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CHLOROPLAST BIOGENESIS

38. QUANTITATIVE DETECTION OF A CHLOROPHYLLIDE *b* POOL IN HIGHER PLANTS

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A pool of chlorophyllide *b* has been detected in greening cucumber cotyledons. This pool exhibited the same spectrofluorometric properties as that of Chl *b* in diethyl ether at 298 and 77 K (Rebeiz, C.A., Belanger, F.C., Freyssinet, G. and Saab, D.G. (1980) Biochim. Biophys. Acta 590, 234–247) but had the chromatographic mobility and solubility of a monocarboxylic phorbin. The presence of a free carboxylic group and of a formyl group in this putative chlorophyllide *b* pool was demonstrated by methylation with diazomethane and by its conversion into chlorophyllide *b* oxime upon treatment with hydroxylamine. The concentration of this chlorophyllide *b* pool in greening tissues was in the same range as those of the protochlorophyllide and chlorophyllide *a* pools. Less than 15% of the chlorophyllide *b* pool could have arisen from chlorophyllase activity in vitro, as confirmed by the extent of hydrolysis of ^{14}C-labeled Chl *b* added to the tissue just before extraction.

Introduction

Chlorophyll *b* (Chl *b*) is a major plant pigment present in virtually all photosynthetic tissues of higher plants; it amounts to about 30% of the total chlorophyll in green plants [1]. It differs from Chl *a* by carrying a formyl group instead of a methyl group at position 3 of the macrocycle (Fig. 1) [1].

The biosynthetic origin of the Chl *b* pool in green plants has been a subject of debate and controversy for the past 30 years. The issue was well described about two decades ago by Smith and French [2]. In the intervening years, mainly through the research effort of Shlyk and co-workers [3], it has been more-or-less accepted that the Chl *b* pool is synthesized from newly formed Chl *a* molecules in the plastid membranes. However, the enzyme(s) responsible for the putative reactions which are responsible for the conversion of the methyl group of Chl *a* into a formyl group have not yet been purified. More recently Oelze-Karow et al. [4,5], after a kinetic analysis of the fluctuation in the chlorophyllide *a* (Chlide *a*) and Chl *b* pool sizes in etiolated mustard cotyledons subjected to various light pretreatments, have suggested that the Chl *b* pool may be formed from Chlide *a* instead of from Chl *a*.

During the course of a detailed spectrofluorometric survey, at 77 K, of the intermediates of the Chl *a* biosynthetic pathway [6] we observed the occurrence of a Chlide *b* pool in ether extracts of greening tissues, which could not be accounted for by an artifactual hydrolysis of Chl *b*. During the revision of this manuscript, it has come to our attention that the conversion of exogenous Chlide *b* into Chl *b* in vitro, has been recently reported [7]. This in turn rein-

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Abbreviations: Chl, chlorophyll; Chlide, chlorophyllide; Pchlide, protochlorophyllide; Chl(ide), chlorophyll and/or chlorophyllide.

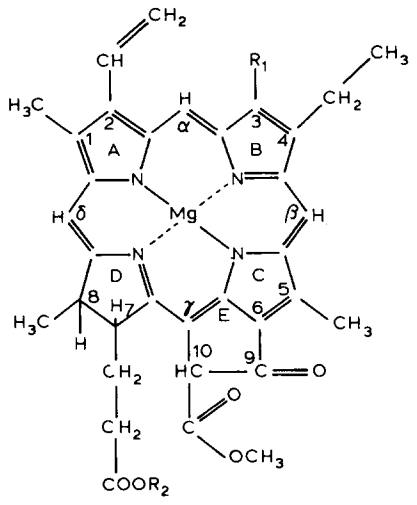

Fig. 1. Structure of some monovinyl chlorophylls and chlorophyllides. a. R_1 = -CH_3, R_2 = $C_{20}H_{39}$, Chl *a*. b. R_1 = -CH_3, R_2 = H; Chlide *a*. c. R_1 = -CHO, R_2 = $C_{20}H_{39}$; Chl *b*. d. R_1 = -CHO, R_2 = H; Chlide *b*. e. R_1 = -CH=NOH, R_2 = $C_{20}H_{39}$; Chl *b* oxime. f. R_1 = -CH=NOH, R_2 = H; Chlide *b* oxime.

forces the importance of the detection of a Chlide *b* pool in greening plants and emphasizes the possible biosynthetic function of this novel metabolic pool. A preliminary communication of these results has been published elsewhere [8].

Materials and Methods

Plant materials and growth conditions. Cucumber seeds (*Cucumis sativus* L. cv. Beit Alpha MR) were sown in moist vermiculite and grown in darkness as previously described [9]. Alternatively, the seeds were grown under a 14 h light/10 h dark photoperiodic regime at 26–28°C [10]. The light intensity was 3.54 mW/cm^2 at the level of the tissue. Etiolated cotyledons were harvested under a dim green safelight after 4 days of growth.

