Chloroplast Biogenesis: Detection of a Magnesium Protoporphyrin Diester Pool in Plants†

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ABSTRACT: The discovery of a novel metalloporphyrin pool in etiolated cucumber cotyledons and in dark-grown *Euglena gracilis* is described. The novel pool exhibited the chromatographic properties of a fully esterified metalloporphyrin, devoid of free carboxylic groups, and the spectrophotometric and spectrofluorometric properties of a magnesium protoporphyrin. Demetalation and hydrolysis indicated that the tetrapyrrole moiety of the metalloporphyrin was a protoporphyrin diester. High-pressure liquid chromatography of the fully esterified metalloporphyrin pool and gas chromatographic/mass spectroscopic analysis of the saponified alcohol fraction revealed that the latter was made up of three major long-chain alcohols. None of those alcohols was identifiable, however, with known isoprenoids such as geraniol, farnesol, or phytol. Similar analysis of the saponified alcohol fraction of the protochlorophyllide ester pool likewise revealed the presence of three major long-chain alcohols none of which was identifiable with known isoprenoid alcohols or with the alcohols of the novel metalloporphyrin pool. On the basis of the above observations, the novel metalloporphyrin pool was tentatively identified as a magnesium protoporphyrin diester pool. It is suggested that this pool is a metabolic intermeidate of the fully esterified branch of the chlorophyll biosynthetic pathway [Rebeiz, C. A., Smith, B. B., Matthesis, I. 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] and is probably the precursor of the protochlorophyllide ester pool in plants.

The chlorophyll of green plants originates in the protochlorophyll(ide) pool. The latter consists of a monocarboxylic protochlorophyll, i.e., of a protochlorophyllide a[1] pool, and of a fully esterified protochlorophyll, i.e., of a protochlorophyllide ester a[1] pool, (Rebeiz et al., 1978). Upon illumination with actinic light of an etiolated tissue lacking chlorophyll but containing protochlorophyll, the protochlorophyllide *a* pool is photoconverted into a chlorophyllide *a* pool by the addition of two trans hydrogens at the 7, 8 positions of the macrocycle. The chlorophyllide *a* is subsequently converted into chlorophyll *a* by esterification (Rebeiz et al., 1978; Schoch et al., 1977). On the other hand, a fraction of the fully esterified protochlorophyllide ester *a* pool is directly photoconverted into chlorophyll *a* by illumination (Rebeiz et al., 1978; Belanger & Rebeiz, 1980c).

It has been suggested that, contrary to previous beliefs, the protochlorophyllide ester *a* pool is not synthesized from the protochlorophyllide *a* pool by esterification of the latter (Rebeiz et al., 1970). Instead, it has been proposed that protochlorophyllide *a* and protochlorophyllide ester *a* are synthesized in parallel from a common precursor via two parallel biosynthetic branches: (a) via a fully esterified biosynthetic branch that results in the formation of protochlorophyllide ester *a* and (b) via a partially esterified (monocarboxylic) biosynthetic branch that results in the formation of protochlorophyllide *a* (Rebeiz et al., 1978).

It is therefore reasonable to surmise that if two separate biosynthetic pathways are indeed involved in the biosynthesis of protochlorophyllide *a* and of protochlorophyllide ester *a*, respectively, then some fully esterified intermediates of the neutral biosynthetic branch may accumulate under certain conditions. Such a phenomenon has already been observed, with the accumulation of some of the intermediates of the monocarboxylic biosynthetic branch during the greening of etiolated tissues (Rebeiz et al., 1975a). This paper reports the discovery of a fully esterified magnesium protoporphyrin pool in plants which may well be a metabolic intermediate of the neutral chlorophyll biosynthetic branch in higher and lower plants.

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. The seeds were germinated directly in moist vermiculite at approximately 30 °C for 4 days in the dark (Rebeiz et al., 1975b).

Dark-grown *Euglena gracilis* cells were a gift of Dr. Dennis Buetow and Richard Eicholz, Cell Biology Program, University of Illinois at Urbana. The cells were grown on a modified Hutner, Greenblatt, and Schiff medium (Mielenz, 1976).

Chemicals. Geraniol and *N*-nitrosomethylurea were purchased from K and K Rare and Fine Chemicals, Plainview, N.Y., while farnesol and phytol were purchased from Sigma, St. Louis, MO.

Tetrapyrrole Standards. Protoporphyrin IX dimethyl ester was purchased from Sigma, St. Louis, MO, and protoporphyrin IX was purchased from Porphyrin Products, Logan, UT. Protochlorophyllide ester was prepared as described elsewhere (Rebeiz et al., 1975a). Magnesium protoporphyrin monoester (MPE)[2] was prepared biosynthetically as described

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[1] The protochlorophyllide pool of etiolated tissues is a mixed pool and consistes of monovinyl- and, divinylprotochlorophyllide (Belanger & Rebeiz, 1980a). Likewise, the protochlorophyllide ester pool consists of monovinyl- and divinylprotochlorophyllide components (Belanger & Rebeiz, 1980b).

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earlier (Rebeiz et al., 1975b). Zn protoporphyrin was synthesized from standard protoporphyrin IX (Rebeiz et al., 1975b). Protopheophytin ester and protoporphyrin monoester were prepared from standard protochlorophyllide ester and MPE, respectively, by demetalation as described under Materials and Methods (vide infra).

Incubation of Cucumber Cotyledons with α,α′-Dipyridyl. The small-scale incubation of 4-day-old etiolated cucumber cotyledons with α,α′-dipyridyl was described elsewhere (Mattheis & Rebeiz, 1977). Large-scale incubations were performed with 60 g of etiolated cucumber cotyledons, in a Pyrex tray (30.5 × 22 × 4 cm) containing 72 mL of the incubation medium. The latter contained 270 μmol of α,α′-dipyridyl dissolved in 0.65 mL of methanol and 325 μmol of δ-aminolevulinic acid and was adjusted to volume with distilled water. The incubation tray was covered with an inverted Pyrex tray of identical dimensions, was wrapped in aluminum foil, and was incubated in the dark at 28 °C for 16–20 h.

Extraction of Pigments from Cucumber Cotyledons. For small-scale incubations, 3 g of cotyledons was homogenized for 2 min in 20 mL of acetone and 0.1 N NH_4OH (9:1 v/v) at 0–4 °C in a Sorvall Omni-mixer, and the homogenate was centrifuged at 39000*g* for 10 min. Fully esterified pigments were extracted from the acetone by partitioning with an equal volume of hexane. Extraction and processing of pigments from large-scale incubations were performed as described above, except that homogenization was accomplished by using a Waring blendor operated at approximately 60% of line voltage (115 V) for 5 min.

