475 and 654 nm for the aggregated state.

The time-dependent spectral shift toward longer wavelengths in benzene was reversible upon addition of a trace amount of a more polar solvent such as methanol. Fig. 2B shows such a reversion series. Immediately after 200 μl of methanol were added to 3 ml of the benzene solution, part of the red band shifted toward a shorter wavelength, forming a doublet band, and the minor band at 546 nm shifted back to 537 nm. In the blue region, the 472 nm band decreased to a shoulder and the 440 nm band increased. At approx. 16 h after methanol addition, the spectrum reversed completely, with the major blue and red bands occurring at 632 and 440 nm, respectively. In both the red and blue shift series, the spectra intersected at several isosbestic points. However, the wavelength positions of the isosbestic points in the 2 series are not the same (see the legend of Fig. 2 for the actual values). Part of the discrepancy may be attributable to the difference in solvent composition between the 2 series.

Changes in fluorescence intensity and light scattering associated with aggregation

In addition to changes in absorption spectra, changes in fluorescence intensity and light scattering would be expected to accompany molecular aggregation. A qualitative examination of the changes in these properties accompanying aggregation and disaggregation was made and some typical results for aggregation are presented in Fig. 3. The experimental conditions were similar to those used in Fig. 2A, using 440 nm light for excitation. These results showed that the relative fluorescence intensity at 640 nm and the relative scattering of the 440 nm excitation light both changed with time after the protochlorophyllide was dissolved in benzene. In the course of standing, the fluorescence intensity decreased and the amount of scattering increased. Similar to the absorption changes shown in Fig. 2B, the fluorescence and light scattering changes were reversed upon standing after addition of methanol to the aggregates in benzene. During the entire course of aggregation, the observed

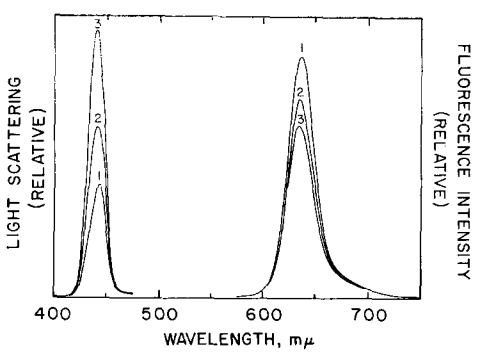

Fig. 3. Relative fluorescence emission at 640 nm and scattering of the excitation beam at 440 nm by protochlorophyllide in benzene upon standing. Curves 1, 2 and 3 were taken immediately, 36 min and 237 min after dissolution of the pigment.

excitation spectra (fluorescence measured at 640 nm) corresponded to the absorption spectra of the pigment in methanol, indicating that only the non-aggregated pigment molecules contributed to the fluorescence.

DISCUSSION

As a result of molecular interaction, the energy difference between the ground state and the excited state usually becomes smaller, the absorption band of the pigment therefore shifts toward a longer wavelength. Such spectral shifts are brought about either when the pigment molecules are adsorbed onto a polymeric substrate or when pigment molecules aggregate into a more condensed state[1]. Many observations on spectral shifts attributed to pigment aggregation have been reported for various chlorophylls (see ref. 1). In the case of chlorophyll *a* and bacteriochlorophyll, for instance, such spectral shifts can be observed by adding water to an acetone solution of the chlorophylls[8,9].

The red shift observed here for protochlorophyllide in solvents of low dielectric constants presumably is also attributable to aggregation of these pigment molecules. The relatively long time necessary for the establishment of the equilibrium, as well as for the disaggregation upon the addition of methanol, suggests that the aggregates are probably polymers consisting of a large but unknown number of pigment molecules, in preference to dimers. However, the aggregation presumably passes through a dimer stage. While the decrease in fluorescence is also consistent with aggregation in general[10,11], the accompanying light scattering observed here also suggests the formation of polymeric aggregates. The reversibility of the aggregation process suggests a physical basis for the process, and that chemical changes were probably not involved. Furthermore, rechromatography of one non-aggregated or aggregated chlorophyll, or a mixture of the two, yielded only one band, namely band 1.

Perhaps the more interesting aspect of protochlorophyllide aggregation is the close correspondence of the spectral properties of the aggregated and non-aggregated pigments to the 2 states of protochlorophyllide *in vivo*. As reported previously by SHIBATA[12] and in our own study[3], the red absorption band of protochlorophyllide in etiolated bean leaves has a maximum at 650 nm with only a shoulder at about 632 nm. Upon a brief illumination, the 650 nm maximum shifts to 684 nm, leaving the shoulder at 632 nm as a maximum. When the etiolated bean leaves were frozen and rapidly thawed, or heated, or incubated in δ-aminolevulinic acid, the major red absorption band had a maximum at about 632 nm instead of 650 nm. Furthermore, the 632 nm protochlorophyllide could neither undergo the light-induced spectral transformation to 684 nm nor participate in chlorophyll biosynthesis. Whereas in normal etiolated leaves a subsequent carotenoid-to-chlorophyll energy transfer can be demonstrated[13,3], in all treated leaves where the major red absorption band was at 632 nm, no such energy transfer could occur[3].

Spectrophotometric examination of the *in vivo* 650 nm and 632 nm pigments yielded identical absorption spectra when extracted into an organic solvent[3]. These observations led to the suggestion[3,4] that the differences arise from the mode of binding of the protochlorophyllide molecules in the lipoprotein complexes. Heat denaturation or freezing followed by rapid thawing would disrupt the matrix organization and the binding mode and could lead to the spectral shifts. However, in view of

the close correspondence of the spectral changes seen for the pigment *in vivo* and for protochlorophyllide in solution, a contribution by aggregation to the *in vivo* spectral properties also appears possible.

Recently SEELY AND TALMADGE[14] studied the mechanism of photoreduction of zinc porphin by ascorbic acid and suggested an analogy between this reaction and the conversion of protochlorophyll to chlorophyll. Although no successful attempt has been reported in the literature[15] on the photoreduction of protochlorophyll outside of the intact leaves or holochrome, the protochlorophyllide aggregates may provide a suitable system for further examination of the problem.

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