Chloroplast Biogenesis: Detection of Divinylprotochlorophyllide Ester in Higher Plants†

Faith C. Belanger and Constantin A. Rebeiz*

ABSTRACT: It is shown that the protochlorophyllide ester pool of etiolated higher plants is a faithful copy of the protochlorophyllide pool. It is made up of both monovinyl- and divinylprotochlorophyllide esters. Although the two tetrapyrroles exhibited similar emission maxima, they were distinguishable by their Soret excitation maxima, which were found at 436–437 and 443–444 nm, respectively, in ether at 77 K. The two pigments were partially separated on thin layers of polyethylene. They were accompanied by two unknown fluorescent compounds. It was also shown that during greening, the protochlorophyllide ester pool maintained a constant qualitative composition. This was in sharp contrast with the drastic qualitative changes undergone by the protochlorophyllide pool of etiolated tissues grown under identical conditions.

The two immediate precursors of the chlorophyll of green plants are considered to be the two protochlorophylls:[1] protochlorophyllide (Pchlide)[2] and its esterified analogue, Pchlide ester (Rebeiz & Castelfranco, 1973). Both of these tetrapyrroles are considered to be monovinylprotochlorophylls, i.e., 2-vinyl-4-ethylprotochlorophylls (Figure 1). Depending on the species and the age of the tissue, the Pchlide fraction usually constitutes ~90–95% of the total protochlorophyll pool while Pchlide ester accounts for the balance.

It was recently reported by Rebeiz et al. (1979) and by Belanger & Rebeiz (1979) that, contrary to previous beliefs, the Pchlide pool of etiolated plants was actually heterogeneous and consisted of divinyl-Pchlide, i.e., 2,4-divinyl-Pchlide in addition to MV-Pchlide (Figure 1). It was also shown to contain two Pchlide-like fluorescent species of unknown structure (Belanger & Rebeiz, 1980a). The two Pchlides were distinguished by their Soret excitation maxima which were found at 436 nm for MV-Pchlide and at 443 nm for DV-Pchlide at 77 K in ether (Belanger & Rebeiz, 1979, 1980a,b).

More recent investigations of the chlorophyll *a* and *b* pools of green plants have also revealed that these two pools were actually more complex than previously described by Belanger & Rebeiz (1979) and consisted of at least four chemically different chlorophyll *a* chromophores and four different chlorophyll *b* chromophores (Rebeiz et al., 1980). Since all the chlorophyll of green plants finds its ultimate origin in the protochlorophyll precursors, we wondered whether the Pchlide ester pool, which is also phototransformable into chlorophyll (Rebeiz & Castelfranco, 1973), contributed to the chlorophyll chromophoric heterogeneity in the same capacity as the Pchlide pool. This in turn led us to question whether the Pchlide ester pool was as heterogeneous as the Pchlide pool of etiolated tissues.

In this work, it is shown that the Pchlide ester pool of etiolated tissues is as heterogeneous as the Pchlide pool and consists of both DV- and MV-Pchlide esters as well as of two fluorescing species of unknown structure. However, in contrast to the Pchlide pool (Belanger & Rebeiz, 1980a), the Pchlide ester pool was shown to maintain a very constant composition during greening.

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 Corp., Modesto, CA. Beans (*Phaseolus vulgaris* L. var. Red Kidney) were purchased from Maxwell Seed House, Champaign, IL. Hybrid sweet corn (*Zea mays* L.) was obtained from Rogers Bros. Co., Idaho Falls, ID. Barley (*Hordeum vulgare* var. Beacon Spring) was donated by the Department of Agronomy, University of Illinois at Urbana. Hulled pumpkin (*Cucurbita pepo*) seeds were purchased from a local supermarket. Etiolated *Euglena gracilis* cells were obtained from Richard Eichholz, Cell Biology Program, University of Illinois at Urbana. The cells were grown on a modified Hutner, Greenblatt, and Schiff medium (Mielenz, 1976).

Etiolated seedlings were grown in moist vermiculite at 28 °C in total darkness. Etiolated tissues were harvested under a dim green safelight. Only the top 2 cm of primary corn and barley leaves was used.

For photoperiodic tissue, seeds were germinated at 28 °C in moist vermiculite under a 14-h light/10-h dark photoperiodic regime as described earlier (Cohen & Rebeiz, 1978). The light intensity in the growth chamber was about 3538 μW cm^{-2} (900 ft-c).

Incubation with δ-Aminolevulinic Acid. Three grams of 3-day etiolated cucumber cotyledons was incubated overnight (13 h) in a 1.82 mM solution of δ-aminolevulinic acid at 28 °C (Rebeiz et al., 1975a).

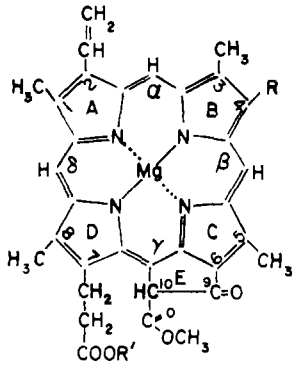
Phototransformation and Dark Regeneration of the Protochlorophyllide Ester Pool. Photoreduction of the Pchlide

† From the Laboratory of Plant Pigment Biochemistry and Photobiology, Department of Horticulture, University of Illinois, Urbana, Illinois 61801. *Received January 22, 1980.* This work was supported by a predoctoral National Science Foundation fellowship to F.C.B. and by National Science Foundation Grant PCM 7811559 and by funds from the Illinois Agricultural Experiment Station to C.A.R.

[1] Protochlorophyll refers to the mixture of protochlorophyllide and protochlorophyllide ester found in plant tissues. Protochlorophyllide ester is used here generically to designate esterified protochlorophylls which may differ from one another by the nature of their side-chain substituents at positions 2 and 4 of the macrocycle. Monovinylprotochlorophyllide ester is defined as that protochlorophyllide ester with a vinyl group at position 2 (or 4) of the macrocycle and an ethyl group at position 4 (or 2) of the macrocycle. Divinylprotochlorophyllide ester is defined as that protochlorophyllide ester with vinyl groups at both the 2 and 4 positions of the macrocycle.

[2] Abbreviations used: Pchlide, protochlorophyllide; MV, monovinyl; DV, divinyl.

0006-2960/80/0419-4875$01.00/0



a. R = $CH_2 \cdot CH_3$; R′ = H; 2-VINYL,4-ETHYL-PROTOCHLOROPHYLLIDE

b. R = $CH_2 \cdot CH_3$; R′ = $C_{20}H_{39}$; 2-VINYL,4-ETHYL-PROTOCHLOROPHYLLIDE ESTER

c. R = CH: CH_2; R′ = H; 2,4-DIVINYL-PROTOCHLOROPHYLLIDE

d. R = CH: CH_2; R′ = $C_{20}H_{39}$; 2,4-DIVINYL-PROTOCHLOROPHYLLIDE ESTER

FIGURE 1: Structure of some protochlorophyll derivatives.

ester pool was achieved by illuminating etiolated cucumber cotyledons for 2 s with 3 mW cm^{-2} of incandescent light (Cohen & Rebeiz, 1978). The tissue was then incubated at 28 °C in the dark for 45 min.

