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SPECTROFLUOROMETRIC CHARACTERIZATION *IN SITU* OF THE PROTOCHLOROPHYLL SPECIES IN ETIOLATED TISSUES OF HIGHER PLANTS¹

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ABSTRACT

The fluorescence emission and excitation properties of protochlorophyll in etiolated cucumber (*Cucumis sativus* L.) cotyledons and primary bean (var. Red Kidney) leaves were characterized at 77 K. Contrary to previous studies, it appears that the short-wavelength protochlorophyll emission band consists of four fluorescent components, instead of only one nonphototransformable protochlorophyll. It was demonstrated that etiolated cucumber cotyledons synthesize and accumulate nontransformable protochlorophyll (E_{430} , F_{630}) as well as short-wavelength phototransformable protochlorophyll (E_{433} , F_{633}), (E_{444} , F_{636}), and (E_{445} , F_{640}). Long-wavelength phototransformable protochlorophyll (E_{450} , F_{657}) is also formed. In this context, E refers to the Soret excitation maxima and F refers to the red emission maxima of the protochlorophylls.

In etiolated bean leaves, the corresponding species were: nontransformable protochlorophyll (E_{440} , F_{650}), short-wavelength phototransformable protochlorophylls (E_{441} , F_{653}), (E_{442} , F_{636}), and (E_{443} , F_{640}), and longwavelength phototransformable protochlorophyll (E_{447} , F_{657}).

It is presently acknowledged that mature etiolated tissues contain two SW³ and one LW Pchl species. One of the SW species is nonphototransformable (nt) and exhibits a red excitation/absorption maximum at 628 nm and a corresponding 77 K fluorescence emission maximum at 630 to 632 nm (10). The other SW species is phototransformable (t) and exhibits a red excitation/absorption maximum at 637 nm. However, it is not considered to be highly fluorescent as it transfers most of its excitation energy to the LW Pchl species (10). Thus, the SW emission band of Pchl *in vivo* has been conventionally attributed solely to the fluorescence of nt-Pchl. On the other hand, the LW Pchl is both phototransformable and highly fluorescent and makes up the bulk of the Pchl pool in mature etiolated tissues (7, 10, 11, 16). It exhibits a red excitation/ absorption maximum at 650 nm and a corresponding fluorescence emission maximum at 655 to 658 nm (9, 10).

The multiplicity of Pchl spectral forms in situ has been conven-

tionally attributed to the differential association of two Pchl chromophores, i.e. MV Pchlide⁴ and MV Pchlide ester, with their lipoprotein macromolecular environment. It has recently been demonstrated, however, that, contrary to previous beliefs, the Pchl pool of etiolated plants consisted of eight different chromophores instead of the two conventional MV Pchlide a and Pchlide ester a components (3, 4). Four of the Pchl chromophores were identified as MV and DV Pchlide a and MV and DV Pchlide ester a (3, 4). The identity of the other four Pchl-like chromophores has not yet been determined, but it was proposed that two of them may represent Pchlide b and Pchlide ester b (3). It was subsequently shown that the MV and DV Pchlide a and MV and DV Pchlide ester a were convertible into MV and DV Chl(ide) a and MV and DV Chl a by a very brief light treatment (2, 5). It now appears that the multiple Chl a chromophoric species detectable in mature green tissues (14) originate from the MV and DV Pchl a and Chl(ide) a precursors (2, 5).

With the discovery of the aforementioned Pchl chromophoric multiplicity, it was reasonable to wonder whether this Pchl chromophoric heterogeneity was also accompanied by a concomitant multiplicity of Pchl spectral forms *in situ*. It was conjectured that a finely tuned spectrofluorometric examination of the Pchl pool of etiolated tissues *in situ* might reveal some additional Pchl spectral species that were previously overlooked. The data reported here suggest that two additional SW Pchl spectral species are detectable in etiolated tissues *in situ*.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Cucumber seeds (*Cucumis sativus* L. cv. Beit Alpha MR) were purchased from the Niagara Chemical Division, FMC Corporation, Modesto, CA. Beans (*Phaseolus vulgaris* L. var. Red Kidney) were purchased from Maxwell Seed House, Champaign, IL. Seeds were germinated at 28 C in moist vermiculite in total darkness as described earlier (6), except that distilled H_2O was used instead of Hoagland nutrient solution.

Harvesting. Etiolated cotyledons and primary bean leaves were harvested under a dim green safelight which did not phototransform Pchl to Chl(ide) a.