Extraction of Pigments from Dark-Grown Euglena. The extraction of *Euglena* cells was modified from the procedure of Cohen & Schiff (1976). Dark-grown *Euglena gracilis* cells were harvested in late-logarithmic or stationary phase. The cells were collected by centrifugation at 1000*g* for 5 min, and the pellet was washed with 0.01 M potassium phosphate buffer, pH 7.0, and 27% sucrose (Freyssinet & Schiff, 1974). The washed cells were extracted with acetone:0.1 N NH_4OH (9:1 v/v) until the pellets were nearly white. The esterified pigments were transferred to hexane by extraction, first with an equal volume of hexane and then with $^1/_3$ volume of hexane.

Purification of the Extracted Pigments. Pigments obtained from small-scale incubations were directly purified by chromatography on thin layers of silica gel H, 500 μm thick. The plates were developed in xylene:ethyl acetate:ethanol (8:2:1 v/v/v) or in toluene:ethyl acetate:ethanol (8:2:1 v/v/v). The pigments obtained from large-scale incubations were first prepurified on a silica column (50–200 μm, 1.5 × 14 cm). The column was first washed with 200 mL of hexane and then with 100 mL of diethyl ether:hexane (1:9 v/v) to remove some of the carotenoids. The fully esterified pigments wee then eluted with 100 mL of diethyl ether:hexane (1:1 v/v). Pchlide ester was separated from MPE by concentrating the column effluent under N_2 to a small volume, and by applying the pigments to thin layers of silica gel H. These plates were then developed in xylene:ethyl acetate:ethanol (8:2:1 v/v/v). The pigments were eluted with ether and rechromatographed, if necessary, until only one fluorescent band was visible. The products of acid hydrolysis were chromatographed on thin layers of silica gel H, developed in 2,6-lutidine:H_2O (10:3 v/v) in an ammonia-saturated atmosphere (Jensen, 1963).

Demetalation of the Metalloporphyrins. Pigments were converted to metal-free tetrapyrroles by mixing ether solutions containing the metalloporphyrins with an equal volume of 6.9 N HCl at 0–4 °C. This acid treatment results in the formation of the acid-soluble free base. The pigments in the acid solution were transferred back to ether by neutralization with solid $NaHCO_3$ as previously described (Rebeiz & Castelfranco, 1971).

Acid Hydrolysis of Tetrapyrroles. Acid hydrolysis of the alcohols esterified to tetrapyrroles was accomplished by dissolving the pigments in a small volume of solvent:ether for Mg-protodiester and in pyridine for protoporphyrin dimethyl ester. The dissolved pigments were then added to 10 mL of 7 N HCl. The reaction was allowed to proceed at room temperature in darkness for 4 h (Smith & Rebeiz, 1977). The pigments were transferred to ether by neutralizing the acid with solid $NaHCO_3$. The ether solution was washed with disilled water until it was free of acid. The porphyrins were then chromatographed, and their spectral characteristics were determined.

Alkaline Hydrolysis of Tetrapyrroles. Alkaline hydrolysis of the esterified tetrapyrroles and separation of the hydrolyzed alcohols were accomplished by a modification of the procedure of Liljenberg & Odham (1969). Purified pigments were dissolved in 0.2 mL of ether and placed in a glass-stoppered conical tube. To this was added 0.8 mL of absolute ethanol and 1 mL of distilled H_2O. Solid KOH was next added until the pigments were dissolved (about 5–10%, w/v). The tube was stoppered and heated for 30 min at 65–70 °C. After hydrolysis, the contents were cooled, and the unsaponifiable components were extracted with 1 mL of hexane. The hexane was washed 5 times with 1 mL of distilled water and was dried over a few pellets of molecular sieve type 13X. The hexane was next evaporated under N_2, and the final residue was redissolved in 20 μL of hexane. This solution was then analyzed by gas chromatography.

Preparation of Diazomethane from N-Nitrosomethylurea. Diazomethane was prepared by adding 1 g of *N*-nitrosomethylurea to an Erlenmeyer flask containing 10 mL of ether and 3 mL of 40% KOH at 0 °C. The flask was swirled on ice until the white solid was completely dissolved and the ether became bright yellow. The ethereal solution was removed and stored over KOH at −20 °C. For methylation purposes, the diazomethane was not separated from the solvent, but was used as an ethereal solution (Feiser & Feiser, 1967).

Methylation of MPE. The hexane-extracted acetone residue from α,α′-dipyridyl-treated cotyledons was enriched in monocarboxylic MPE. The pigments were transferred to ether by the addition of $^1/_{17}$ volume of saturated NaCl and $^1/_{70}$ volume of 0.5 M KH_2PO_4. The ether extract was then washed with H2O and dried under N_2 until a separate water/acetone phase was formed. The latter was reextracted with ether until the aqueous phase became nonfluorescent when viewed under a 366-nm UV light. The combined ether extracts were again concentrated under N_2 and applied to silica gel H plates which were developed in toluene:ethyl acetate:ethanol (8:2:2 v/v/v) in the dark at 4 °C. The MPE band ($R_f \simeq 0.62$) was eluted with methanol:acetone (4:1 v/v) and then was dried under N_2. The final residue was redissolved in methanol, to which was added an excess of the ethereal solution of diazomethane. The mixture was briefly swirled on ice and then completely dried under N_2.

Reverse-Phase High-Pressure Liquid Chromatography. Pigment isolated from α,α′-dipyridyl-treated cucumber cotyledons, which had been prepurified on a silica column, was applied without additional purification to a Spectra Physics

[2] Abbreviations used: MPE, magnesium protoporphyrin monoester; Mg-proto diester, magnesium protoporphyrin diester; GLC, gas–liquid chromatography; HPLC, high-pressure liquid chromatography; MS, mass spectroscopy; Pchlide, protochlorophyllide.