Light treatment. For greening under continuous illumination, cotyledon pairs were excised from dark-grown seedlings with about 2 cm of the hypocotyl hook still attached. About 5 g tissue were placed in a 10 cm × 8 cm petri dish with 6 ml of distilled H_2O, and the samples were irradiated with 320 μW/cm^2 of white fluorescent light at 28°C for various periods of time. Cotyledons from photoperiodically-grown seedlings were excised during the third hour of the fourth light cycle.

Chemicals and radiochemicals. δ-Amino[4-^{14}C]-levulinic acid (46 Ci/mol) was purchased from Research Products International (CEA), Elk Grove Village, Il. [^{14}C]Toluene ($5.67 \cdot 10^5$ dpm/g) was a product of the Packard Instrument Co., Downers Grove, Il. Scintillation grade toluene was obtained from the Fisher Chemical Co., Fairlawn, NJ.

Extraction of the monocarboxylic tetrapyrroles. At the indicate time, 1 g samples of etiolated, greening or green cotyledon tissues were placed in a metal strainer and were frozen by immersion in liquid N_2. The frozen tissue was immediately homogenized in a chilled mortar with 6.7 ml acetone/0.1 N NH_4OH (9 : 1, v/v) and about 1 g acid-washed sand. All further manipulations of homogenates or pigment-containing extracts were performed at 0–4°C under subdued light. The homogenate was clarified by centrifugation at 39 000 × *g*, and the pellet was re-extracted with 2.0 ml cold 80% acetone. The combined 80% acetone extracts were extracted with hexane to remove the fully esterified pigments as previously described [11]. The monocarboxylic tetrapyrroles remained in the hexane-extracted acetone fraction. The volume of the hexane extract was recorded and an aliquot was removed for the determination of chlorophylls (see below).

The volume of the hexane-extracted acetone fraction containing the monocarboxylic tetrapyrroles was also recorded, and an aliquot was removed for determining the amounts of porphyrins and monocarboxylic phorbins as described below. Pigments in the remaining hexane-extracted acetone fraction were then transferred to 1.5 ml peroxide-free diethyl ether after addition of 1/17 vol. saturated aqueous NaCl and 1/70 vol. 0.5 M KH_2PO_4 [11]. The diethyl ether epiphase was removed, and the aqueous residue was re-extracted 2–3 times with 1 ml volumes of diethyl ether until the extract was colorless and non-fluorescent. The fluorescence spectrum of a small aliquot of the combined diethyl ether extract was monitored at 77 K, and the remainder was subjected to chromatographic purification.

Thin-layer chromatography. The diethyl ether extract of the hexane-extracted acetone fraction, containing chlorophyllides and protochlorophyllides as well as small amounts of contaminating chlorophylls

a and *b*, was purified on thin layers of silica gel H as described below. The diethyl ether solution was concentrated under N_2 gas to a small volume, usually less than 1 ml of a turbid aqueous residue. The pigments in this residue were removed by repeated extraction with 0.5 ml volumes of diethyl ether, and the combined diethyl ether extract was dehydrated over 0.5 g/ml granular NaCl [12]. The dehydrated diethyl ether extract was removed, and the pigments adsorbed on the NaCl granules were removed by washing twice with 2 ml volumes of diethyl ether. The diethyl ether extract and washings were combined, concentrated under N_2 gas to about 0.3 ml, then applied quantitatively to a thin-layer plate of silica gel H which was developed in toluene/ethyl acetate/ethanol (8 : 2 : 1, v/v) at 4°C in darkness. Thorough dehydration of the diethyl extract as described above greatly improved the chromatographic resolution.

Elution of the segregated pigments. The segregated tetrapyrroles were visualized on developed chromatograms by their red fluorescence under 366 nm ultraviolet light. The pigment bands were scraped into beakers containing organic solvents before allowing the chromatograms to dry. The chlorophyll band ($R_F \approx 0.8$), actually containing a mixture of Chls *a* and *b*, was eluted in diethyl ether. The chlorophyllide and protochlorophyllide bands ($R_F \approx 0.1$ and 0.2, respectively) were eluted in methanol/acetone (4 : 1, v/v). The eluates were clarified by centrifugation.

In the case of the chlorophyll bands, the silica gel pellet was washed twice with 1 ml volumes of diethyl ether. The combined chlorophyll eluates were evaporated to dryness under N_2, and the residue was redissolved in 1 ml of 80% acetone for fluorometric chlorophyll determinations (see below).

The chlorophyllide eluate (in methanol/acetone, 4 : 1, v/v) was mixed with an equal volume of diethyl ether, and the mixture was passed through 5 vol. 0.37 M potassium phosphate buffer at pH 7.0. The diethyl ether epiphase was collected, and small amounts of water were removed by centrifugation before evaporation to dryness under N_2. The chlorophyllide residue was redissolved in 0.5 ml diethyl ether for spectrofluorometric monitoring at 77 K.