Spectrofluorometry. Corrected fluorescence emission and excitation spectra of Pchl in homogenates and tissue slices were recorded at 77 K in 67% glycerol with a Perkin-Elmer spectroflu-

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³ Abbreviations: SW Pchl, membrane-bound, short-wavelength protochlorophyll having a broad emission maximum *in vivo* or *in situ* between 629 and 643 nm at 77 K; LW Pchl, membrane-bound, long-wavelength Pchl having an emission maximum *in vivo* or *in situ* at 657 nm at 77 K; t-Pchl, phototransformable Pchl; nt-Pchl, nonphototransformable Pchl; MV, monovinyl; DV, divinyl.

⁴ Pchlide and Pchlide ester are used here generically to designate Pchls which may differ from one another by the nature of their side-chain substituents at positions 2 and 4 of the macrocycle. MV Pchl is defined as that Pchl with a vinyl group at position 2 (or 4) of the macrocycle and an ethyl group at position 4 (or 2) of the macrocycle. DV Pchl is defined as that Pchl with vinyl groups at both positions 2 and 4 of the macrocycle.

Crude homogenates of 20- to 24-h-old etiolated or illuminated cotyledons were prepared as follows. One g tissue was gently ground (25 to 30 strokes) in 2.0 ml 0.5 M sucrose, 0.2 M Tris-HCl (pH 8.0) at 0 to 4 C. The suspension was filtered through one layer of nylon mesh, and 0.1 ml of the filtrate was mixed with 0.2 ml glycerol. The mixture was transferred to the lower sample section of a cylindrical glass tube and was immediately frozen by inserting the sample tube into a long stem Dewar filled with liquid N₂ (7).

Tissue slices of 2- and 5-day-old etiolated or illuminated cucumber cotyledons and primary bean leaves were 10×1 mm. They were transferred to the sample tube in the Tris/sucrose bufferglycerol (1:2 v/v) and immediately frozen as described above.

To measure fluorescence emission spectra, the following combination of filters was used to minimize the interference of light scattered by the frozen sample: a blue filter, Pyrex No. 5543, that was transparent in the 350- to 500-nm region, was placed between the excitation monochromator and the sample; a red sharp cut-off filter, Turner No. 25, that excluded light below 600 nm was interposed between the sample and the emission monochromator. When excitation spectra were recorded, the blue filter was removed.

Phototransformation of Pchl. In 20- to 24-h-old etiolated cotyledons, Pchl was phototransformed by illuminating with 3.2 w/m^2 white fluorescent light for 15 s.

In 5-day-old etiolated cotyledons and bean leaves, the Pchl was phototransformed by illumination with 3.2 w/m^2 white fluorescent light for 60 s.

Computer Analysis of Emission/Excitation Matrices. Deconvolution of the emission/excitation matrices of the Pchl profiles *in vivo* was performed according to Weber (18). All computations were programmed either on a Control Data Corp. computer Cyber 175 or on a Hewlett-Packard 9825S microcomputer system.

RESULTS

Build-up of Pchl Heterogeneity during Etiolation. The development of the complete Pchl profile during etiolation has been described as an age-dependent, stepwise process (1, 11). SW Pchl is formed first; as tissue development proceeds in darkness, LW Pchl accumulates faster than the SW Pchl and becomes the predominant species. This process is manifested by a shift in the *in vivo* absorption maximum of the Pchl from 636 nm in juvenile etiolated tissues to 650 nm in older etiolated tissues (1, 11). A study of the 77 K fluorescence characteristics of Pchl revealed a parallel shift in the main emission maximum from about 633 to 657 nm in cucumber cotyledons grown in complete darkness (7). Data obtained during this latter investigation suggested that the SW Pchl emission band in aging etiolated tissues was complex, possibly containing some previously uncharacterized Pchl component(s). Evidence for this hypothesis is presented below.