Table I: Relative Chromatographic Mobilities of Various Tetrapyrroles on Thin Layers of Silica Gel H[a]

experiment	pigment	approximate R_f values: xylene:ethyl acetate:ethanol (8:2:1 v/v/v)	toluene:ethyl acetate:ethanol (8:2:2 v/v/v)	2,6-lutidine:H_2O:NH_3 vapors (10:3 v/v)
A	protochlorophyllide ester	0.82	0.85	
	unknown metalloporphyrin	0.75	0.76	
	Mg protoporphyrin monoester	0.28	0.62	
	protochlorophyllide	0.13	0.45	
B	demetalated protochlorophyllide ester		0.86	
	demetalated unknown metalloporphyrin		0.79	
	demetalated Mg protoporphyrin monoester		0.56	
C	protoporphyrin dimethyl ester			1.0
	protoporphyrin dimethyl ester after total hydrolysis			0.33
	unknown metalloporphyrin after total hydrolysis			0.39
	standard protoporphyrin IX			0.33[b]

[a] Pigments were applied to 5 × 20 cm plates, 500 μm thick. The plates were developed for 18–19 cm in at least one of three different solvent systems. [b] Average value of several determinations

Model SP 8000 liquid chromatograph. Separations were performed on a 25-cm Spherisorb ODS (5-μm) column which was eluted isocratically with water:methanol:acetone (5:85:10 v/v/v). A Schoeffel FS 970 spectrofluorometric monitor, set at an excitation wavelength of 420 nm, was used for the detection of the tetrapyrroles.

Pigments in the collected fractions were transferred to ether by mixing the fractions with an equal volume of ether and then passing the solution through a large volume of water saturated with $MgCO_3$ (Freyssinet et al, 1980). The resulting ether phase was washed with saturated NaCl and evaporated to dryness under N_2. The residue was redissolved in ether.

Gas–Liquid Chromatography. Gas chromatographic separation of the isolated alcohols was performed on a Tracor Model 550 gas chromatograph. An all-glass column (6 ft × 0.25 in.) with a solid support of Chromosorb W (80/100 mesh) and a stationary phase of 2% OV-17 was used. The detector was heated to 320 °C, and the injection port was held at 220 °C. The column temperature was programmed to increase linearly from 70 to 250 °C at a rate of 5 °C/min. After a 2-μL sample injection, the column temperature was held at 70 °C for approximately 2 min before starting the temperature program. The carrier gas flow rate (N_2) was adjusted to 38–40 mL/min.

Gas Chromatography/Mass Spectrometry. Mass spectrometry was performed in the Mass Spectrometry Laboratory of the School of Chemical Sciences, University of Illinois, on a Varian-MAT 311A mass spectrometer interfaced with a Watson-Beimann two-stage separator and connected to a Varian 2740 gas chromatograph. A capillary column with a stationary phase of OV-17 was used. The conditions for the GLC separation were as described above for the Tracor Model 550 chromatograph, except that the detector was held at 300 °C. The mass spectrometer was operated at an accelerating potential of 3 kV. The source pressure was 3×10^{-6} torr, and the temperature was held at 250 °C. The interface and probe temperatures were both held at 290 °C. The electron-impact mass spectra were recorded with an electron energy of 90 eV and a resolution of 600.

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

Spectrofluorometric Measurements. Corrected fluorescence emission and excitation spectra of the extracted pigments were recorded at room temperature on a Perkin-Elmer spectrofluorometer, Model MPF-3, equipped with a corrected spectra accessory (Rebeiz et al., 1975b). Some spectra were also recorded on a fully corrected, photon counting SLM spectrofluorometer, Model 8000 DS, equipped with two red-sensitive, extended S20 photomultipliers (type EMI 9658). The spectrofluorometer was interfaced with a Hewlett-Packard microcomputer system, Model 9825 S, and a Hewlett-Packard plotter, Model 7225 A (Belanger & Rebeiz, 1980c).

Results

Accumulation of an Apolar Metalloporphyrin in Etiolated Cucumber Cotyledons Incubated with δ-Aminolevulinic Acid and α,α′-Dipyridyl. α,α′-Dipyridyl is a potent activator of the biosynthesis of Mg porphyrins in plants, as well as an inhibitor of their conversion into protochlorophyll (Granick, 1961; Duggan & Gassman, 1974; Vlcek & Gassman, 1979). It was therefore conjectured that incubation of etiolated cucumber cotyledons with α,α′-dipyridyl may result in the accumulation of some of the fully esterified Mg porphyrin intermediates which supposedly populate the putative neutral branch of the chlorophyll biosynthetic pathway. The metalloporphyrins which accumulated during a dark incubation of etiolated cucumber cotyledons with α,α′-dipyridyl and δ-aminolevulinic acid were therefore extracted into acetone and 0.1 N NH_4OH. The lipophilic fully esterified pigments were transferred to hexane as described under Materials and Methods. The acidic tetrapyrroles remained in the hexane-extracted acetone fraction.

Chromatography of the hexane extract, which contained the fully esterified tetrapyrroles, was carried out on thin layers of silica gel developed in xylene:ethyl acetate:ethanol (8:2:1 v/v/v). Two bands exhibiting red fluorescence under 366-nm UV light migrated with R_f's consistent with those for fully esterified tetrapyrroles. The fast-moving band had an R_f of 0.82, similar to that of Pchlide ester, while the second band moved just below the Pchlide ester band and migrated with an R_f of about 0.75. Under the same chromatographic conditions, standard MPE, a monocarboxycyclic tetrapyrrole, exhibited an R_f of 0.28 (Table IA). These results suggested that a fluorescent pigment nearly as apolar as Pchlide ester may have been formed during the dark incubation.

Tentative Identification of the Apolar Metalloporphyrin as a Mg Protoporphyrin. In order to gain additional information about the putative esterified tetrapyrroles, the latter were segregated on thin layers of silica gel H, developed in xylene:ethyl acetate:ethanol (8:2:1 v/v/v); the segregated pigments (Table IA) were eluted in ether, and their spectroscopic properties were determined at room temperature.