Extraction of the Protochlorophyllide Ester Pool from Higher Plant Tissues. Three grams of tissue was homogenized in 20 mL of acetone–0.1 N NH_4OH (9:1 v/v) at 0–4 °C for 2 min in a Sorvall Omni-mixer (Rebeiz et al., 1975b) and then centrifuged at 39000*g* for 10 min. The pigments were partitioned into a hexane fraction containing Pchlide ester and a hexane-extracted acetone fraction containing Pchlide by extracting the 80% acetone solution first with an equal volume of hexane and then with a 1/3 volume of hexane (Rebeiz et al., 1975b). The hexane extracts containing the Pchlide ester fraction were combined and concentrated under N_2 gas to ~1 mL before chromatography.

Extraction of the Protochlorophyllide Ester Pool from the Inner Seed Coats of Pumpkin. The extraction of DV-Pchlide ester was essentially as described by Houssier & Sauer (1969). Five grams of seeds was soaked in distilled water for ~10 min and then blotted dry. The seeds were next soaked in 15 mL of acetone for 5 min. In this manner the pigments in the inner seed coats were extracted into the acetone. The acetone was poured off and the seeds were reextracted with another 15 mL of acetone. The acetone extracts were combined and adjusted to 75% acetone with water. The Pchlide ester pool was transferred to hexane by extraction, first with an equal volume of hexane and then with 1/3 volume of hexane.

Extraction of the Protochlorophyllide Ester Pool from Etiolated Euglena. The extraction of *Euglena* cells was modified from Cohen & Schiff (1976). Etiolated *E. gracilis* cells were harvested in late logarithmic or stationary phase. The cultures were centrifuged at 1000*g* for 5 min to pellet the cells. The pellets were combined and washed with 0.01 M potassium phosphate buffer, pH 7.0, and 27% sucrose (Freyssinet & Schiff, 1974) and recentrifuged. The pigments were extracted from the cells by repeated extractions with acetone–0.1 N NH_4OH (9:1 v/v) until the pellets were nearly white. The Pchlide ester pool was transferred to hexane by extraction, first with an equal volume of hexane and then with 1/3 volume of hexane.

Separation of the Protochlorophyllide Ester Pool from the Protochlorophyllide and the Chlorophyll Pools. The protochlorophyllide ester pool (R_f = 0.9) was separated from the Pchlide (R_f = 0.5) and the chlorophyll *a* and *b* pools (R_f = 0.8) by chromatography on thin layers of silica gel H developed in toluene–ethyl acetate–ethanol (8:2:2 v/v) at 4 °C in the dark. The Pchlide ester fraction was eluted into ether and dried under N_2 gas in order to remove traces of developing solvent. The pigment was then redissolved in ether.

Separation of the Divinylprotochlorophyllide Ester and Monovinylprotochlorophyllide Ester Pools of Etiolated Tissues. Divinylprotochlorophyllide ester was partially segregated from MV-Pchlide ester by chromatography on thin layers of polyethylene in the dark at room temperature. Polyethylene powder was purchased from Polysciences, Inc., Warrington, PA. The preparation of the thin-layer plates was modified from Jeffrey's (1969). Fifty grams of polyethylene was homogenized with 150 mL of acetone for 1 min in a blender. The plates were spread by using a Brinkman spreader. The plates became ready for use after drying at room temperature. They were developed in 2-propanol–acetone (1:1 v/v). The pigments were eluted into ether, dried under N_2, and then redissolved in ether prior to spectroscopic determinations.

Preparation of Standard Monovinyl- and Divinylprotochlorophyllide Ester from the Inner Seed Coat of Pumpkin. Standard DV-Pchlide ester was purified from the inner seed coat extract of pumpkin (vide supra) by chromatography on thin layers of sucrose. The preparation of the sucrose plates (500 μm thick) was modified from Jeffrey's et al. (1975). Commercial powdered sugar was dried at 90 °C for 30 min. Sixty grams of dried sugar was mixed with 100 mL of chloroform for 2 min in a blender, and the thin layers were spread by using a Brinkman spreader. The plates became ready for use after drying at room temperature. They were developed in 1.2% 1-propanol in isooctane (Houssier & Sauer, 1969). The pigment was eluted into ether, dried under N_2, and then redissolved in ether prior to spectroscopic monitoring.

Spectrophotometry. Absorption spectra were recorded with an Aminco dual-wavelength spectrophotometer, Model DW-2, operated in the split-beam mode.

Spectrofluorometry. Corrected fluorescence emission and excitation spectra were recorded on a Perkin-Elmer spectrofluorometer, Model MPF-3, equipped with a corrected spectra accessory (Rebeiz et al., 1975a). Pigment solutions either were monitored at room temperature in cylindrical microcells 4 mm in diameter or were transferred to cylindrical sample tubes and monitored at 77 K as described by Cohen & Rebeiz (1978). Emission spectra were recorded at an excitation bandwidth of 6 nm and an emission bandwidth of 3 nm. Excitation spectra were recorded at an emission bandwidth of 6 nm and an excitation bandwidth of 3 nm. At 77 K the following combination of filters was used to eliminate interference by scattered light: a blue filter, Pyrex No. 5543, which is transparent in the 350–500-nm region, was placed between the excitation monochromator and the sample; a pyrex yellow sharp cut-off filter, that excluded light below 520 nm, was interposed between the sample and the emission monochromator. when the excitation spectra were recorded, the blue filter was removed from the excitation beam.

The fluorescence spectra reported in Figures 5 and 6 were recorded on a fully corrected, photon-counting spectrofluorometer, Model SLM 8000 DS, equipped with two red-sen-

Table I: Spectral Properties of Monovinyl- and Divinylprotochlorophyllide Ester in Ether, at Room Temperature

pigment or chromatographic band	source of inner seed coat	R_f	absorption max (nm)				soret excitation max (nm)	ref
MV-Pchlide ester	pumpkin		432	535	571	623		Jones (1966)
MV-Pchlide ester	pumpkin		432	533	570	622		Houssier & Sauer (1969)
band 1	pumpkin	0.61	431	530	568	620	432	this work
DV-Pchlide ester	squash		438	532.5	571	622		Stanier & Smith (1959)
DV-Pchlide ester	pumpkin		438	537	574	624		Jones (1966)
DV-Pchlide ester	pumpkin		437	536	572	622		Houssier & Sauer (1969)
band 2	pumpkin	0.51	437	530	570	622	438	this work
band 3	pumpkin	0.37	436	528	572	622	438	this work
band 4	pumpkin	0.18	436	528	570	622	437	this work

sitive, extended S20 photomultipliers (EMI 9658) and interfaced with a Hewlett-Packard microputer system, Model 9825 S.

Computer Analysis of the Emission/Excitation Matrices. Deconvolution of the emission/excitation matrices of the Pchlide ester pool was performed according to Weber (1961). All computations were programmed on a Control Data Corp. Computer Cyber 175 or on a Hewlett-Packard 9825S microcomputer system.

Results

Spectrofluorometric Profile of the Protochlorophyllide Ester Pool of Etiolated Tissues at Room Temperature. It is well established that the Pchl pool of etiolated tissues consists of an acidic (Pchlide) and of a neutral, fully esterified component (Pchlide ester) (Löeffler, 1955; Wolff & Price, 1957). Both components exhibit similar spectral properties but differ widely in their chromatographic properties (Virgin, 1960; Rebeiz, 1968; Rebeiz et al., 1970). The chemical heterogeneity of the Pchlide pool was recently described (Belanger & Rebeiz, 1980a), while in this work, we endeavor to describe the chemical heterogeneity of the Pchlide ester pool.