The build-up of complexity in the SW Pchl emission band of etiolated cucumber cotyledons as a function of seedling age is depicted in Figure 1. Since the level of Pchl was low during the first 24 h (6), spectra at this developmental stage were recorded using concentrated homogenates prepared in Tris-sucrose buffer rather than tissue slices. In 2- and 5-day-old cotyledons, spectra were recorded using single cotyledonary slices. This had no influence on the results, however, since it was shown earlier that the

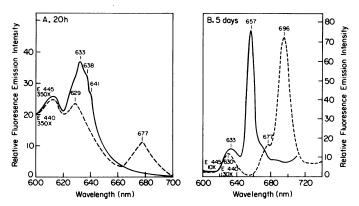


FIG. 1. Fluorescence emission spectra at 77 K of the Pchl in etiolated cucumber cotyledons, before (----) and after (---) phototransformation. A, homogenate of 20-h-old cotyledons; B, slices of a single 5-day-old cotyledon. Pchl was phototransformed with 3.2 w/m² white fluorescent light. Twenty-h-old cotyledons were illuminated for 15 s, whereas 5-day-old cotyledons were illuminated for 60 s. Pre- and postillumination spectra were normalized at 600 nm; the ordinate scale attenuation is indicated on the spectra, \times 350 being the least attenuated ordinate scale.

fluorescence emission spectra of Pchl in homogenates and slices of etiolated cucumber cotyledons were similar (7). Recent spectra of cotyledonary slices prepared from 20-h-old etiolated cotyledons and recorded on a very sensitive spectrofluorometer (SLM 8000 DS) were identical to spectra recorded on concentrated homogenates prepared from the same tissue (C. A. Rebeiz, unpublished). Homogenates of cotyledons grown in darkness for 20 h exhibited a well-defined and skewed Gaussian fluorescence emission band with a maximum at 632 to 633 nm and emission shoulders at about 636 to 638 nm and 640 to 642 nm (Fig. 1A). This suggested that this band was composed of more than one Pchl species. LW Pchl was not detectable at this stage of development.

Partial deconvolution of the SW band was achieved by phototransforming the Pchl. A light treatment of 15 s converted some of the Pchl to Chl(ide) a, as evidenced by the appearance of a new emission band with a maximum at about 677 nm (Fig. 1A). The remaining nt-Pchl exhibited an emission band with a maximum at 628 to 629 nm; the emission shoulders at about 638 and 641 nm disappeared (Fig. 1A). These results indicated that the SW Pchl band was made up of more than one component even at this early stage of etiolation: (a) a nt-Pchl species with an emission maximum at about 629 nm, and (b) at least one or two SW t-Pchl species, apparently emitting at a longer wavelength than the nt-Pchl component.

During subsequent growth of the cotyledons in darkness, the SW Pchl band broadened considerably and exhibited a bellshaped emission maximum between 629 and 642 nm (Fig. 1B). By 5 days, the Pchl profile became dominated by the LW t-Pchl, having an emission maximum at 657 nm (Fig. 1B). The absolute amount of SW Pchls also increased significantly. Upon illumination, the SW Pchl band underwent a decrease in amplitude and a blue shift in its emission maximum. It became narrower and assumed a more symmetrical Gaussian configuration centered around the nt-Pchl emission maximum at 630 nm (Fig. 1B). This was consistent with the results shown in Figure 1A and the notion that the SW Pchl band is made up of one or more fluorescent, t-Pchl components in addition to nt-Pchl. Further analysis of the SW Pchl emission band was undertaken as described below.

Heterogeneity of the SW Pchl Emission Band of Etiolated Cotyledons as Revealed by Differential Excitation. The position of the emission maximum of a given substance excited with light of different wavelengths is considered to be a constant (18). The appearance of new emission maxima and shoulders as a function of excitation wavelength is usually attributed to the presence of a mixture of fluorescent compounds, each with its own distinct emission and excitation characteristics. This phenomenom was exploited in an attempt to characterize further the Pchl component(s) of the SW Pchl emission band. This was accomplished by recording 77 K fluorescence emission spectra of the Pchl in 5-dayold etiolated cucumber cotyledons excited at 5-nm intervals between 430 and 460 nm. We hoped that, at some excitation wavelengths, we would encounter some special absorption wavelengths at which the emission from a particular tetrapyrrole in the Pchl pool would be greatly maximized, thereby facilitating its detection. Of course, the same is also true for excitation spectra recorded at different emission wavelengths.

In 5-day-old etiolated cotyledons, the emission maximum of the SW Pchl band shifted from about 630 nm to about 633, 637, and 640 nm as the excitation wavelength was varied from 440 to 445, 450, and 455 nm, respectively (Fig. 2A, solid curves). This suggested that the SW Pchl band may have contained several SW Pchl species.