Spectrophotometry. Absorption spectra were recorded on an Aminco model DW-2 spectrophotometer operated in the split-beam mode.

Spectrofluorometry. Fully corrected fluorescence excitation and emission spectra were recorded either with a Perkin Elmer spectrofluorometer, model MPF-3, or with an SLM spectrofluorometer, model 8000 DS interfaced with a Hewlett-Packard microcomputer model 9825S, as described elsewhere [13]. Emission spectra were recorded on the Perkin Elmer spectrofluorometer with an excitation slit width of 6 nm and an emission slit width of 3 nm, while excitation spectra were recorded with an excitation slit width of 3 nm and an emission slit width of 6 nm. Emission spectra recorded on the SLM spectrofluorometer employed an excitation slit width of 4 nm and an emission slit width of 2 nm, while excitation spectra were recorded with an excitation slit width of 2 nm and an emission slit width of 4 nm. Under these conditions the spectral accuracy of the reported maxima is about 1 nm. Low-temperature spectra were recorded in diethyl ether by previously published methods [14].

Quantitative determination of pigments. The concentrations of Chl(ide) *a* and Chl(ide) *b* were calculated from fluorescence excitation spectra recorded in hexane-extracted acetone at room temperature as described elsewhere [15]. The concentrations of protochlorophyllide and Mg-protoporphyrin monoester were calculated from fluorescence emission spectra recorded in hexane-extracted acetone at room temperature as described previously [16,17].

All spectra used for quantitative determinations were recorded on the SLM spectrofluorometer which was interfaced with a Hewlett-Packard 9825S microcomputer system. The on-line calculations that converted digital spectral data into quantitative values were automatically performed by the computer.

Determination of the amounts of chlorophyllides a and b. The hexane-extracted acetone fraction of green tissues usually contains monocarboxylic tetrapyrroles such as chlorophyllides, protochlorophyllides and Mg-porphyrins as well as small amounts of Chls *a* and *b* which were not removed by the hexane extraction. Thus, for precise determination of the Chlide *a* and Chlide *b* concentrations in the hexane-extracted acetone fraction of such tissues it is essential to correct these fractions for Chl *a* and *b* contamination. This was accomplished by the three-step process described below.

First, the total quantities of Chl(ide) *a* and

Chl(ide) *b* present in the hexane-extracted acetone fraction were determined spectrofluorometrically [15]. The contaminating Chls *a* and *b* were then separated from the chlorophyllide and protochlorophyllide pools by thin-layer chromatography on silica gel H as described above. The chlorophylls were eluted in diethyl ether and transferred to 80% acetone for spectrofluorometric estimation [15]. Finally, the amounts of contaminating chlorophylls, corrected for chromatographic recovery (28–30%) were subtracted from the Chl(ide) values determined in the first step to yield the net amount of Chlides *a* and *b* present in the hexane-extracted acetone fraction.

Preparation of diazomethane. An ethereal solution of diazomethane was prepared as described by Fieser and Fieser [18]. The solution was stored in a tightly sealed vessel at −20°C in darkness.

Preparation of Chl(ide) b oximes. The oxime derrivatives of Chl *b* and of the putative Chlide *b* pools were prepared by the method of Ogawa and Shibata [19] as described below.

A diethyl ether solution, containing 0.2 to 1 nmol standard Chl *b* (see below) or chromatographically purified putative Chlide *b*, was evaporated to dryness under N_2. The residue was redissolved in 0.95 ml methanol; 50 µl 5.76 M NH_2OH at pH 4.0 were added, and the mixture was incubated for 10 min at room temperature in the dark. Diethyl ether (1 ml) was then added, and the resulting solution was passed through 10 ml 0.37 M potassium phosphate buffer, pH 7.0, by slowly releasing the diethyl ether solution from a pipette submerged in the buffer. The diethyl ether phase was collected, evaporated to dryness under N_2, and redissolved in diethyl ether for spectrofluorometric analysis. The standard Chl *b* oxime thus prepared exhibited the same spectrophotometric properties at room temperature as those reported by Ogawa and Shibata [19].

Preparation of ^{14}C-labeled chlorophyll b. [^{14}C]-Chl *b* was formed by incorporation of exogenous δ-amino[^{14}C]levulinic acid into the Chl *b* pool of greening cucumber cotyledons. Cucumber seedlings were grown photoperiodically for 2 days as described above. At this stage of growth, the shoots had just emerged from the vermiculite, and the cotyledons appeared light green; their seed coats were still partially attached. The seed coats were removed 8 h after the beginning of the second light cycle and, 12 µl of an aqueous solution of δ-amino[^{14}C]levulinic acid were applied directly to the adaxial surfaces of the cotyledons of each of 18 seedlings. Each ml of the δ-amino-[^{14}C]levulinic acid solution contained 60 µCi δ-amino[4-^{14}C]levulinic acid (46 Ci/mol), 35 µmol potassium phosphate buffer, pH 6.0, and 48 µg Tween 20. The plants were returned to the growth chamber and were administered an additional 10 µl of the substrate solution 8 h after the beginning of the third light cycle. The cotyledons were harvested 8 h after the beginning of the fourth light cycle.