In our quest for the presence of DV-Pchlide ester in etiolated tissues, the fluorescence properties, at room temperature, of the Pchlide ester pool of etiolated leaves of barley, corn, and bean and of cucumber cotyledons were compared to those of standard MV- and DV-Pchlide ester. It was conjectured that if any of those Pchlide ester pools contained enough DV-Pchlide ester in addition to MV-Pchlide ester, this may be manifested by a distinct DV-Pchlide ester Soret excitation maximum at ~438 nm in addition to the MV-Pchlide ester Soret at 432 nm (Belanger & Rebeiz, 1979).

Standard MV- and DV-Pchlide esters were purified from the inner seed coat of pumpkin as described under Materials and Methods. However, upon chromatography of the extracted DV-Pchlide ester pool on thin layers of sucrose, three DV Pchlide ester bands with very similar spectroscopic properties were observed in addition to MV-Pchlide ester (Table I). As will be discussed later (see Discussion), these DV-Pchlide esters probably differed only by their esterifying long-chain alcohols (Ellsworth & Nowak, 1974). Throughout this work, however, only the most prominent of the DV-Pchlide ester bands, i.e., band 2, which moved with an R_f of 0.51 was used as our DV-Pchlide ester standard. Both the MV- and DV-Pchlide ester standards exhibited spectroscopic properties at room temperature that were similar to those published by others (Table I; Figure 2, lines a and b).

The protochlorophyllide ester pools of the various etiolated tissues were separated from the Pchlide pool by chromatography on thin layers of silica gel H; they were eluted in ether as described under Materials and Methods, and their fluorescence emission and Soret excitation spectra in ether were recorded at room temperature. The fluorescence emission of the purified Pchlide ester pool of all the tissues that were examined exhibited a protochlorophyll emission maximum at 624.0–626.0 nm (Figure 2A). As expected (Belanger & Rebeiz, 1979), the Soret excitation spectrum was more revealing and suggested the presence of some DV-Pchlide ester in addition to MV-Pchlide ester. This was very obvious, for example, in barley which exhibited a distinct DV-Pchlide ester Soret excitation maximum at 437 nm in addition to the MV-Pchlide ester maximum at 432 nm(Figure 2B, lines a, b, and c). The DV Pchlide ester component was slightly less obvious in corn than in barley and was only noticeable as a weak Soret excitation shoulder in bean and cucumber (Figure 2B, lines e and f).

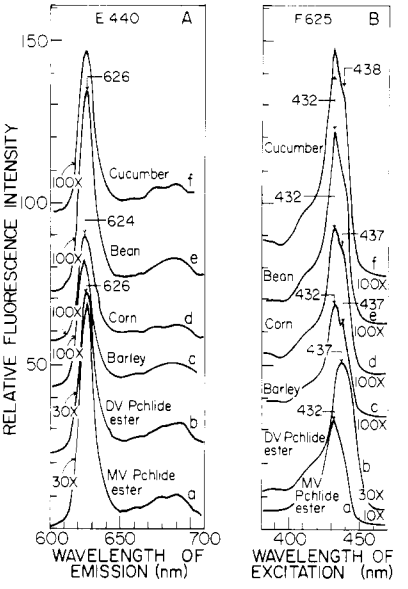


FIGURE 2: Fluorescence emission (A) and excitation (B) spectra of the extracted Pchlide ester pool of etiolated barley, corn, and bean leaves, of etiolated cucumber cotyledons, and of standard MV- and DV-Pchlide esters, in ether at room temperature. The emission spectra were elicited by an excitation wavelength E of 440 nm while the excitation spectra were recorded at an emission wavelength F of 625 nm. Ordinate scale attenuation is indicated on the spectra, 350× being the highest sensitivity used. Base lines were arbitrarily adjusted to avoid overlap of the spectra. Arrows point to wavelengths of interest.

Altogether, the above results suggested that, depending on the plant species, various amounts of DV-Pchlide ester may be present in the Pchlide ester pool of etiolated tissues.

Detection of Divinylprotochlorophyllide Ester Fluorescence in the Protochlorophyllide Ester Pool of Etiolated Tissues at 77 K. It was demonstrated earlier that the recording of fluorescence emission and excitation spectra at 77 K resulted in a considerable improvement in peak resolution (Belanger & Rebeiz, 1979, 1980a). It was, therefore, decided to confirm

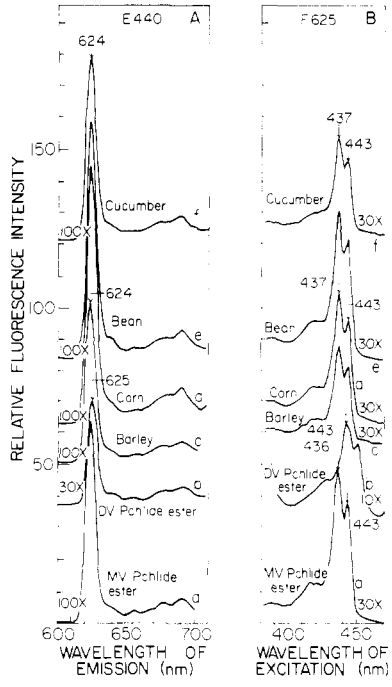


FIGURE 3: Fluorescence emission (A) and excitation (B) spectra at 77 K in ether of the extracted Pchlide ester pool of etiolated barley, corn, and bean leaves, of etiolated cucumber cotyledons, and of standard MV- and DV-Pchlide esters. The emission spectra were elicited by excitation at E440 nm. The excitation spectra were recorded at an emission wavelength of 625 nm. All symbols are as in Figure 2.

the conclusions drawn from the room temperature fluorescence spectra by examining the fluorescence emission and excitation spectra of the extracted Pchlide ester pools recorded at 77 K.

The fluorescence emission and excitation spectra of the standard MV- and DV-Pchlide esters purified from the inner seed coat of pumpkin are depicted in Figure 3, lines a and b. MV-Pchlide ester exhibited an emission maximum at 624.0 nm, while the emission maximum of DV-Pchlide ester was observed at 625 nm (Figure 3A, lines a and b). The Soret excitation profiles revealed, however, that the MV-Pchlide ester was partially contaminated by DV-Pchlide ester as evidenced by the detection of a 443.0-nm DV-Pchlide ester Soret excitation maximum in addition to the 436-nm MV-Pchlide ester Soret maximum (Figure 3B, line a). Likewise, the standard DV-Pchlide ester with a Soret excitation maximum at 442.5 nm was also partially contaminated by a fluorescent compound having a Soret excitation maximum at 450 nm (Figure 3B, line b). Further characterization of this compound will be described later (vide infra).

The fluorescence emission spectra of the Pchlide ester pools extracted from different etiolated tissues all exhibited a Pchlide ester emission maximum at 623.5–625.0 nm (Figure 3A, lines c–f). On the other hand, the Soret excitation profiles recorded at or near the emission maxima all exhibited distinct MV-Pchlide ester Soret excitation maxima at 436.5 nm and DV-Pchlide ester Soret excitation maxima at 443.5 nm (Figure 3B, lines c–f). These results in turn suggested that the Pchlide ester pool of etiolated tissues very likely contained both MV- and DV-Pchlide esters.