The fast-moving band (R_f 0.82) exhibited absorption and fluorescence emission and excitation properties at room tem-

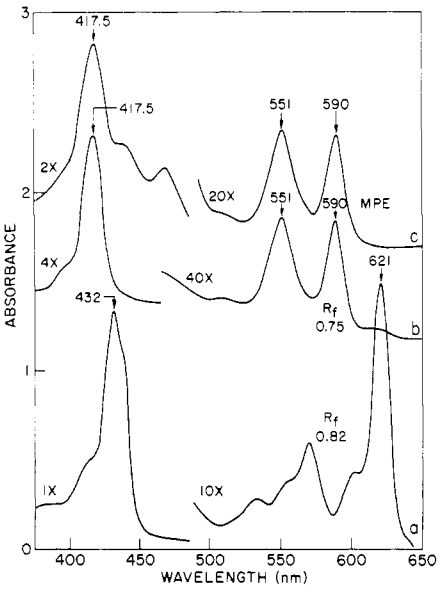


FIGURE 1: Absorption spectra of the segregated protochlorophyllide ester and of the unknown metalloporphyrin pool. The pigments were separated on thin layers of silica gel H as described in Table IA, and the segregated pools were eluted in ether. The absorption spectra were recorded at room temperature and at the sensitivities indicated, 40X being the highest sensitivity. In order to avoid overlapping of the spectra, the base lines were arbitrarily adjusted on the ordinate scale as evident from the absorbance values at 650 nm. The R_f values refer to those values reported in Table IA. Arrows point to wavelengths of interest. MPE = standard Mg protoporphyrin monoester.

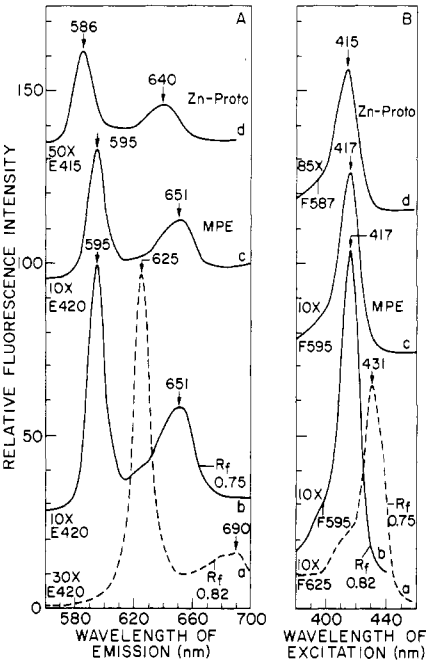


FIGURE 2: Fluorescence emission (A) and excitation (B) spectra of the segregated protochlorophyllide ester and of the unknown metalloporphyrin pools. The pigments were separated as described in Figure 1, and their spectra were recorded in ether at room temperature. Ordinate scale attenuation is indicated on the spectra, 350X being the highest sensitivity used. The emission spectra were elicited by the E wavelengths indicated. Likewsie, the excitation spectra were recorded at the F wavelengths indicated. The arrangement of the spectra on the ordinate scale is arbitrary. Arrows point to wavelengths of interest. Zn-Proto = standard Zn protoporphyrin. Other symbols are as in Figure 1.

perature, in ether, that were identical with those of standard protochlorophyllide ester extracted from etiolated cucumber cotyledons (Belanger & Rebeiz, 1980b). Its Soret and red absorption maxima which were found at 432 and 621 nm, respectively (Figure 1, curve a), were similar to values reported by others (Falk, 1964). It also exhibited protochlorophyllide ester emission maxima at 625 and 690 nm and a Soret excitation maximum at 431 nm (Figure 2, curve a).

The slower moving band (R_f 0.75) exhibited an absorption spectrum in ether at room temperature which was identical with that of standard Mg protoporphyrin monoester (MPE) except for the contaminating xanthophyll absorption bands observed in the later at about 437 and 468 nm (Rebeiz, 1968). Its Soret absorption maximum was recorded at 417.5 nm with α and β absorption bands at 551 and 590 nm, respectively (Figure 1, curve b). Its fluorescence emission spectrum was identical with that of standard MPE with emission maxima at 595 and 651 nm (Figure 2, curves a–c). It exhibited a Soret excitation maximum at 417 nm which was identical with that of standard MPE (Figure 2B, curves b,c). These spectral properties were distinctly different from those of standard Zn protoporphyrin. Under the same conditions, the latter exhibited blue-shifted absorption maxima at 414, 543.5, and 580 nm. Its red emission maxima at 586 and 640 nm and Soret excitation maximum at 415 nm were also blue-shifted by about 9, 11, and 2 nm, respectively, in comparison to the novel apolar Mg protoporphyrin like compound described in this work (Figure 2, curve d).

These results indicated that during dark incubation with α,α'-dipyridyl and δ-aminolevulinic acid, etiolated cucumber cotyledons synthesized and accumulated a tetrapyrrole with a chromatographic mobility close to that of a fully esterified protochlorophyll and with the spectroscopic properties of Mg protoporphyrin.

Conversion of the Apolar Metalloporphyrin into an Esterified Protoporphyrin by Demetalation. Further characterization of the apolar metalloporphyrin was undertaken after converting it into a free base by demetalation. The pigment was demetalated as described under Materials and Methods. The demetalated porphyrin was then chromatographed on thin layers of silica gel, developed in toluene:ethyl acetate:ethanol (8:2:2 v/v/v), along with standard protopheophytin ester and protoporphyrin monoester. The unknown tetrapyrrole migrated with an R_f of 0.79 while protopheophytin ester had an R_f of 0.86 (Table IB). Standard protoporphyrin monoester, with one free carboxyl group, migrated with an R_f of 0.56 (Table IB). These results indicated that the apolar porphyrin still retained the chromatographic mobility of a fully esterified tetrapyrrole, after demetalation.

Fluorescence emission and excitation spectra of the demetalated apolar porphyrin were also recorded. The fluorescence emission spectrum was identical with that of standard protoporphyrin dimethyl ester with maxima at 635 and 706 nm (Figure 3A). The fluorescence excitation spectrum, recorded at 635 n,, gave a Soret excitation maximum at 403 nm with additional maxima at 502, 536, and 580 nm. These maxima were also identical with those of authentic protoporphyrin dimethyl ester (Figure 3B). Taken together, the chromatographic and spectral results suggested that the apolar metalloporphyrin was most probably a fully esterified Mg protoporphyrin.

Conversion of the Apolar Metalloporphyrin into Protoporphyrin by Acid Hydrolysis. For further establishment of the chemical nature of the porphyrin moiety of the apolar Mg porphyrin pool, the latter was subjected to total acid hydrolysis. This treatment removes the metal and cleaves the ester linkages. Hydrolysis of the ester groups can then be monitored by conventional chromatographic techniques since the loss of such groups alters the chromatographic mobility of the resulting tetrapyrrole.