To determine the phototransformability of these SW spectroscopic species, emission spectra of Pchl in 5-day-old etiolated cotyledons were recorded after briefly illuminating the tissue. The light treatment phototransformed completely the LW t-Pchl with an emission maximum at 657 nm (Fig. 2A). After illumination, the SW Pchl fluorescence bands elicited by excitation at 440, 445, 450, and 455 nm all exhibited a significant decrease in amplitude, which could not be accounted for by the disappearance of the LW t-Pchl (Fig. 2A, dashed curves). This, in turn, suggested that some components of the SW Pchl band have been phototransformed to Chl(ide) *a*. Furthermore, the emission maxima at 630, 633, 636 to 638, and 640 to 642 nm, which were elicited by excitation at 440, 445, 450, and 455 nm, respectively, and which were clearly discernable before phototransformation (Fig. 2A, solid curves), either

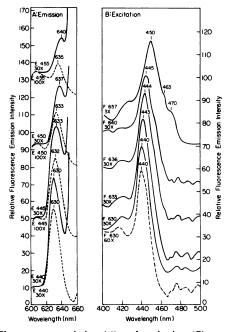


FIG. 2. Fluorescence emission (A) and excitation (B) spectra at 77 K of the Pchl in slices from a single 5-day-old etiolated cucumber cotyledon. One slice was monitored before illumination (---) and another slice was monitored after illumination (---) with 3.2 w/m^2 white fluorescent light for 60 s. Pre- and postillumination emission spectra were elicited by excitation at 5-nm intervals between 440 and 455 nm; they were normalized at 600 nm. Excitation spectra were recorded by positioning the emission monochromator at the emission maxima of the various Pchl species and by scanning the region between 400 and 500 nm with the excitation monochromator. All other symbols are as in Figure 1.

disappeared and were replaced by shorter wavelength emissions (*i.e.* the 640-, 637-, and 633-nm emissions) or decreased significantly in amplitude (the 630-nm emission) (Fig. 2A, dashed curves). The postillumination decrease in fluorescence emission amplitude observed at 630 nm, after excitation at 440 nm, was probably due to the phototransformation of the neighboring Pchl species emitting at about 633, 637, and 640 nm. We propose that the Pchl species emitting at 630 nm is probably nonphototransformable, whereas the Pchl species emitting at 640, 637, and 633 nm are partially phototransformable.

Further Characterization of SW Pchl Species in Etiolated Cotyledons: Soret Excitation Spectra. Further characterization of the components of the SW emission band in etiolated cucumber cotyledons was achieved by recording their corresponding Soret excitation spectra. These were obtained by positioning the emission monochromator at the emission maxima of the putative SW Pchl species (Fig. 2A, solid curves) and scanning the wavelength relation between 400 and 500 nm with the excitation monochromator.

The Soret excitation spectrum of the nt-Pchl species was recorded using both etiolated and briefly illuminated, etiolated cotyledons. With the emission monochromator positioned at 630 nm, *i.e.* at the emission maximum of nt-Pchl, the Soret excitation spectrum exhibited a maximum at about 440 nm (Fig. 2B). We propose that the Soret excitation and red emission maxima at about 440 and 630 nm, respectively, belong to nt-Pchl. This species will be referred to as nt-Pchl (E_{440} , F_{630}), where E refers to the Soret excitation maximum and F refers to the fluorescence emission maximum of the pigment.

The Soret excitation spectra of the other SW Pchl species were recorded using etiolated cotyledons. The Soret excitation maximum for the 633-nm emission was observed at 443 nm (Fig. 2B). The Soret excitation maxima of the species emitting at 636 to 638 nm and 640 to 642 nm were observed at about 444 and 445 nm, respectively. These Pchls will therefore be referred to as t-Pchl (E_{443} , F_{633}), t-Pchl (E_{444} , F_{636}), and t-Pchl (E_{445} , F_{640}).

The Soret excitation spectrum of the t-LW Pchl emitting at 657 nm (Fig. 1B) exhibited a maximum at 449 to 450 nm (Fig. 2B). This is identical to the Soret excitation/absorption maximum of Pchl in etiolated bean leaves reported by Shibata (16). We therefore propose that the Soret excitation and red emission maxima at about 450 and 657 nm, respectively, belong to the LW t-Pchl. This species will be referred to as t-Pchl (E_{450} , F_{657}). A further peculiar feature of the Soret excitation spectrum of Pchl (E_{450} , F_{657}) was the presence of pronounced excitation shoulders at about 463 and 470 nm (Fig. 2B). The origin of these excitation shoulders will be discussed elsewhere.