Detection of Components E452F639 Ester, an Esterified Fluorescent Compound of Unknown Structure, in the Protochlorophyllide Ester Pool of Etiolated Tissues. Since the Pchlide and Pchlide ester pools of etiolated tissues appeared to be very similar in their MV- and DV-protochlorophyll constituents (Belanger & Rebeiz, 1979, 1980a; Figure 3), we

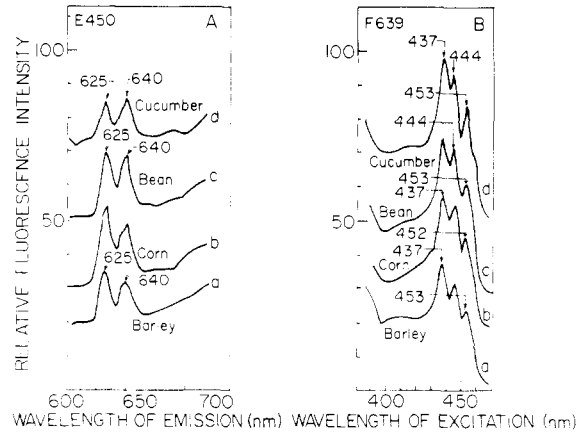


FIGURE 4: Detection of compound E452F639 ester in the fluorescence emission (A) and excitation (B) spectra of the Pchlide ester pool of etiolated barley, corn, bean, and cucumber. The spectra were recorded in ether at 77 K. The emission spectra were elicited by excitation at E450 nm. The excitation spectra were recorded at an emission wavelength of 639 nm. All spectra were recorded at an attenuation scale of 350×. All symbols are as in Figure 2.

wondered whether this similarity between the two pools extended also to the presence of an esterified analogue of compound E453F640 in the Pchlide ester pool. Compound E453F640 was detected earlier in the Pchlide pool of etiolated tissues (Belanger & Rebeiz, 1980a). In this context "E" refers to the Soret excitation maximum while "F" refers to fluorescence emission maximum in ether at 77 K of the unknown tetrapyrrole. Compound E453F640 was detected by its emission maximum at 640 nm which was elicited by a 450-nm excitation and by its Soret excitation maximum at 453 nm when the excitation spectrum was recorded at or near the emission maximum, i.e., near 640 nm (Belanger & Rebeiz, 1980a).

The fluorescence emission spectra of the Pchlide ester pools extracted from the different etiolated tissues and elicited by a 450-nm excitation are depicted in Figure 4A. In addition to the Pchlide ester emission at ~625 nm, they all exhibited well-resolved emission maxima at 639–640 nm (Figure 4A, lines a–d). The Soret excitation spectra recorded at 639 nm exhibited, in addition to the MV- and DV-Pchlide ester Soret excitation maxima at 437 and 444 nm, respectively, a well-resolved Soret excitation maximum at 452–453 nm (Figure 4B, lines a–d).

Altogether the above results suggested that the Pchlide ester pool of etiolated tissues contained an esterified analogue of the unknown fluorescent compound E453F640 which was detected earlier in the Pchlide pool of etiolated tissues (Belanger & Rebeiz, 1980a). This esterified compound will be referred to as compound E452F639 ester. From a comparison of the emission amplitudes at 625 and 639 nm, and assuming equal quantum yield of fluorescence, it was estimated that compound E452F639 ester constituted ~6–7% of the Pchlide ester pool in etiolated tissues.

Partial Segregation of the Protochlorophyllide Ester Pool of Cucumber Cotyledons into Divinyl- and Monovinylprotochlorophyllide Ester Components. The partial segregation of MV-Pchlide ester and DV-Pchlide ester from pumpkin seed coat was described earlier by Houssier & Sauer (1969). A modification of their procedure was successfully used in this work to prepare MV- and DV-Pchlide ester standards from pumpkin seed coat (Table I; Figure 2 lines a and b). However, these same procedures failed to segregate the putative MV- and DV-Pchlide ester components of the Pchlide ester pool of etiolated higher plant tissues. It now appears that the

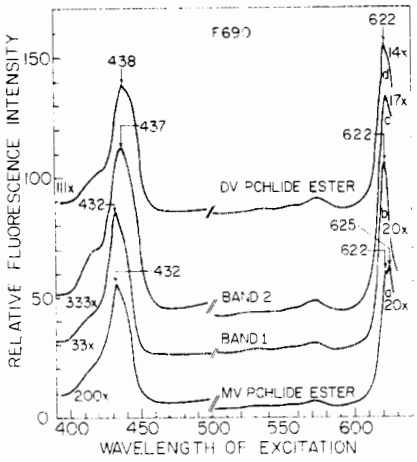


FIGURE 5: Fluorescence excitation spectra in ether at room temperature of standard MV- and DV-Pchlide esters and of the two fluorescent bands segregated from the Pchlide ester pool of cucumber cotyledons by chromatography on thin layers of polyethylene. The spectra were recorded in the ratio mode on an SLM spectrofluorometer, Model 8000 DS at an emission wavelength of 690 nm. From 390 to 500 nm,the spectra were recorded with an excitation slit width of 2 nm and an emission slit width of 4 nm. From 500 to 630 nm, the excitation and emission slits were 4 and 8 nm, respectively, and the recorded spectra were the sum of five successive scans. The photon count was integrated for 2 s at each 1-nm increment. The relative sensitivity at which the various spectra were recorded are indicated on the spectra. All symbols are as in Figure 2.

procedure described by Houssier & Sauer (1969) is successful in separating the MV and DV components of the Pchlide ester pool when these components are esterified with different alcohols (see Discussion). In etiolated tissues of higher plants where the nature of the alcohol heterogeneity seems to be different (see Discussion), a different chromatographic system had to be evolved, as described below, for etiolated cucumber cotyledons.

The protochlorophyllide ester pool of etiolated cucumber cotyledons was first freed from carotenoids by prepurification on thin layers of silica gel H developed in toluene–ethyl acetate–ethanol (8:2:2 v/v) at 4 °C in the dark (see Materials and Methods). The protochlorophyllide ester pool moved with an R_f of ~0.9 and was eluted in ether. The ether eluate was washed with H_2O and dried completely under N_2 gas in order to get rid of traces of the chromatographic solvents. This procedure was essential for the success of the subsequent chromatographic steps. The dried Pchlide ester pool was dissolved in ether and applied to thin layers of polyethylene. The spotted Pchlide ester was dried completely by holding the plates at room temperature in subdued light for 10 min before developing in 2-propanol–acetone (1:1 v/v) in the dark at room temperature.

Two chromatographic bands were eluted from the developed plates. One band (band 1) moved with an R_f of ~0.52 and was eluted in ether. It exhibited a bright red fluorescence under UV light of 360 nm. The second band (band 2) moved with an R_f of ~0.3 and was scraped into ether beginning at least 1.5 cm below the lower rim of band 1. At the medium degree of loading of the thin-layer plates adopted in most instances, the concentration of band 2 was low enough that no distinct fluorescence was detected by the naked eye. The eluted bands were dried under N_2 gas and were redissolved in ether for spectroscopic determinations.