Excised cotyledons were washed with distilled water and blotted dry; then the chlorophyll was extracted with ammoniacal acetone as described for the extraction of the monocarboxylic tetrapyrroles. The chlorophylls in the 80% acetone extract were transferred to hexane [21], and this hexane was evaporated to dryness under N_2 at room temperature. The chlorophyll residue was redissolved in 5.0 ml of diethyl ether and chromatographed on thin-layers of cellulose MN300 developed in ligroine (60–90)/*n*-propanol (99 : 1, v/v) in darkness at room temperature [20]. Chl *b* (R_F = 0.25) was eluted in diethyl ether and its specific radioactivity was determined. Spectrofluorometric analysis of this fraction indicated that it was free of Chl *a* and pheophytin *b*. The total amount of Chl *b* recovered was 855 nmol and had a specific radioactivity of 1.1 Ci/mol. The labeled pigment was used immediately or was evaporated to dryness under N_2 and stored in darkness at −20°C for later use.

Preparation of Chl a and Chl b spectrofluorometric standards. A mixture of Chls *a* and *b* was extracted from 4-day-old photoperiodically grown cucumber cotyledons into 80% acetone (see below). The Chl was transferred to hexane, then purified on thin layers of cellulose as described above. The Chl *a* (R_F = 0.58) and Chl *b* (R_F = 0.25) bands were eluted with diethyl ether. The clarified eluates were evaporated to dryness under N_2, then the purified chlorophyll residue was redissolved in dry diethyl ether for spectrofluorometric monitoring at 77 K.

Preparation of a chromatographic monocarboxylic phorbin standard. A chromatographic phorbin standard, containing Chlide *a* and Pchlide *a*, was prepared from 4-day-old etiolated cucumber cotyledons which had been illuminated for 5 min with white fluorescent light (320 µW/cm^2). Such a treatment usually

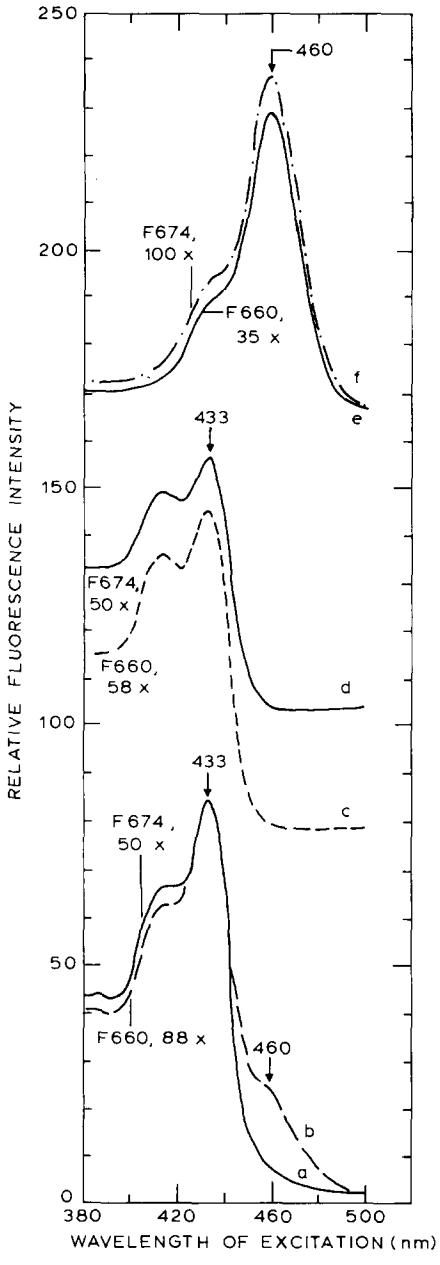

Fig. 2. Fluorescence excitation spectra, of the monocarboxylic phorbin pool (a, b) of 4-day-old photoperiodically-grown cucumber cotyledons, recorded at room temperature in hexane extracted acetone. The excitation spectra of standard Chl *a* (c, d) and Chl *b* (e, f) are also shown for comparative purposes. The spectra were recorded on the SLM spectrofluorometer. The wavelength setting in nm of the emission monochromator is indicated by the appropriate fluorescence emission (*F*) value. Ordinate scale attenuation is indicated on the individual spectra, 350X being the highest sensitivity used. The spectra have been vertically arranged for the purpose of clarity. Arrows point to wavelengths of interest.

results in the conversion of most of the Pchlide *a* into Chlide *a*, while a small fraction of the Pchlide *a* pool remains unphototransformed and is recovered with the Chlide *a*. The tissue was homogenized in ammoniacal acetone, and the monocarboxylic phorbin pool was transferred to diethyl ether as described under 'extraction of the monocarboxylic tetrapyrroles'. The diethyl ether extract, containing Chlide *a* + Pchlide *a*, was used immediately, as a monocarboxylic phorbin chromatographic marker or was evaporated to dryness under N_2 and stored in darkness at −20°C for later use.