Determination of Minimum Number of Cucumber Pchl Species Detected in Vivo by Matrix Analysis of Pchl Profile. At this stage, it should be emphasized that, although the detection of multiple emission maxima induced by differential excitation of a sample containing two emitting species is highly suggestive, it does not constitute a proof for the presence of more than two emitting species in the sample. The same is true for the detection of multiple excitation maxima upon recording excitation spectra at different emission wavelengths. For example, the differential excitation of a sample containing two fluorescent species will certainly result in different emission amplitudes of the two emitters, depending, of course, on the wavelength of excitation. The overlap of two fluorescent spectra of changing relative amplitudes will very likely shift the apparent emission maxima of the mixture to shorter or longer wavelengths. This, in turn, will depend on the degree of spectral overlap and on the relative amplitudes of the two overlapping emission spectra. Since the fluorescence emission/excitation data suggested that five fluorescent Pchls were present in etiolated cucumber cotyledons, it was desirable to determine, by some independent means, the minimum number of Pchls that

could have given rise to the multiple emission and excitation maxima that were just described. This was achieved by using Weber's matrix technique (18). Weber (18) demonstrated that, for an emission/excitation matrix (M), whose elements $(M_{i,j})$ represented the fluorescence intensity measured at wavelength λ_{j} , for an excitation λ_{i} , the number of fluorescent components in the matrix was equal to the rank of the minors for which the following inequality was obeyed.

$$\frac{\Delta}{P} \ge 3 \frac{(\delta F)}{\bar{F}} \tag{1}$$

where Δ is the value of the determinant for a minor of any particular rank, *P* is the value of the permanent [*i.e.* the sum of all products in the determinant (13) for the same minor], δF is the noise level or minimum detectable fluorescence, and \overline{F} is the mean fluorescence intensity of the $M_{i,j}$ elements of the minor under consideration.

Such a treatment of fluorescent data is valid only for samples which fall within the linear response range of the measuring instrument. It should also be emphasized that, although this technique will detect all the fluorescent species in a mixture, when they have reasonably well-resolved excitation bands, it fails in cases of extreme excitation-band overlap (17). It also fails to differentiate between two fluorescent species if both species possess the same excitation/absorption spectrum (18).

With these limitations in mind, the excitation/emission profile of the cucumber Pchl was submitted to matrix analysis. To this effect, 10 emission spectra were recorded at 77 K on the same sample. The emission spectra were elicited by the following excitations: 430, 435, 440, 442, 445, 447, 450, 452, 455, and 460 nm. The fluorescence emission amplitudes at 621, 624, 630, 633, 636, 640, 652, 657, 662, and 670 nm then were determined from every emission spectrum. All fluorescence emission amplitudes were normalized to the same attenuation scale. The 10×10 matrix of the cucumber Pchl profile depicted in Table I was next assembled from the normalized emission amplitudes. The determination of all the minors of a given rank which obeyed equation 1 was achieved by means of the computer. The latter was programmed to report any minor of a given rank *n* with

$$\frac{\Delta}{P} > 3 \, \frac{\delta F}{\bar{F}}$$

Table I. Emission/Excitation Matrix of Pchl Pool of Etiolated Cucumber Cotyledons

The $M_{i,j}$ elements represent the fluorescence intensity measured at wavelength F_{λ} for an excitation at wavelength E_{λ} . In this matrix, a column represents an emission spectrum, whereas a row represents an excitation spectrum. The matrix was assembled as described in the text. All fluorescence amplitudes were normalized to a \times 100 ordinate scale attenuation and are measured in arbitrary units.

Emission Observa-	Fluo	rescen	ce Em		-	itudes ths (E		lowing	g Excit	citation		
tion Wave- lengths $(F_{\lambda nm})$	E ₄₃₀	E ₄₃₅	E ₄₄₀	E ₄₄₂	E ₄₄₅	E447	E ₄₅₀	E ₄₅₂	E ₄₅₅	E ₄₆₀		
F ₆₂₁	5	8	9	6	4	3	1	0	0	0		
F_{624}	11	16	17	13	9	7	3	1	0	0		
F_{630}	16	23	29	25	18	14	11	8	4	2		
F_{633}	16	22	28	26	20	16	13	11	7	2		
F_{636}	14	19	25	24	19	16	14	12	8	2		
F ₆₄₀	11	15	18	19	17	14	12	11	9	3		
F_{652}	77	79	83	80	73	70	73	77	60	50		
F_{657}	140	140	143	147	143	140	157	153	120	97		
F_{662}	80	70	67	70	77	63	77	73	63	53		
F_{670}	31	37	37	37	35	41	38	38	30	26		