Because of the low concentration of the eluted bands and the high sensitivity of spectrofluorometry, excitation spectra from 390 to 630 nm were recorded at room temperature in lieu of conventional absorption spectra. The room temperature excitation spectra thus recorded (Figure 5) were very similar to the absorption spectra of MV- and DV-Pchlide ester reported by others (Table I). They were also very similar to the excitation spectra of the standard MV- and DV-Pchlide ester of pumpkin seed coat recorded under similar conditions (Figure 5). Band 1 exhibited MV-Pchlide ester Soret and red excitation maxima at 432 and 622 nm, respectively (Figure 5, line b). Its overall excitation spectrum was very similar to that of the standard MV-Pchlide ester of pumpkin seed coat (Figure 5, line a). The latter exhibited an additional red excitation maximum at 625 nm which was not observed in the absorption spectrum of the same sample, however, and the origin of which is presently unknown (Figure 5, line a). The detection of this second red excitation maximum was probably made possible by the high resolution of the holographic excitation double monochromators with which the SLM spectrofluorometer is equipped. On the other hand, band 2 exhibited DV-Pchlide ester Soret and red excitation maxima at 437 and 623 nm, respectively, which were very similar to those of the standard DV-Pchlide ester (Figure 5, lines c and d).

Similar DV- and MV-Pchlide ester components were also segregated from an extract of etiolated *Euglena*. However, in this case the DV- and MV-Pchlide ester components separated into two fluorescent bands of approximately equal intensity.

Altogether, the above data indicated that the Pchlide ester pool of etiolated cucumber cotyledons and etiolated *Euglena* contained MV- and DV-Pchlide ester components which are separable by chromatography on thin layers of polyethylene.

Detection of Additional Fluorescent Components in the Monovinyl- and Divinylprotochlorophyllide Ester Fractions of Cucumber Cotyledons. When the Pchlide pool of etiolated tissues was segregated earlier into MV- and DV-Pchlide components, spectrofluorometric monitoring of the MV and DV fractions at 77 K revealed the presence of other fluorescent constituents in these fractions (Belanger & Rebeiz, 1980a). Thus, the fluorescence emission and excitation profiles of the segregated MV- and DV-Pchlide ester components of the cucumber Pchlide ester pool were reexamined at 77 K, in order to determine the purity of these fractions.

As was observed with the segregated MV- and DV-Pchlide components (Belanger & Rebeiz, 1980a), it was observed that the segregated MV- and DV-Pchlide ester fractions were also contaminated by other fluorescent compounds (Figure 6). Band 1 exhibited an emission maximum at 623 nm (Figure 6A, line a) and appeared to be made up mainly of MV-Pchlide ester. This was evidenced by the very pronounced MV-Pchlide ester Soret excitation maximum at 436 nm (Figure 6B, line a). It was contaminated by lesser amounts of DV-Pchlide ester as evidenced by the DV-Pchlide ester Soret excitation maximum at 443 nm (Figure 6B, line a). Its degree of contamination was a little higher than the contamination of the standard MV-Pchlide ester of pumpkin seed coat by DV-Pchlide ester (Figure 6B, line b). In addition, band 1 appeared to be contaminated by compound E452F639 ester. This was apparent from the emission maximum at 639 nm which was elicited by a 450-nm excitation (Figure 6A, line c) and from the corresponding Soret excitation maximum at 452 nm which was recorded at an emission maximum of 640 nm (Figure 6B, line c). Band 1 appeared to be more contaminated by this compound than the Pchlide ester band of pumpkin seed coat (Figure 6A,B, line d).

Band 2 exhibited a slightly red shifted emission maximum at 624 nm (Figure 6A, line e) and appeared to be made up mainly of DV-Pchlide ester. This was evidenced from the

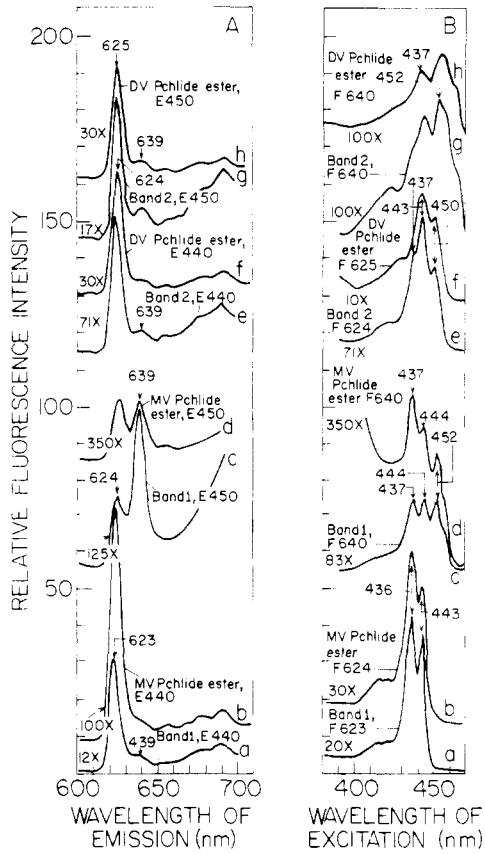


FIGURE 6: Comparison of the fluorescence emission (A) and excitation (B) spectra in ether at 77 K of the individual Pchlide ester species segregated on thin layers of polyethylene, with the MV- and DV-Pchlide ester fractions of pumpkin seed coat. The latter were recorded on a Perkin-Elmer spectrofluorometer, Model MPF3. The spectra of bands 1 and 2 were recorded on an SLM spectrofluorometer, Model 8000 DS, operated in the ratio mode. In this case the emission spectra were recorded at an emission slit width of 2 nm and an excitation slit width of 4 nm, while excitation spectra were recorded at an emission slit width of 4 nm and an excitation slit width of 2 nm. All symbols are as in Figure 2.

major DV-Pchlide ester Soret excitation maximum at 443 nm (Figure 6B, line e). It was contaminated by very small amounts of MV-Pchlide ester as denoted by the Soret excitation shoulder at 436–437 nm (Figure 6B, line e).

As was observed for the DV-Pchlide fraction of etiolated cucumber cotyledons (Belanger & Rebeiz, 1980a), band 2 contained significant amounts of an esterified analogue of compound E451F626. In this work this compound will be referred to as E450F625 ester. Its presence was indicated by a pronounced Soret excitation maximum at 450 nm which was elicited by recording the excitation spectrum at the emission maximum of band 2, i.e., at 624 nm (Figure 6B, line e). This compound was also present in the standard DV-Pchlide ester purified from pumpkin seed coat (Figure 6B, line f). Compound E450F625 ester was considered to be different from compound E452F639 ester, because its emission maximum was located at ~624–625 instead of at 639 nm as in the case for compound E452F639 ester [also see Belanger & Rebeiz (1980a)]. This was also obvious from the Soret excitation amplitudes recorded at 640 and 624 nm, respectively, as described below.

Band 2 was also slightly contaminated by compound E452F639 ester as was just mentioned. This was evidenced by the relatively low emission amplitude at 639 nm, which was elicited by a 450-nm excitation (Figure 6A, line g). The corresponding Soret excitation maximum recorded at 640 nm exhibited an excitation amplitude at 452–453 nm which was commensurate with the amplitude of the emission amplitude at 639 nm (Figure 6B, line g). A similar degree of contamination was also observed in the standard DV-Pchlide ester purified on thin layers of sucrose from pumpkin seed coat (Figure 6A,B, line h).