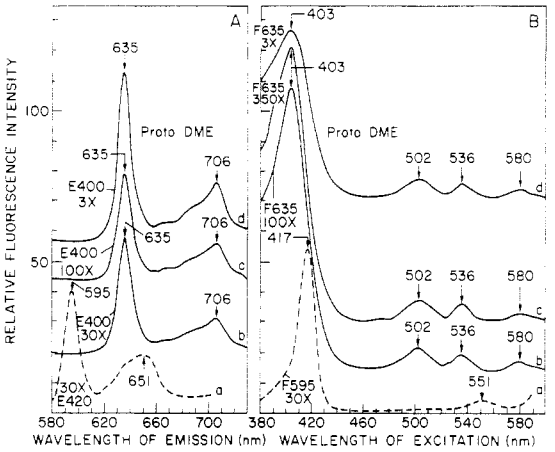


FIGURE 3: Fluorescence emission (A) and excitation (B) spectra of the unknown metalloporphyrin before and after demetalation and total acid hydrolysis. The spectra were recorded in ether at room temperature. (a) Unknown metalloporphyrin; (b) unknown metalloporphyrin after demetalation; (c) unknown metalloporphyrin after total acid hydrolysis; (d) Proto DME = standard protoporphyrin dimethyl ester. All symbols are as in Figure 2.

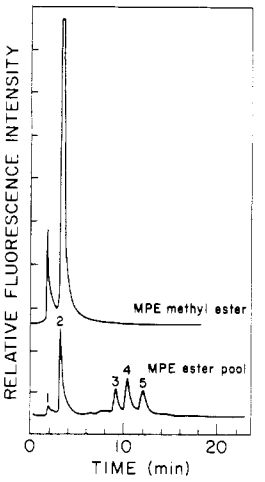


FIGURE 4: HPLC profile of the partially purified Mg-proto diester pool. The Mg-proto diester pool was partically prepurified on a silica column before HPLC analysis. Detection was by fluorescence emission elicited by excitation at 420 nm. Segregation was on a 25-cm Spherisorb ODS (5-μm) column, eluted isocratically with water: methanol:acetone (5:85:10 v/v/v) at a flow rate of 1 mL/min. MPE ester = Mg-proto diester; MPE methyl ester = standard Mg-proto dimethyl ester.

The purified apolar Mg porphyrin was dissolved in ether and then hydrolyzed in 7 N HCl and chromatographed as described under Materials and Methods. In order to check the effectiveness of the hydrolysis, standard protoporphyrin dimethyl ester was also treated with HCl exactly as described for the apolar Mg porphyrin.

In 2,6-lutidine:H_2O (10:3 v/v), the R_f values of the various tetrapyrroles that were monitored appeared to be inversely related to the number of free carboxyl groups of the tetrapyrrole (Table IC). For example, before hydrolysis protoporphyrin dimethyl ester migrated with the solvent front, while standard protoporphyrin IX, a dicarboylic tetrapyrrole, migrated with an average R_f of 0.33. Following total acid hydrolysis of the unknown metalloporphyrin and of standard protoporphyrin dimethyl ester, the hydrolyzed tetrapyrroles exhibited the chromatographic mobilities of dicarboxylic porphyrins with R_f values of about 0.39 and 0.33, respectively (Table IC). The fluorescence emission and excitation spectra in ether and at room temperature of the product of total hydrolysis of the unknown metalloporphyrin were identical with those of standard protoporphyrin IX dimethyl ester (Figure 3). The latter exhibited identical spectral properties before and after total hydrolysis and conversion into protoporphyrin IX.

Altogether, the above results lent further support to the notion that the porphyrin moiety of the unknown apolar metalloporphyrin was a fully esterified protoporphyrin IX.

Heterogeneity of the Apolar Metalloporphyrin Pool As Evidenced by Reversed-Phase HPLC. So far, the spectral data suggested that the unknown apolar metalloporphyrin was a Mg complex (Figures 1 and 2). On the other hand, demetalation of the metalloporphyrin identified the porphyrin nucleus as a protoporphyrin type tetrapyrrole, while total acid hydrolysis suggested a fully esterified protoporphyrin (Table I, Figure 3). Thus, the unknown metalloporphyrin appears to be a fully esterified Mg protoporphyrin and will therefore be tentatively referred to as Mg protoporphyrin diester (Mg-proto diester).

In order to further probe the nature of the esterifying alcohol(s), the putative Mg-proto diester pool was further investigated by reversed-phase HPLC. It was conjectured that if only one type of alcohol esterified the propionate residue at the seventh position of the macrocycle, while the same alcohol or a different one esterified the propionate residue at the sixth position of the macrocycle, then HPLC analysis of the Mg-proto diester pool should reveal the presence of only one Mg-proto diester species. On the other hand, if two or more different species of alcohols esterified the residues at the seventh and/or sixth positions of the macrocycle, then more than one Mg-proto diester species may become evident after HPLC separation.

The Mg-proto diester pool of α,α'-dipyridyl-treated cucumber cotyledons was extracted and partially purified on a silica column as described under Materials and Methods. Spectrofluorometric analysis of the column eluate indicated that it was made up of about equal amounts of Mg-proto diester and protochlorophyllide ester. The crude eluate was dissolved in hexane:acetone (1:1 v/v) and was subjected to HPLC analysis on a C-18-bonded reversed-phase column eluted isocratically with water:methanol:acetone (5:85:10 v/v/v) at a flow rate of 1 mL/min. Five fluorescent peaks, with retention times of about 116, 203, 575, 656, and 758 s, respectively, were resolved (Figure 4). The different peaks were collected and transferred to ether for spectrofluorometric analysis at 77 K.

Peak 1 (retention time = (t_R) = 116 s) exhibited an MPE emission at 592 nm (Figure 5), as well as the mobility of standard MPE. Therefore, it was ascribed to small amounts of MPE that contaminated the partially purified, fully esterified tetrapyrrole pigments eluted from the silica column.

Peak 2 (t_R = 203 s) exhibited a mobility similar to that of standard Mg-proto dimethyl ester (Figure 4) and had an MPE fluorescence emission spectrum with a maximum at 592.5 nm (Figure 5). Peak 2 was, therefore, assigned to a fully esteriried Mg protoporphyrin.