Radiochemical determinations. Samples were counted in 15 ml toluene scintillation fluid (0.6 g 1,4-bis[2-(5-phenyloxazolyl)]benzene and 7.0 g 2,5-diphenyloxazole in 1 liter toluene). The counting was done in a Packard Tri-Carb scintillation counter, Model 3375, to an S.D. of ±2% or less. Absolute radioactivity was determined by the addition of a [^{14}C]toluene internal standard.

Results

Detection of Chl(ide) b fluorescence in the monocarboxylic tetrapyrrole pool of cucumber cotyledons

During spectrofluorometric investigations of the various monocarboxylic tetrapyrrole pools present in the hexane-extracted acetone extracts of greening tissues (see Materials and Methods), we have consistently observed a Soret excitation peak at about 460 nm, at room temperature, similar to that of standard Chl *b* (Fig. 2) [15]. Since this observation suggested the occurrence of Chlide *b* (Fig. 1 c, d) in these extracts, we decided to probe this possibility further.

Better spectral resolution of the putative Chl(ide) *b* component was achieved by transferring the monocarboxylic tetrapyrroles to diethyl ether and by recording their spectral properties at 77 K: at this temperature, the resolution is greatly improved due to a narrowing of the fluorescence bandwidths [21] as shown in the past by the detection of small amounts of Chl *b* in the presence of karge amounts of Chl *a* [22,23] and of several metabolic intermedi-

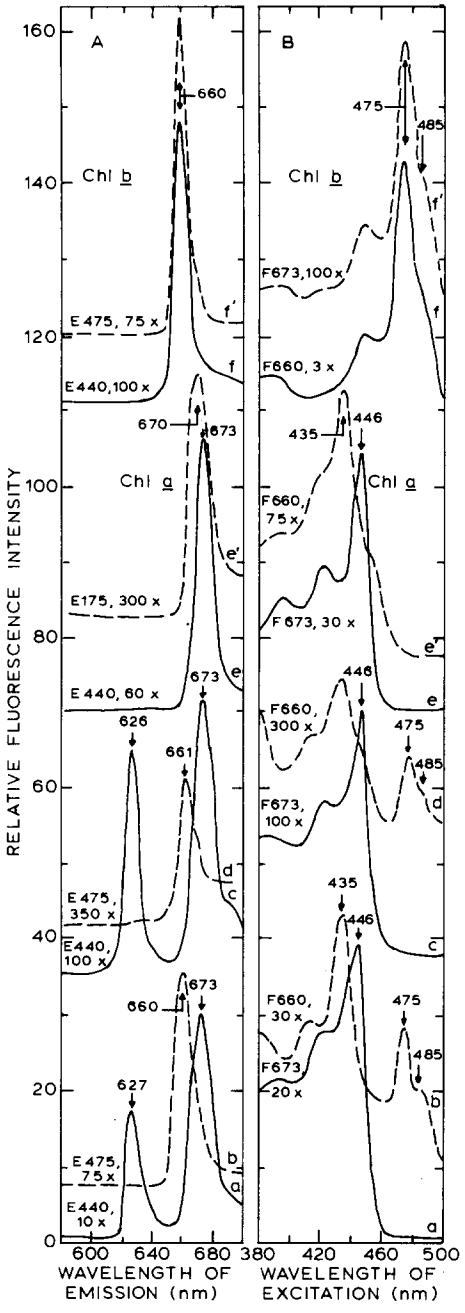

Fig. 3. Fluorescence emission (A) and excitation (B) spectra of the crude monocarboxylic phorbin pool of greening cucumber cotyledons, recorded in diethyl ether at 77 K. (a, b) 4-day-old, photoperiodically-grown cucumber cotyledons; (c, d) 4-day-old etiolated cucumber cotyledons greened for 5 h under continuous illumination, (e, e'): purified Chl *a* standard; (f, f') purified Chl *b* standard. All spectra were recorded on the Perkin-Elmer spectrofluorometer model MPF-3. The emission spectra were elicited by the indicated excitation (*E*) wavelengths. All other symbols are as in Fig. 1.

ates of the chlorophyll biosynthetic pathway [9,13, 24].