Altogether these results suggested that the Pchlide ester pool of etiolated tissues consisted of four fluorescent species, namely, MV-Pchlide ester E436F623, DV-Pchlide ester E443F624, compound E450F625 ester, and compound E452F639 ester. In this respect these compounds were similar to the unesterified fluorescent species detected in the Pchlide pool of etiolated tissues (Belanger & Rebeiz, 1980a) but were blue shifted in their emission and excitation maxima by ~1 nm.

Determination of the Minimum Number of Fluorescent Species in the Protochlorophyllide Ester Pool of Etiolated Tissues. The fluorescence spectra described in previous sections were analyzed by Weber's matrix technique (Weber, 1961) to obtain an independent estimate of the number of fluorescent components in the Pchlide ester pool of etiolated tissues. This method was successfully applied earlier to determine the minimum number of fluorescent species in the extracted chlorophyll *a* and *b* pools of green tissues (Rebeiz et al., 1980) and in the extracted Pchlide pool of etiolated tissues (Belanger & Rebeiz, 1980a).

According to Weber (1961), for an emission/excitation matrix "**M**" whose elements $M(i,j)$ represent the fluorescence intensity measured at wavelength $\lambda\, j$, for an excitation $\lambda\, i$, the number of fluorescent components depicted in the matrix is equal to the rank of the minors for which the following inequality is obeyed:

$$\Delta P \geq 3\delta F/\bar{F} \qquad (1)$$

where Δ = value of the determinant for a minor of any particular rank; P = value of the permanent (i.e., the sum of all diagonal products of the matrix) (Minc, 1978); δF = noise level or minimum detectable fluorescence; $\bar{F}$ = mean fluorescence intensity of the $M(i,j)$ elements of the minor under consideration.

Weber's technique was therefore used for determining the minimum number of fluorescent chromophores in the extracted Pchlide ester pool of several etiolated tissues. To this effect 10 Soret excitation spectra were recorded on the same aliquot of the Pchlide ester pool. The excitation spectra were recorded at the following fluorescence emission wavelengths: 615, 617, 621, 625, 631, 635, 637, 641, 643, and 645 nm. The Soret excitation amplitudes at 407, 415, 423, 430, 437, 440, 443, 450, 453, and 460 nm were then determined for every excitation spectrum. All excitation amplitudes were normalized to the same attenuation scale. The 10 × 10 matrix from cucumber, depicted in Table II, was then assembled from the normalized Soret excitation amplitudes. In this matrix a row represented an emission spectrum while a column represented an excitation spectrum. The determination of all the minors of a given rank that obeyed eq 1 was achieved by means of the computer. The latter was programmed to report any minor of a given rank "n" with $\Delta/P \geq 3\delta F/\bar{F}$. According to Minc (1978), for a 10 × 10 matrix, all minors of rank n are given by $[10!/[n!(10-n)!]]^2$.

All possible 63 504 minors of rank 5 of the cucumber Pchlide ester pool had Δ/P values that were below the $3\delta F/\bar{F}$ threshold. However, several hundred minors of rank 4 had Δ/P values that were higher than the $3\delta F/\bar{F}$ threshold. Nine significant Δ/P values for nine minors that spanned the emission wavelengths from 615 to 637 nm and the Soret excitation wavelengths from 430 to 460 nm are given in Table

Table II: Excitation/Emission Matrix Construct[a] of the Pchlide Ester Pool of Etiolated Cucumber Cotyledons

Soret observation wavelength, Eλ (nm)	Soret excitation amplitudes (arbitrary units) at fluorescence emission wavelengths, Fλ (nm)									
	615	617	621	625	631	635	637	641	643	645
407	5	14	55	50	13	10	10	19	9	9
415	9	24	76	82	19	9	10	20	8	9
423	9	24	76	82	23	12	14	20	12	9
430	14	40	128	128	24	16	17	23	13	10
437	26	102	303	350	73	31	28	35	24	17
440	20	74	222	245	61	25	24	30	17	17
443	24	80	257	268	57	26	24	31	16	15
450	0	0	23	27	19	12	11	21	11	9
453	2	1	12	12	6	12	12	25	17	12
460	0	0	3	6	1	4	5	7	6	5

[a] The $M(i,j)$ elements represent the excitation intensity measured at Eλ (nm) for an emission at Fλ (nm). In this matrix, a column represents an excitation spectrum and a row represents an emission spectrum. The matrix was assembled as described in the text. All fluorescence amplitudes were normalized to a 350X ordinate scale attenuation.

Table III: Δ/P[a] Values for Some of the 4 × 4 Minors of the Pchlide Ester Matrix Depicted in Table II

observation wavelengths, Eλ (nm)	emission wavelengths, Fλ (nm)					
	615-617-621-637		615-617-621-643		615-617-625-631	
	Δ/P	$\delta F/\bar{F}$	Δ/P	$\delta F/\bar{F}$	Δ/P	$\delta F/\bar{F}$
430-450-453-460	0.390	0.060	0.431	0.059	0.433	0.057
437-450-453-460	0.429	0.030	0.475	0.030	0.478	0.026
440-450-453-460	0.422	0.039	0.468	0.039	0.470	0.034
443-450-453-460	0.410	0.035	0.454	0.035	0.456	0.032

[a] For more details, consult the text.

III. Similar results were obtained for the Pchlide ester pool of etiolated bean, corn, and barley.

Altogether, these results confirmed our earlier interpretation of the fluorescence data and indicated that there appeared to be present at least four different fluorescent chromophores in the Pchlide ester pool of etiolated tissues.

Constancy of the Protochlorophyllide Ester Pool Composition under Different Growth Conditions. We had reported earlier that the composition of the Pchlide pool of etiolated tissues changed drastically under different growth conditions. For example, while MV-Pchlide was the major constituent of the Pchlide pool in etiolated tissues, it was DV-Pchlide that became the predominant Pchlide species after incubating the tissue with δ-aminolevulinic acid and during continuous and photoperiodic greening (Belanger & Rebeiz, 1980a). It was therefore desirable to investigate the composition of the Pchlide ester pool under the same growth conditions that caused profound changes in the composition of the Pchlide pool of etiolated tissues.

In the first experiment, the Pchlide ester profile of etiolated cucumber cotyledons was monitored after incubating the tissue overnight either with H_2O or with δ-aminolevulinic acid as described under Materials and Methods. As shown in Figure 7, lines a and b, the δ-aminolevulinic acid treatment had no effect on the qualitative composition of the Pchlide ester pool.

In the second experiment, part of the endogenous Pchlide ester pool and most of the Pchlide pool were photoreduced by illumination for 2 s with 3 mW cm^{-2} of incandescent light. The protochlorophylls were then allowed to re-form by transferring the tissue back to darkness for 45 min. In this case too, the Pchlide ester pool remained qualitatively unchanged and was not affected by this treatment (Figure 7, line c).

In the third experiment, the Pchlide ester profile of green photoperiodically grown 4-day-old cucumber cotyledons was examined. In this tissue too, the Pchlide ester pool remained qualitatively unchanged (Figure 7, line c). However, it appeared as if the proportion of MV- to DV-Pchlide ester slightly increased in this photoperiodic tissue (Figure 7B, line d).