Peaks 3, 4, and 5 (t_R = 575, 656, and 758 s, respectively) all exhibited a protochlorophyllide ester fluorescence emission profile with an emission maximum at 624 nm in ether at 77 K (Figure 5) (Belanger & Rebeiz, 1980b). By analogy to the observations of Sudyina (1963) and those of Belanger & Rebeiz (1980b), the different retention times of the three protochlorphyllide ester peaks probably reflected esterification by different alcohols of the propionate residue, at the seventh position of the macrocycle. More interestingly however, fluorescence emission analysis at 77 K revealed that all three

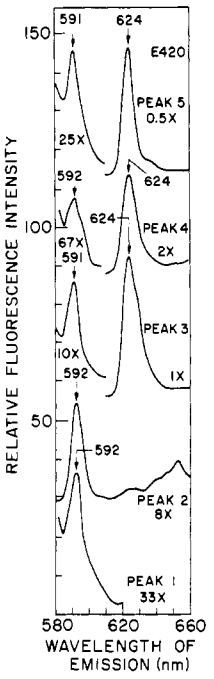


FIGURE 5: Fluorescence emission spectra of the eluted HPLC peaks depicted in Figure 4. The pigments in the collected fractions were transferred to ether, and their spectra were recorded in ether at 77 K on an SLM spectrofluorometer, Model 8000 DS, at an emission slit width of 2 nm. The fluorescence emission was elicited by excitation at 420 nm, at an excitation slit width of 4 nm; the spectral data was smoothened once by the microcomputer before replotting. Relative scale attenuations are indicated on the spectra, 67X and 0.5 X being the highest and lowest sensitivities used, respectively. The break in the spectra denotes a change in the attenuation at which the data were being recorded. All symbols are as in Figure 2.

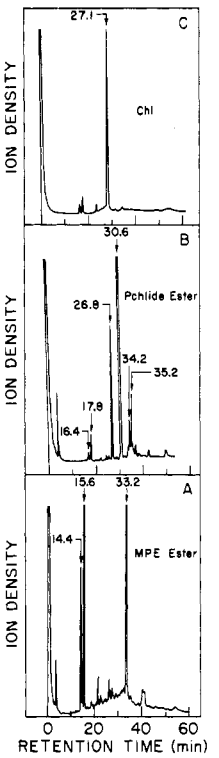


FIGURE 6: Gas–liquid chromatography of the alcohol fraction of the Mg-proto diester, protochlorophyllide ester, and chlorophyll pools. The pigments were saponified as described under Materials and Methods, and the hydrolyzed alcohols were transferred to hexane. Separations were performed on an OV-17 column. The temperature was programmed to increase linearly from 70 to 250 °C at a rate of 5 °C/min. MPE Ester = alcohol fraction of the Mg-proto diester pool; Pchlide Ester = alcohol fraction of the protochlorophyllide ester pool; Chl = alcohol fraction of the chlorophyll pool. Arrows indicate retention times of relevant peaks.

protochlorophyllide ester bands also contained smaller amounts of tetrapyrroles which exhibited a Mg protoporphyrin emission maximum at 591–592 nm (Figure 5). This observation implied that those slow-moving apolar Mg protoporphyrin tetrapyrroles that cochromatographed with the heterogeneous protochlorophyllide ester species probably corresponded to fully esterified Mg-proto diester tetrapyrroles which differed from the main, faster moving Mg-proto diester component (i.e., from peak 2, t_R = 203 s) by esterification with different alcohols of the propionate residue at the seventh and/or sixth position of the macrocycle. This in turn suggested that the fully esterified Mg-proto diester pool was heterogeneous, and its various components probably differed from one another by the nature of the esterifying alcohols at the seventh and/or sixth position of the macrocycle.

Gas Chromatographic/Mass Spectroscopic Analysis of the Esterifying Alcohols of the Mg-proto Diester Pool. If the Mg-proto diester pool was indeed heterogeneous due to esterification of the Mg tetrapyrrole with different alcohols, then gas–liquid chromatographic/mass spectroscopic (GLC/MS) analysis of the alcohol fraction following saponification should provide additional information about these alcohols.

The Mg-proto diester pool from α,α'-dipyridyl-treated cucumber cotyledons was purified on a silica column and then on thin layers of silica gel H as described under Materials and Methods. Spectrofluorometric analysis indicated that about 25% of the total purified pool was still made up of protochlorophyllide ester. Following alkaline hydrolysis, the apolar fraction containing the esterifying alcohols was subjected to GLC/MS analysis. Three major peaks with t_R values of 14.4, 15.6, and 33.2 min, respectively, were resolved by GLC (Figure 6A). The first two major peaks (t_R = 14.4 and 15.6 min, respectively) exhibited t_R values that fell between those of standard geraniol (a C-10 isoprenoid alcohol that yielded a GLC doublet at t_R = 9.6 and 10.8 min) and farnesol (a C-15 isoprenoid alcohol, t_R = 21.2 min). Both peaks exhibited similar but not identical MS profiles with a base peak at m/e 57 and major ions at m/e 41 and 205. Although the third major peak (t_R = 33.2 min) (Figure 6A) exhibited the retention time of a C-20-type alcohol, it was retained longer than standard phytol (a C-20 diterpenoid alcohol, t_R = 27.1 min). It exhibited a base peak at m/e 176 and a different MS profile than that of standard phytol and than that of the first two major peaks of Figure 6A.

Since repeated chromatography of the Mg-proto diester pool failed to eliminate the protchlorophyllide ester contamination which amounted to about 25% of the total pigment pool, it was important to determine if any one of the three major peaks resolved by GLC in Figure 6A could be attributed to contamination of the Mg-proto diester pool by protchlorophyllide ester. To this end, the hydrolyzed alcohol fraction of freshly purified protochlorphyllide ester was likewise sugjected to GLC/MS analysis. As depicted in Figure 6B, two major peaks (t_R = 26.8 min, base peak m/e 88 and t_R = 30.6 min, base peak m/e 55) as well as several minor components were revealed by GLC. None of the major or minor peaks that were derived from the hydrolysis of the protochlorophyllide ester pool was identical in every respect with the peaks derived from the Mg-proto diester pool. Furthermore, none of the peaks depicted in Figure 6B cochromatographed with standard phytol (t_R = 27.1 min). However, one of the minor peaks (t_R = 17.8 min) exhibited a retention time (t_R = 17.8 min) and an MS profile that were very similar to those of one of the peaks (t_R = 15.6 min) of the Mg-proto diester pool depicted in Figure

6A. It exhibited a base peak at m/e 57 and major ions at m/e 41 and 205.