When the diethyl ether extract containing the monocarboxylic phorbin pool of photoperiodically grown tissues was excited at 440 nm, near the Soret absorption maximum of Chl(ide) *a*, two emission maxima were observed at about 673 and 627 nm which corresponded to Chl(ide) *a* and Pchl(ide), respectively [9,25] (Fig. 3Aa, e). To elicit the fluorescence emission of any Chl(ide) *b* that may have been be present, the same diethyl ether extract was excited at 77 K at 475 nm, the Soret absorption maximum of Chl(ide) *b* at 77 K in diethyl ether [25]. The Pchlide *a* and Chl(ide) *a* emissions at 627 and 673 nm, respectively, disappeared, and a Chl(ide) *b* emission with a maximum at 660 nm appeared (Fig. 3Ab, f) [23,25]. Similar results were observed in diethyl ether extracts of the monocarboxylic phorbin pools of etiolated cucumber cotyledons which were illuminated for 5 h with white fluorescent light before extraction (Fig. 3Ac, d). That the emission maximum at 660 nm (Fig. 3Ab, d) did not emanate from the Chl(ide) *a* pool was confirmed by the absence of such an emission maximum upon exciting a Chl *a* standard at 475 nm (Fig. 3Ae'). It is noteworthy that Chl *a* and Chlide *a* exhibit very similar fluorescence emission and excitation properties, as is also true for Chl *b* and Chlide *b* [15].

Further characterization of the putative Chl(ide) *b* fluorescence was achieved by comparing its Soret excitation peaks with those of the Chl *a* and *b* standards and with those of the Pchlide *a* and Chlide *a* components of the monocarboxylic phorbin pool. The Soret excitation spectrum of the Pchlide *a* pool recorded at its emission maximum (627 nm) exhibited a divinyl excitation peak with a maximum at 443 nm (data not shown), as was reported for the Pchlide *a* pool of photoperidocally grown tissues [9]. The excitation spectrum recorded at the Chl(ide) *a* emission maximum, at 673 nm, exhibited an excitation maximum at 446 nm (Fig. 3Ba, e) consistent with that of monovinyl Chl *a* [25]. On the other hand, the Soret excitation spectrum of the putative Chl(ide) *b* recorded at its emission maximum (i.e., 660 nm) exhibited a Soret excitation peak at 475 nm and a

shoulder at about 485 nm, identical to that of standard Chl *b* (Fig. 3Bb, f) [25]. The additional Soret excitation maximum observed at about 435 nm (Fig. 3Bb) is that of a blue-shifted Chl *a* component of the Chl *a* pool, which is usually observed at this wavelenth when the emission monochromator is positioned at 658–660 nm (Fig. 3B,e′) [25]. Similar results were observed in extracts of etiolated cucumber cotyledons that were illuminated for 5 h with white fluorescent light prior to extraction (Fig. 3Bc, d).

These results indicate the presence of a compound with the spectrofluorometric properties of Chl(ide) *b* in the monocarboxylic tetrapyrrole pool of greening cucumber cotyledons. Since the detection of such a compound may have been caused by contamination of the monocarboxylic tetrapyrrole pool by small amounts of Chl *b*, this possibility was examined as described below.

Partial purification of Chlide b and Chlide a from the monocarboxylic tetrapyrrole pool of greening tissues

Separation of the putative Chl(ide) *b* from any possible Chl *b* contamination of the monocarboxylic tetrapyrrole pool was achieved by TLC on silica gel H in toluene/ethyl acetate/ethanol (8 : 2 : 1, v/v). In this solvent, the fully esterified Chl *a* and *b* components migrate rapidly with a relative R_F of about 0.85–0.95, while the monocarboxylic Chlides *a* and *b* migrate much slower, with an R_F of about 0.1–0.25. In this chromatographic system, however, Pchlide *a* and Chlides *a* and *b* are not always resolved, but their partial separation can be achieved when the thin layers are lightly loaded with a thoroughly dried diethyl ether extract (see Materials and Methods). In such cases, the Pchlide (R_F = 0.24) move just ahead of the Chlide *a* + *b* band (R_F = 0.19). No effort was made at this stage to separate the Chlide *a* from the Chlide *b* components, since these can be readily resolved by fluorescence excitation spectra recorded at 77 K at different emission wavelengths (Fig. 3) [23].

Chromatography of the diethyl ether extract of the monocarboxylic tetrapyrrole pool of greening cucumber cotyledons easily revealed a minor polar band (R_F = 0.9) with the fluorescence properties of Chls *a* and *b* and another band that moved with the R_F of the Chlide *a* chromatographic standard (R_F = 0.19). Upon elution and transfer to diethyl ether (see Methods) the latter band exhibited the emission properties of Chlide *a* and Chlide *b* with fluorescence emission maxima at about 673 and 660 nm and Soret excitation maxima at 442 and 475 nm, respectively (Fig. 4). Small amounts of contaminating Pchlide *a* with an emission maximum at about 625 nm can still be observed (Fig. 4Aa). The additional Soret excitation maximum of the Chlide *a* + *b* pools observed at about 434 nm (Fig. 4Ba) is that of a 'blue-shifted' component of the Chlide *a* pool which is usually observed when the emission monochromator is positioned at the short-wavelength tail of the Chlide *a* emission band [25].