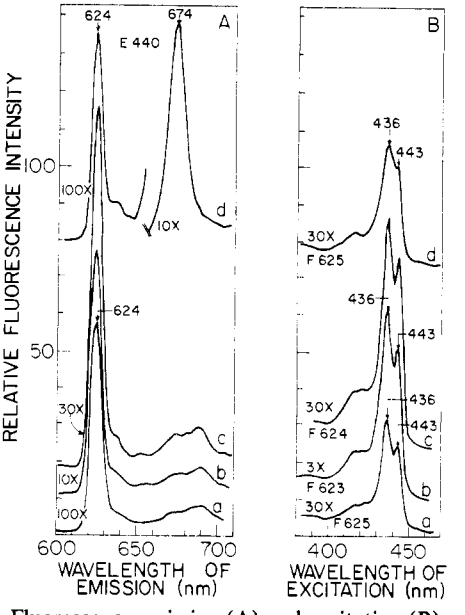


FIGURE 7: Fluorescence emission (A) and excitation (B) spectra in ether at 77 K of the extracted Pchlide ester pool of (a) etiolated cucumber cotyledons incubated overnight in H_2O or (b) after incubation overnight with δ-aminolevulinic acid, (c) etiolated cucumber cotyledons given 2 s of light and then returned to darkness for 45 min, and (d) green cucumber cotyledons grown photoperiodically for 4 days. All symbols are as in Figure 2.

Altogether, the above results indicated that contrary to the Pchlide pool (Belanger & Rebeiz, 1980a) the qualitative composition of the Pchlide ester pool tends to remain constant irrespective of the growth and greening conditions.

Discussion

It appears from this work that the Pchlide ester pool of etiolated tissues is essentially a faithful copy of the Pchlide

pool. Furthermore, the Pchlide ester pool appeared to be slightly more enriched in one of the fluorescent species, namely, compound E452F639 ester, than the Pchlide pool. The fluorescent components are DV-Pchlide ester, MV-Pchlide ester, and two esterified compounds of unknown structure, namely, compound E452F639 ester and compound E450F625 ester. Previously Belanger & Rebeiz (1980a) had reported that by analyzing the Pchlide pool of etiolated tissues by Weber's matrix technique, only three fluorescent components were detected in 7 × 7 matrix constructs prior to segregating the MV-Pchlide from the DV-Pchlide. At that time this was shown to be due to extreme Soret excitation overlap which interfered with the detection by matrix analysis of all the fluorescent compounds in the unsegregated Pchlide pool (Belanger & Rebeiz, 1980a). By expanding the emission/excitation matrix from a 7 × 7 order to a 10 × 10 order, spanning a broader wavelength interval, it was possible to circumvent this problem and to detect the fourth fluorescent component of the Pchlide ester pool prior to chromatographic separation. This in turn indicated that the four fluorescent components of the Pchlide ester pool were not chromatographic artifacts.

Of the four fluorescent compounds detected in the Pchlide ester pool of etiolated tissues, only MV Pchlide ester was previously thought to occur in etiolated tissues, while DV-Pchlide ester was reported to occur only in the inner seed coat of pumpkin (Inada & Shibata, 1960; Jones, 1966; Houssier & Sauer, 1969). To our knowledge, this is, therefore, the first report that describes the contribution of DV-Pchlide ester and compounds E452F639 ester and E450F625 ester to the chemical heterogeneity of the Pchlide ester pool of etiolated tissues.

The nature of the esterifying alcohols in the Pchlide ester pool is presently under investigation. Preliminary results from high-pressure liquid chromatography and from gas chromatographic–mass spectroscopic determinations (S. A. McCarthy and C. A. Rebeiz, unpublished experiments) suggest that (a) the fatty alcohols of the Pchlide ester pool of *Cucurbita* seed coat are different from those of the Pchlide ester pool of etiolated tissues and (b) in both *Cucurbita* seed coat and in etiolated tissues, the Pchlide ester pool is esterified with several different long-chain alcohols. This in turn may explain the differences in the chromatographic behavior of the Pchlide ester pool of *Cucurbita* seed coat and of etiolated cucumber cotyledons (Table I). The chemical heterogeneity of the alcohols of the Pchlide ester pool of *Cucurbita* seed coat was also reported by other investigators. For example, Sudyina (1963) segregated the Pchlide ester pool of the inner seed coat of mature pumpkin seeds into four different chromatographic bands having identical spectrophotometric profiles. On the other hand, Ellsworth & Nowak (1974) reported that the MV- and DV-Pchlide esters of pumpkin seed coat, which were isolated as the protopheophytin esters, were each esterified with several different isoprenoid alcohols, including phytol. However, the mixture of esterifying alcohols obtained from DV protopheophytin ester contained only 50% phytol and was considerably more heterogeneous than that obtained from MV-protopheophytin ester which was made up of ~90% phytol.

An important question that remains to be elucidated pertains to the function of the different components of the Pchlide ester pool that were described in this report. It is now well documented that the chlorophyll biosynthetic chain is split into two parallel biosynthetic branches, namely, the unesterified (or Pchlide) branch and the esterified (or Pchlide ester) branch (Rebeiz et al., 1978). Therefore, we now propose that MV-Pchlide ester and DV-Pchlide ester as well as compound E452F639 and E450F625 esters are metabolic intermediates of the esterified biosynthetic branch in the same capacity as their unesterified analogues are intermediates of the unesterified biosynthetic branch. It is further proposed that these esterified intermediates do not necessarily bear a precursor–product relationship to each other but may belong to more than one subchain of the esterified biosynthetic branch. This seems to be borne out by recent observations which indicate that in etiolated tissues both the DV- and MV-Pchlide esters are directly photoconvertible by a very brief light pulse into DV- and MV-chlorophyll *a* chromophores (Belanger & Rebeiz, 1980b). The latter exhibited Soret excitation maxima at 459 and 449 nm, respectively, which were red shifted in comparison to the chlorophyll *a* species that originate from MV- and DV-chlorophyllide (Belanger & Rebeiz, 1979; Rebeiz et al., 1980). This in turn suggests that DV- and MV-Pchlide esters may play distinct roles as the specific precursors of DV- and MV-chlorophyll *a* in higher plants.

As mentioned elsewhere, the exact structure of compounds E453F640 and E450F626 (Belanger & Rebeiz, 1980a) and of their esterified analogues which were described here has not yet been determined. Preliminary evidence suggests, however, that compound E452F639 ester is not a Pchlide ester *b* with a formyl group instead of a methyl group at position 3 of the macrocycle (Figure 1). As for compound E450F625 ester, its structure is presently under investigation, along with the other components of the Pchlide ester pool. To this effect, NMR, infra red, and mass spectroscopic analysis is in progress.

Finally, the observation that the Pchlide ester pool of etiolated tissues maintained a constant qualitative composition during greening (Figure 7), in contrast to the behavior of the Pchlide pool (Belanger & Rebeiz, 1980a), had interesting implications. Perhaps the most obvious one suggests that the esterified and nonesterified branches of the chlorophyll biosynthetic pathway are regulated differently during greening. Furthermore, this observation tends to confirm the aforementioned suggestion that the DV- and MV-Pchlide ester components of the Pchlide ester pool contribute in a different capacity to the greening process than the MV- and DV-Pchlide pools of etiolated tissues.