Finally, as a control on the hydrolysis and extraction procedures used in this work, chlorophyll was purified and then saponified, and the apolar alcohol fraction was extracted and subjected to GLC/MS analysis. Only one major peak that cochromatographed with standard phytol (t_R = 27.1 min) was resolved (Figure 6C). It exhibited an MS profile that was nearly identical with that of standard phytol with an ion corresponding to loss of water ($M^+ - 18$) at m/e 278 as well as major ions at m/e 43, 57, 68, 71, 82, and 95.

Altogether, these results suggested that the small fraction of the protochlorophyllide ester pool that copurified with the Mg-proto diester pool was esterified with alcohols essentially different from those present in the bulk of the protochlorophyllide ester pool which in turn migrated independently of the Mg-proto diester pool, upon column and thin-layer chromatography. On the other hand, the bulk of the protochlorophyllide ester pool appeared to be esterified with two major and several minor alcohols, none of which was identifiable with standard phytol or farnesol, but one of which (t_R = 17.8 min, Figure 6B) appeared to be related to one of the major peaks of the Mg-proto diester alcohol profile (t_R = 15.6 min, Figure 6A).

Detection of a Mg-proto Diester Pool in Etiolated Cucumber Cotyledons and in Dark-Grown Euglena Not Treated with Inhibitors. Although the accumulation of a Mg-proto diester pool in α,α'-dipyridyl-treated higher plants which were enhanced in their rate of Mg porphyrin biosynthesis and were inhibited in their rate of Mg porphyrin conversion to protochlorophyll argued in favor of a functional metabolic role for Mg-proto diester, it was highly desirable to derive some independent evidence for such an assigned metabolic role. A systematic effort was therefore undertaken to detect such a pool in higher and lower plants that were not treated with inhibitors. Particular attention was paid to plants known to accumulate relatively high amounts of protochlorophyllide ester, such as *Euglena* (Cohen & Schiff, 1976). It was conjectured that if the Mg-proto diester pool was indeed a precursor of the protochlorophyllide ester pool and belonged to the fully esterified chlorophyll biosynthetic branch (Rebeiz et al., 1978), then the accumulation of small amounts of Mg-proto diester in these plants may be a real possiblity.

Two dark-grown cultures of *Euglena*, *Euglena gracilis* strain He (Wurtz & Buetow, 1981) and *Euglena gracilis* Klebs strain Z (Pringsheim), were found to contain Mg-proto diester. In both cases, it was observed that about equal amounts of Mg-proto diester and protochlorophyllide ester accumulated in the etiolated cultures grown in the absence of added inhibitors. The Mg-proto diester pool of dark-grown *Euglena* exhibited similar thin-layer chromatographic mobility and spectrophotometric and spectrofluorometric properties as those of the Mg-proto diester pool of cucumber cotyledons before and after demetalation (Figure 7).

Very recently, a Mg-proto diester pool was also detected in etiolated cucumber cotyledons incubated for 16 h with an aqueous solution (25 mM) of δ-aminolevulinic acid in the dark at 28 °C.

Discussion

This paper documents the detection of a novel metalloporphyrin in plants. The metalloporphyrin has been identified as a fully esterified Mg protoporphyrin (Figure 8). This pigment has been detected in higher plant tissues treated with α,α'-dipyridyl and has also been observed in untreated dark-grown *Euglena* cultures, as well as in untreated etiolated

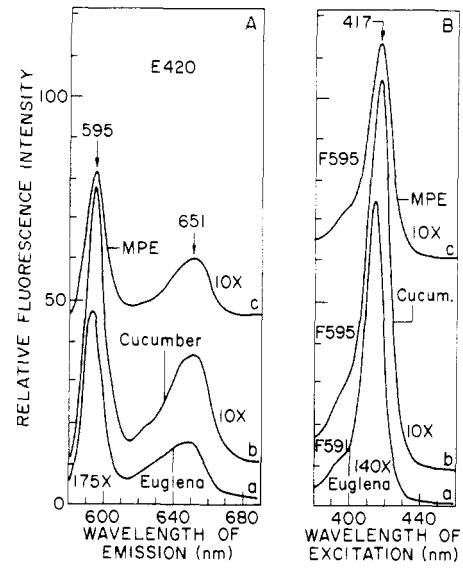
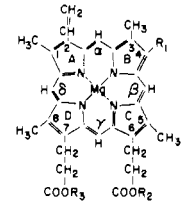


FIGURE 7: Fluorescence emission (A) and excitation (B) spectra of the Mg-proto diester pools of etiolated cucumber cotyledons and dark-grown *Euglena*. The spectra were recorded in ether at room temperature. Fluorescence emission was elicited by excitation at 420 nm. (a) Putative Mg-proto diester pool of etiolated *Euglena* grown in the absence of added inhibitors to the medium; (b) putative Mg-proto diester pool of etiolated cucumber cotyledons incubated with δ-aminolevulinic acid and α,α'-dipyridyl in the dark; (c) standard Mg protoporphyrin monoester. All symbols are as in Figure 2.



a. R_1 = -CH=CH_2; R_2 = -CH_3; R_3 = long chain fatty alcohol; = DV. Mg-PROTO DIESTER
b. R_1 = -CH_2-CH_3; R_2 = -CH_3; R_3 = long chain fatty alcohol; = MV. Mg-PROTO DIESTER
c. R_1 = -CH=CH_2; R_2 = -CH_3; R_3 = H; = DV, MPE
d. R_1 = -CH_2-CH_3; R_2 = -CH_3; R_3 = H; = MV. MPE

FIGURE 8: Structure of the putative Mg-proto diester pool and of the standard Mg protoporphyrin monoester pool. The Mg-proto diester and MPE pools are depicted as made up mainly of divinyl (DV) components and of smaller amounts of monovinyl (MV) components as reported elsewhere (Belanger & Rebeiz, 1980d). A full account of the MV components of these pools is in preparation.

cucumber cotyledons incubated with δ-aminolevulinic acid. These observations argue, in turn, for the ubiquitious occurrence of this pool in plants. During the preparation of this manuscript, it has been brought to our attention that a Mg protoporphyrin IX pool, of yet uncertain esterification state, has also been found in *Euglena gracilis* var. *bacillaris* (Frey et al., 1979).