Altogether, the above results strongly suggested the occurrence of both Chlide *b* and Chlide *a* pools in greening plant tissues.

Methylation of the putative Chlide b pool of photoperiodically grown cucumber cotyledons

If the spectral entity tentatively identified as Chlide *b* differs in solubility and chromatographic mobility from Chl *b* only by possession of a free carboxyl group at position 7 of the macrocycle (Fig. 1), it should be possible to esterify this group chemically, thus converting the putative Chlide *b* into methyl Chlide *b*. The latter should then exhibit the spectral properties of Chlide *b* but the solubility and chromatographic behavior of a fully esterified tetrapyrrole (i.e., Chl *b*).

To test this hypothesis, the monocarboxylic phorbin pool from 14 g 4-day-old photoperiodically grown cucumber cotyledons was extracted into diethyl ether as described in Materials and Methods. Traces of chlorophyll contamination were removed by chromatography on thin layers of silica gel developed in toluene/ethyl acetate/ethanol (8 : 2 : 2, v/v) (R_F Chls *a* + *b* = 0.88). The chlorophyllide-containing band (R_F = 0.48) was eluted and transferred to diethyl ether as previously described. Fluorescence spectroscopy at 77 K revealed that this preparation contained Chlide *a*, putative Chlide *b* and some Pchlide *a*.

A 3 ml aliquot of this purified chlorophyllide preparation was methylated by slow addition of 0.5 ml of a freshly prepared solution of diazomethane in diethyl ether (see Materials and Methods) at 0–4°C with continuous stirring which was continued for an

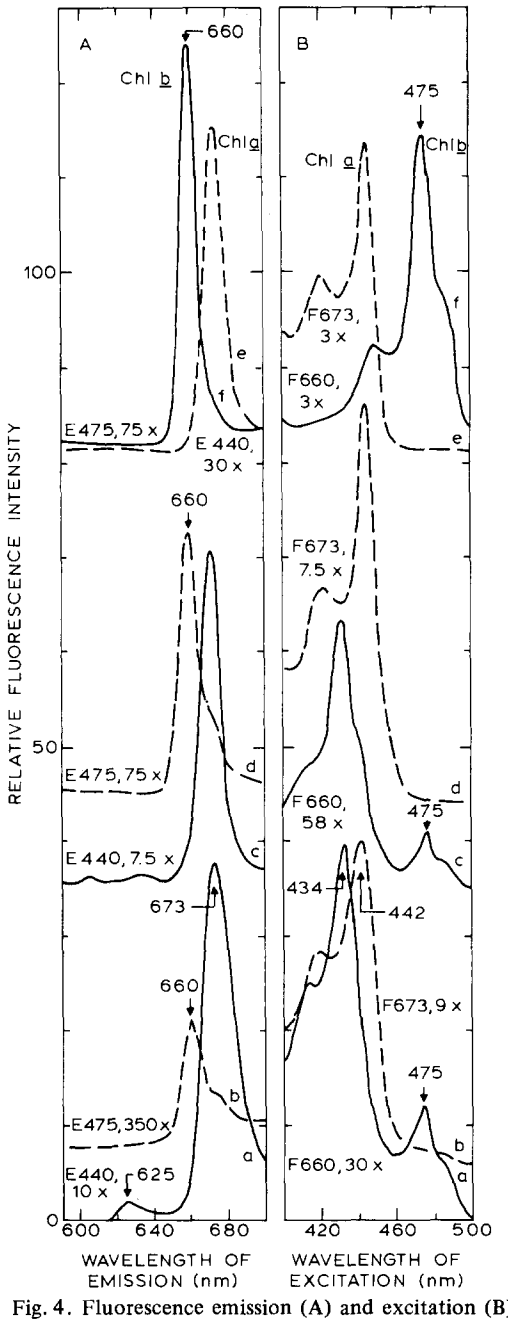

Fig. 4. Fluorescence emission (A) and excitation (B) spectra of the monocarboxylic phorbin pool of greening cotyledons after purification on thin layers of silica gel H. The spectra were recorded in ether at 77 K. (a, b) 4-day-old, photoperiodically-grown cucumber cotyledons; (c, d) 4-day-old etiolated cucumber cotyledons greened for 5 h under continuous illumination; (e) purified Chl *a* pool; (f) purified Chl *b* pool. Spectrum Bb was recorded on the SLM spectrofluorometer, while the remaining spectra were recorded on the Perkin-Elmer instrument. All other symbols are as in Fig. 1 and 2.