According to Minc (13), for a 10×10 matrix, all minors of rank *n* are given by:

$$\left[\frac{10!}{n! (10-n)!}\right]^2$$
(2)

All possible 63504 minors of rank 5 of the cucumber Pchl in vivo had Δ/P values that were below the 3 $(\delta F/\bar{F})$ threshold. However, several thousands of the 44,100 minors of rank 4 had Δ/P values which were higher than the 3 $(\delta F/\bar{F})$ threshold. Sixteen significant Δ/P values of 16 minors that spanned the excitation wavelengths from 435 to 460 nm, and the emission wavelengths from 621 to 670 nm are given in Table II.

Altogether, the above data indicated that the Pchl pool of cucumber cotyledons *in vivo* was made up of at least four Pchl species. The failure of matrix analysis to detect the fifth Pchl component will be examined under "Discussion."

Fluorescence Emission Characteristics of Pchl in Etiolated Bean Leaves. At this point, it became desirable to determine whether the different SW Pchl species observed in etiolated cucumber cotyledons were detectable only in this particular tissue or whether their occurrence was more ubiquitous as was found to be the case for the multiple Pchl-chromophoric species of etiolated tissues (3, 4). Thus, the fluorescence profile of Pchl in etiolated bean leaves was also investigated.

As shown in Figure 3A (solid curves), the emission maximum of the SW Pchl band of 5-day-old etiolated bean leaves shifted from about 630 nm to 633, 637, and 640 nm as the excitation wavelength was varied from 435 to 455 nm at 5-nm intervals. The LW Pchl form exhibited an emission maximum at about 657 nm as was observed in cucumber cotyledons (Fig. 3A, E_{440} excitation).

A brief light treatment triggered photoconversion phenomena similar to those observed in etiolated cotyledons (Figs. 1A and 2A). The LW Pchl emitting at 657 nm was completely photoreduced; the fluorescence integral of the SW Pchl band decreased significantly, and the emission maxima at 633, 637, and 640 nm either disappeared or were replaced by much reduced and blueshifted emissions (Fig. 3A, dashed curves). The emission maxima at 677 and 696 nm are those of Chl(ide) a.

These results suggested that similar SW and LW Pchl species were present in both 5-day-old etiolated bean leaves and cucumber cotyledons. The Pchls in both tissues exhibited similar phototransformability. In contrast to cucumber cotyledons, the Pchl profile in etiolated bean leaves underwent more pronounced changes during tissue development in the dark: as the proportion of the SW Pchl forms changed, the Pchl species emitting at 640 to 642 nm dominated the Pchl emission profile in 5- to 9-day-old leaves and then became less pronounced in older leaves.

Soret Excitation Characteristics of SW and LW Pchl Species in Bean Leaves. The Soret excitation maxima of the fluorescing Pchl species detected in etiolated bean leaves were determined as in cucumber cotyledons (see above).

Table II. Δ/P Values for Some of 4×4 Minors Extracted from Matrix Depicted in Table I

Observation Wave-	Δ/P at Following Excitation Wavelengths $(E_{\lambda \text{ nm}})$					
lengths ($F_{\lambda nm}$)	435, 452, 455, 460	440, 452, 455, 460	442, 452, 455, 460	445, 452 455, 460		
621, 624, 636, 652	0.54	0.54	0.54	0.54		
621, 624, 636, 657	0.53	0.53	0.53	0.53		
621, 624, 636, 662	0.54	0.54	0.54	0.54		
621, 624, 636, 670	0.55*	0.55*	0.55ª	0.55*		

^a The corresponding $\delta F/\bar{F}$ values amounted to 0.02; for the other minors, the $\delta F/\bar{F}$ values amounted to 0.01.

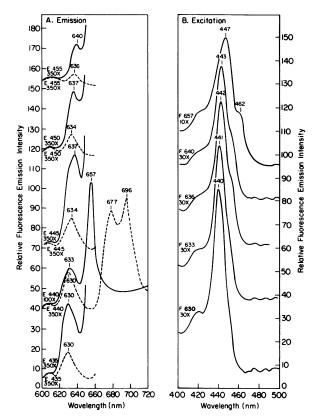


FIG. 3. Fluorescence emission (A) and excitation (B) spectra at 77 K of the Pchl in slices from a single 5-day-old etiolated bean leaf. One slice was monitored before illumination (---) and another slice was monitored after illumination (---) with 3.2 w/m² white fluorescent light for 60 s. Pre- and postillumination emission spectra were elicited by excitation at 5-nm intervals between 435 and 455 nm; they were normalized at 600 nm. Excitation spectra were recorded by positioning the emission monochromator at the emission maxima of the various Pchl species and by scanning the region between 400 and 500 nm with the excitation monochromator. All other symbols are as in Figures 1 and 2.