α,α'-Dipyridyl has been successfully used in the past to induce the accumulation of other metabolic intermediates of the chlorophyll biosynthetic pathway. For example, Granick (1961) used this inhibitor successfully in order to induce the accumulation of MPE in etiolated barley seedlings. Later, the metabolic role of MPE was established when its conversion into protochlorophyllide was unambiguously demonstrated in vitro (Mattheis & Rebeiz, 1977). Likewise, at this stage, we have no reason to believe that the α,α'-dipyridyl-induced Mg-proto diester pool is an artifact. This conclusion seems to be borne out by the detection of this pool in etiolated cucumber cotyledons and in dark-grown *Euglena* cultures that were not treated with α,α'-dipyridyl.

It is not presently clear whether the carboxylic group at position 6 of the Mg-proto diester macrocycle is esterified with methyl alcohol or with another alcohol. At present, it is assumed that all the intermediates of the chlorophyll biosynthetic pathway past MPE are esterified with a methyl group at the sixth position of the macrocycle (tenth position in phorbins) (Granick, 1961; Ellsworth & St. Pierre, 1975). If one assumes that in the Mg-proto diester pool the sixth position is likewise esterified with a methyl group, then the alcohol heterogeneity unraveled by HPLC and by GLC analyses (Figures 4 and 6) must be attributed solely to the alcohols that esterify the seventh position of the macrocycle. Presently, the chemical nature of these alcohols is not yet precisely known as their chromatographic properties differed from those of authentic isoprenoid alcohols such as geraniol, farnesol, and phytol. Furthermore, more work is needed before their relationship to the alcohol intermediates involved in the conversion of chlorophyllide *a* to chlorophyll *a* (Schoch et al., 1977) is determined.

At the present time, the reason for the alcohol heterogeneity of the Mg-proto diester pool is not well understood. However, if it is assumed that the Mg-proto diester pool is a precursor of the protochlorophyllide ester pool, then one is led to the conclusion that the alcohol heterogeneity of the protochlorophyllide ester pool, which was reported in Figure 6B, probably finds its origin in the alcohol heterogeneity of the Mg-proto diester pool (Figure 6A). However, since the esterifying alcohols of the protochlorophyllide ester pool appear to be essentially different from those of the Mg-proto diester pool, it is further suggested that during the conversion of the Mg-proto diester pool into protochlorophyllide ester the formation of the cyclopentanone ring is also accompanied by chemical modifications of the Mg-proto diester alcohols at position 7 of the macrocycle.

Finally, phytol was not identified as one of the esterifying alcohols of protochlorophyllide ester. This is in agreement with earlier observations (Rebeiz & Castelfranco, 1973; Liljenberg, 1974; Griffiths, 1980). It is also compatible with the notion that protochlorophyllide ester is not derived from protochlorophyllide by phytylation (Rebeiz et al., 1978). This in turn calls into question the biosynthetic origin of protochlorophyllide ester in plants. As a working hypothesis, we therefore propose that the protochlorophyllide ester pool is derived from the Mg-proto diester pool. The precursor–product relationship of these two pools is presently under investigation.

References

Belanger, F. C., & Rebeiz, C. A. (1980a) *J. Biol. Chem. 255*, 1266–1277.

Belanger, F. C., & Rebeiz, C. A. (1980b) *Biochemistry 19*, 4875–4883.

Belanger, F. C., & Rebeiz, C. A. (1980c) *Plant Sci. Lett. 18*, 343–350.

Belanger, F. C., & Rebeiz, C. A. (1980d) *Plant Physiol. 65*, 106S.

Cohen, C. E., & Schiff, J. A. (1976) *Photochem. Photobiol. 24*, 555–566.

Duggan, J. X., & Gassman, M. L. (1974) *Plant Physiol. 53*, 206–215.

Ellsworth, R. K., & St. Pierre, L. A. (1975) *Photosynthetica 9*, 340–342.

Falk, J. E. (1964) *Porphyrins and Metalloporphyrins*, Elsevier, New York.

Feiser, L. F., & Feiser, M. (1967) *Reagents for Organic Synthesis*, Vol. 1, p 192, Wiley-Interscience, New York.

Frey, M. A., Alberte, R. S., & Schiff, J. A. (1979) *Biol. Bull. (Woods Hole, Mass.) 157*, 368–369.

Freyssinet, G., & Schiff, J. A. (1974) *Plant Physiol. 53*, 543–554.

Freyssinet, G., Rebeiz, C. A., Fenton, J. M., Khanna, R., & Govindjee (1980) *Photobiochem. Photobiophys. 1*, 203–212.

Granick, S. (1961) *J. Biol. Chem. 236*, 1168–1172.

Griffiths, T. W. (1980) *Biochem. J. 186*, 267–278.

Jensen, J. (1963) *J. Chromatogr. 10*, 236–238.

Liljenberg, C. (1974) *Physiol. Plant. 32*, 208–213.

Liljenberg, C., & Odham, J. (1969) *Physiol. Plant* 22, 686–693.

Mattheis, J. R., & Rebeiz, C. A. (1977) *J. Biol. Chem. 252*, 4022–4024.

Mielenz, J. R. (1976) Ph. D. Thesis, University of Illinois at Urbana–Champaign.

Rebeiz, C. A. (1968) *Magon, Ser. Sci. 21*, 1–25.

Rebeiz, C. A., & Castelfranco, P. A. (1971) *Plant Physiol. 47*, 24–32.

Rebeiz, C. A., & Castelfranco, P. A. (1973) *Annu. Rev. Plant Physiol. 24*, 129–172.

Rebeiz, C. A., Yaghi, M., Abou-Haidar, M., & Castelfranco, P. A. (1970) *Plant Physiol. 46*, 57–63.

Rebeiz, C. A., Mattheis, J. R., Smith, B. B., Rebeiz, C. C., & Dayton, D. F. (1975a) *Arch. Biochem. Biophys. 171*, 549–567.

Rebeiz, C. A., Mattheis, J. R., Smith, B. B., Rebeiz, C. C., & Dayton, D. F. (1975b) *Arch. Biochem. Biophys. 166*, 446–465.

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.

Schoch, S., Lampert, M., & Rudiger, W. (1977) *Z. Pflanzenphysiol. 83*, 427–436.

Smith, B. B., & Rebeiz, C. A. (1977) *Arch. Biochem. Biophys. 180*, 178–185.

Sudyina, E. G. (1963) *Photochem. Photobiol. 2*, 181–190.

Vlcek, L. M., & Gassman, M. L. (1979) *Plant Physiol. 64*, 393–397.

Wurtz, E. A., & Buetow, D. E. (1981) *Curr. Genet.* (in press).