Acknowledgments

The authors thank Daniel G. Saab for writing the computer program used in the matrix analysis of the data and Richard Eichholz for growing the etiolated *Euglena*. The toluene–ethyl acetate–ethanol (8:2:2 v/v) solvent system was developed in our laboratory by S. McCarthy.

References

Belanger, F. C., & Rebeiz, C. A. (1979) *Biochem. Biophys. Res. Commun. 88*, 365.

Belanger, F. C., & Rebeiz, C. A. (1980a) *J. Biol. Chem. 255*, 1266.

Belanger, F. C., & Rebeiz, C. A. (1980b) *Plant Sci. Lett. 18*, 343.

Cohen, C. E., & Schiff, J. A. (1976) *Photochem. Photobiol. 24*, 555.

Cohen, C. E., & Rebeiz, C. A. (1978) *Plant Physiol. 61*, 824.

Ellsworth, R. K., & Nowak, C. A. (1974) *Anal. Biochem. 57*, 534.

Freyssinet, G., & Schiff, J. A. (1974) *Plant Physiol. 53*, 543.

Houssier, C., & Sauer, K. (1969) *Biochim. Biophys. Acta 172*, 476.

Inada, Y., & Shibata, K. (1960) *Plant Cell Physiol. 1*, 311.

Jeffrey, S. W. (1969) *Biochim. Biophys. Acta 177*, 456.
Jeffrey, S. W., Sielicki, M., & Haxo, F. T. (1975) *J. Phycol. 11*, 374.
Jones, O. T. G. (1966) *Biochem. J. 101*, 153.
Löeffler, J. E. (1955) *Year Book–Carnegie Inst. Washington 54*, 159.
Mielenz, J. R. (1976) Thesis, University of Illinois at Urbana–Champaign.
Minc, H. (1978) *Encyclopedia of Mathematics and its Applications* (Rota, G. C., Ed.), Vol. 6, pp 1–14. Addison-Wesley, Reading, MA.
Rebeiz, C. A. (1968) *Magon, Ser. Sci. 21*, 1.
Rebeiz, C. A., & Castelfranco, P. A. (1973) *Annu. Rev. Plant Physiol. 24*, 129.
Rebeiz, C. A., Yaghi, M., Abou-Haidar, M., & Castelfranco, P. A. (1970) *Plant Physiol. 46*, 57.
Rebeiz, C. A., Mattheis, J. R., Smith, B. B., Rebeiz, C. C., & Dayton, D. F. (1975a) *Arch. Biochem. Biophys. 166*, 446.
Rebeiz, C. A., Mattheis, J. R., Smith, B. B., Rebeiz, C. C., & Dayton, D. F. (1975b) *Arch. Biochem. Biophys. 171*, 549.
Rebeiz, C. A., Smith, B. B., Mattheis, J. R., Cohen, C. E., & McCarthy, S. A. (1978) in *Chloroplast Development* (Akoyunoglou, G., & Argyroudi-Akoyunoglou, J. H., Eds.) pp 59–76, Elsevier/North-Holland Bio-Medical Press, Amsterdam.
Rebeiz, C. A., Belanger, F. C., Cohen, C. E., & McCarthy, S. A. (1979) *Ill. Res. 21*, 3.
Rebeiz, C. A., Belanger, F. C., Freyssinet, G., & Saab, D. G. (1980) *Biochim. Biophys. Acta 590*, 234.
Sudyina, E. G. (1963) *Photochem. Photobiol. 2*, 181.
Virgin, H. (1960) *Physiol. Plant. 13*, 155.
Weber, G. (1961) *Nature (London) 190*, 27.
Wolff, J. B., & Price, L. (1957) *Arch. Biochem. Biophys. 72*, 293.

Molecular Properties, Partial Purification, and Assay by Incubation Period Measurements of the Hamster Scrapie Agent†

Stanley B. Prusiner,* Darlene F. Groth, S. Patricia Cochran, Frank R. Masiarz, Michael P. McKinley, and Hugo M. Martinez

ABSTRACT: The scrapie agent causes a progressive degeneration of the central nervous system of animals after a prolonged incubation period. Measurements of incubation period length, defined as the time from inoculation to the onset of clinical signs of neurological dysfunction, were related to the titer of the agent and the dilution of the inoculated sample. Equations defining the relationship provide a new assay for the agent requiring fewer animals than end point titrations. By use of this incubation period assay, the scrapie agent from hamster brain was found to have an $s_{20,w}$ of <300 S but >30 S assuming $\rho_p = 1.2$ g/cm^3. A partially purified fraction P_3 was obtained by differential centrifugation and sodium deoxycholate extraction. When P_3 was extracted with phenol, virtually no infectivity was found in the aqueous phase even after examining such variables as pH, salt concentration, and predigestion of samples with proteinase K. Nonionic and nondenaturing, anionic detergents did not inactivate the scrapie agent; in contrast, denaturing detergents inactivated the agent. Sodium dodecyl sulfate ($NaDodSO_4$) inactivated greater than 90% of the agent at a $NaDodSO_4$ to protein ratio of 1.8 g/g. Inactivation by $NaDodSO_4$ appears to be a cooperative process. Addition of a nonionic detergent to form mixed micelles with $NaDodSO_4$ prevented inactivation of the agent by $NaDodSO_4$. Weak chaotropic ions do not inactivate the scrapie agent while strong chaotropic ions like SCN^- and Cl_3CCOO^- destroy infectivity at concentrations of 0.2 M. These data provide evidence in support of a protein component within the scrapie agent which is essential for maintenance of infectivity. Thus, it is unlikely that the scrapie agent is composed only of a "naked" nucleic acid as is the case for the plant viroids.

The scrapie agent causes a progressive deterioration of the central nervous system (CNS) of infected animals after a prolonged incubation period during which the animals exhibit no signs of neurological dysfunction (Sigurdsson, 1954; Eklund et al., 1967). The CNS degeneration is characterized pathologically by neuronal vacuolation and astroglial proliferation. Most striking is the lack of any sign of inflammation in an infectious process which has devastated the CNS. Two similar disorders in humans, kuru and Creutzfeldt-Jakob disease (CJD), have been identified (Gajdusek, 1977). The causative agents of scrapie, kuru, and CJD all appear to have many unusual biological properties. The unusual properties of the scrapie agent seem, in large part, to be a consequence of its small size and apparent hydrophobicity (Alper et al., 1966; Prusiner et al., 1978c, 1979).

To date, the scrapie agent has eluded isolation and identification. This appears to be due mainly to the lack of rapid assay for the agent and the hydrophobic nature of the agent itself.

In this communication, we describe an assay for the scrapie agent in hamsters based on the length of the incubation period and the development of equations relating titer, dilution, and incubation period. This assay substantially reduces the number of animals needed for quantitating the agent in a given sample when compared to the end point titration method. Using this new assay procedure, we have been able to develop a partial purification protocol and to define some biochemical and

† From the Howard Hughes Medical Institute Laboratory, Departments of Neurology and Biochemistry and Biophysics, University of California, San Francisco, California 94143. *Received February 28, 1980.* This work was supported in part by research grants from the National Institutes of Health (NS 14069) and the National Science Foundation (PCM 77 24096). M.P.M. was supported by U.S. Public Health Service Training Grant AG-00047.

* Correspondence should be addressed to this author. He is an Investigator of the Howard Hughes Medical Institute.

0006-2960/80/0419-4883$01.00/0