Corrected excitation spectra exhibited a maximum at about 440 nm when the emission monochromator was positioned at 630 nm, *i.e.* at the emission maximum of nt-Pchl (Fig. 3B). This was in close agreement with the value of 439 nm reported by Kahn *et al.* (10). It seems that the nt-SW Pchl species in etiolated cucumber cotyledons and bean leaves were quite similar and may be referred to as nt-Pchl (E_{440} , F_{630}).

When the emission monochromator was positioned at the fluorescence maximum of the 633-nm Pchl species, the corresponding Soret excitation maximum was observed at about 441 nm (Fig. 3B). This maximum was blue-shifted by about 2 nm in comparison to the Soret maximum of the 633-nm Pchl form of cucumber cotyledons. Likewise, the Soret excitation maximum of the bean SW Pchl form emitting at about 636 nm was also blue-shifted by about 2 nm and was observed at 442 nm instead of at 444 nm, as in cucumber (Fig. 3B). Similarly, the Soret excitation maximum of the 640- to 642-nm Pchl emitting species in bean was observed at about 443 nm (Fig. 3B), a blue shift of about 2 nm when compared to its counterpart in cucumber. The Soret excitation maximum of the bean LW Pchl form emitting at 657 nm was blue-shifted by about 3 nm and was observed at 447 nm instead of at 450 nm as in cucumber (Fig. 3B). An additional difference in the Soret excitation properties of the LW Pchl forms in cucumber and bean was manifested by the absence of a Soret excitation shoulder at 470 nm in the latter: a pronounced excitation shoulder was observed only at about 462 nm in bean (Figs. 2B and 3B).

The data presented above indicated that the SW and LW Pchl emitting species in 5-day-old etiolated bean leaves and cucumber cotyledons exhibited similar emission maxima, whereas the Soret excitation maxima of the transformable species in bean were blueshifted by 2 to 3 nm. In addition, the Soret excitation spectra of the LW Pchl of the two plant species exhibited distinct difference.

DISCUSSION

In this study, the Pchl species of etiolated cucumber cotyledons and primary bean leaves were characterized at 77 K by their shortwavelength red fluorescence emission maxima (F) between 629 and 660 nm, and by their Soret excitation maxima (E) between 440 and 470 nm. Selection of the Soret excitation maxima as well as the red emission maxima as a means of characterizing the Pchls was motivated by the high reliability of recording corrected excitation spectra in the Soret region and corrected emission spectra in the red region. This was due to: (a) the high molar extinction coefficient of Pchl in the blue region; (b) the superior electronic correction of excitation spectra in the blue region of the spectrum in instruments, such as the Perkin-Elmer MPF-3, that rely on the use of chemical quantum counters for correction (rhodamine B in this case); (c) excellent potentiometric correction of the emission spectra in the red region of the spectrum; and (d) the lack of interference by other fluorescent pigments in vivo between 630 and 660 nm.

Inasmuch as the different emission and excitation maxima reported here may have been fluorescence artifacts, this possibility is now examined. Selective scattering and sieve effects have been reported to distort *in vivo* absorption spectra (8, 12). However, we have ruled out these two sources of artifacts in this work. Selective light scattering, which causes apparent red shifts in absorption maxima (12), could not have distorted the emission spectra reported here because these were recorded scatter-free as described. Latimer and Rabinowitch (12) reached the same conclusion with respect to the fluorescence of Chl a in highly scattering suspension of green algae and diatoms. As for the sieve effect, which may have distorted the Soret excitation maxima, the work of Das *et al.* (8) demonstrated that it causes only a flattening of absorption bands rather than a red shift in the absorption maxima.

The data presented here suggested that the SW Pchl emission band of etiolated tissues was more complex than previously reported, and the deconvolution of the spectrofluorometric data by matrix calculations suggested the occurrence of at least four Pchls in etiolated cucumber cotyledons. However, a close examination of the spectrofluorometric data suggested that a fifth Pchl species may be present. Our assignment for the Pchls of cucumber cotyledons is as follows: one nt-SW Pchl [*i.e.* Pchl (E_{440} , F_{630})], three partially t-SW Pchls [*i.e.* Pchl (E_{443} , F_{633}), Pchl (E_{444} , F_{636})], and Pchl (E_{445} , F_{640})], and one LW t-Pchl [*i.e.* Pchl (E_{450} , F_{657})] (Table III). In etiolated bean leaves, the Soret excitation maxima of the Pchls were blue-shifted by 2 to 3 nm, and the Pchl band appeared to consist of nt-SW Pchl (E_{440} , F_{630}), three partially t-SW Pchls

 Table III. Fluorescent Pchl Species Observed at 77 K in Slices of 5-Dayold Etiolated Cucumber Cotyledon and Bean Leaves

	Pchl Species			
Tissue –	SW	LW		
Cucumber	nt-(E440, F630)			
	t-(<i>E</i> 443, <i>F</i> 633)	t-(<i>E</i> 450, <i>F</i> 657)		
	t-(<i>E</i> 444, <i>F</i> 636)			
	t-(<i>E</i> 445, <i>F</i> 640)			
Bean	nt-(<i>E</i> 440, <i>F</i> 630)			
	t-(<i>E</i> 441, <i>F</i> 633)	t-(<i>E</i> 447, <i>F</i> 657)		
	t-(<i>E</i> 442, <i>F</i> 636)			
	t-(E443, F640)			

[*i.e.* Pchl (E_{441} , F_{633}), Pchl (E_{442} , F_{636}), and Pchl (E_{443} , F_{640})], and LW t-Pchl (E_{447} , F_{657}) (Table III).

The extreme Soret excitation band overlap of the four putative SW Pchl species within a wavelength range of 10 nm (Table III) may explain the failure of matrix analysis to detect the fifth Pchl. This hypothesis was tested elsewhere (3) as follows. Coproporphyrin tetramethyl ester with a Soret excitation maximum at 399 nm (E_{399}), uroporphyrin octamethyl ester (E_{402}), protoporphyrin IX dimethyl ester (E_{409}) , and Zn-uroporphyrin octamethyl ester (E_{414}) were dissolved together in ether and the Soret excitation/ emission spectra of the mixture were recorded at 77 K. The four Soret excitation maxima of this mixture fell within a wavelength range of 15 nm, *i.e.* about 5 nm more than was the case for the cucumber Pchls in vivo. Deconvolution of the excitation/emission matrix according to Weber (18) indicated the presence of only three fluorescent components in the mixture. However, when the wavelength range separating the Soret excitation maxima in the mixture was increased to 26 nm by substituting Zn-protoporphyrin dimethyl ester (E_{425}) for Zn-uroporphyrin tetramethyl ester, the matrix analysis of the data detected all four fluorescent components.

The results presented here are compatible with the newly discovered Pchl chromophoric heterogeneity of etiolated tissues and suggest that the Pchl spectral multiplicity detected *in situ* may be due to the interaction of several chemically different Pchl chromophores with different macromolecular environment(s). It is noteworthy to point out that four different Pchlide-binding polypeptides were recently separated electrophoretically on polyacrylamide gels (15). However, the low temperature fluorescence properties of these Pchlide-protein complexes have not yet been determined. The foregoing results are also compatible with the observations of Virgin and French (19). These workers reported that the Chl(ide) *a* species apparent immediately after irradiation of etiolated barley leaves consisted of the same four major components reported to be present in all mature tissues of higher plants and algae.

Finally, the nt-SW Pchl appeared to be almost identical in both etiolated cucumber cotyledons and bean leaves, exhibiting a Soret excitation maximum at about 440 nm and an emission maximum at about 630 nm. The t-SW Pchls and t-LW Pchl exhibited emission maxima at 633, 636 to 638, 640 to 642, and 657 nm, respectively, in both tissues. The Soret excitation maxima of the phototransformable SW and LW Pchl species in etiolated bean leaves were blue-shifted, however, by 2 to 3 nm, as compared to their counterparts in etiolated cotyledons (Figs. 2B and 3B). Furthermore, although the LW Pchl in both tissues exhibited an excitation shoulder at about 463 nm, that in etiolated bean leaves lacked completely the prominent excitation shoulder at 470 nm which was observed in cucumber cotyledons. Differences were also observed between the Soret excitation spectra of the SW and LW Pchls within the same species, notably the lack of excitation shoulders at 463 nm (and at 463 and 470 nm in cucumber) in the SW Pchl species. The reasons for these differences will be discussed elsewhere.

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