additional 2 min prior to evaporation to dryness under N_2. The residue was dissolved in 0.5 ml diethyl ether and subjected to TLC using the solvent system given above. An intensely fluorescent band was observed with an R_F of 0.88, corresponding to the Chl *a* and Chl *b* standards which migrated as a single band in this solvent system. When this band was eluted in diethyl ether and its fluorescence properties recorded at 77 K, typical Chl *b* spectra were observed with emission and excitation maxima at 660 and 474 nm, respectively; these maxima were identical to those observed in the preparation before reaction with diazomethane and to those previously reported for standard Chl *b* (Fig. 3). As a further control, an aliquot of the original chlorophyllide preparation which had not been treated with diazomethane, was rechromatographed on silica gel H in the same solvent system. It yielded a single red fluorescent band in the monocarboxylic tetrapyrrole region of the chromatogram (R_F = 0.49), thus indicating that it contained only monocarboxylic tetrapyrroles.

These results indicate as expected that the putative Chlide *b* does indeed possess a free carboxylic group.

Conversion of the putative Chlide b into a Chlide b oxime

Ogawa and Shibata have demonstrated that the formyl group of Chl *b* (Fig. 1c) will react quantitatively with hydroxylamine in 95% methanol to yield an oxime derivative (Fig. 1e) [19]. In this solvent Chl *b* oxime has absorption peaks at 453 nm and 663 nm at 293 K [19]. Since treatment with hydroxylamine had no effect on the absorption properties of Chl *a*, this technique has been used successfully to measure small quantities of Chl *b* in the presence of high concentrations of Chl *a* [4,19,28].

Thus the putative Chlide *b* reported in these studies should react with hydroxylamine to produce an oxime with spectral properties identical to those of standard Chl *b* oxime (Fig. 1e, f). To test this hypothesis, authentic Chl *b* and putative Chlide *b* were treated with hydroxylamine (see Materials and Methods), and the spectral properties in diethyl ether at 77 K of authentic Chl *b* and of the Chl *b* oxime were compared with those of the putative, partially purified Chlide *b* and Chlide *b* oxime. The results of this experiment are depicted in Fig. 5.

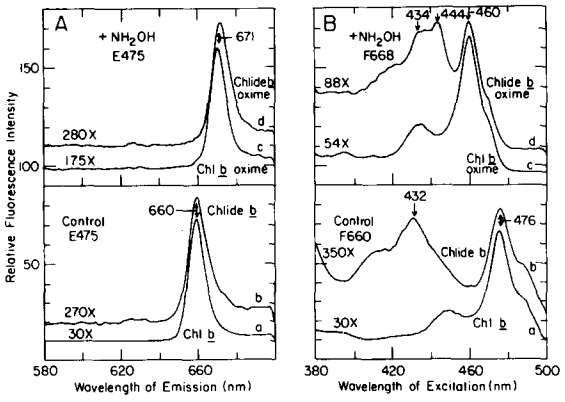

Fig. 5. Fluorescence emission (A) and excitation (B) spectra of standard Chl *b* and of the putative Chlide *b* pool before and after treatment with hydroxylamine. The spectra were recorded in diethyl ether at 77 K. All spectra were recorded on the SLM spectrofluorometer. (a, b) control: samples incubated in 95% methanol for 10 min at room temperature; (c, d) treated: samples incubated in 95% methanol containing 0.288 M NH_2OH at pH 5.8 for 10 min. See Materials and Methods for details. Chlide *b* refers to the partially purified putative Chlide *b* pool; Chl *b* = standard Chl *b*. All other symbols are as in Fig. 2.

The Chl *b* standard (Fig. 5a) and the putative Chlide *b* pool (Fig. 5b) exhibited nearly identical Soret excitation and emission maxima at about 476 nm and 660 nm, respectively. The presence of Chlide *a* in the Chlide *b* pool was indicated by the short-wavelength Chlide *a* Soret excitation maximum at about 432 nm (Fig. 5Bb) [25] which was absent in the purified Chl *b* sample (Fig. 5Ba). However, treatment with hydroxylamine caused fluorescence emission and excitation shifts due to the formation of authentic Chl *b* oxime, and identical shifts were observed in the hydroxylamine-treated putative Chlide *b* pool. Both samples exhibited an 11 nm bathochromic shift in their emission maxima (from 660 to 671 nm) (Fig. 5A) and a 16 nm hypsochromic shift in their Soret excitation maxima (from 476 to 460 nm) (Fig. 5B). The emission spectra reported in Fig. 5A were deliberately elicited by excitation on the long-wavelength tail of the Chl *b*-oxime excitation band (i.e., at 475 nm) in order to detect traces of unreacted Chl(ide) *b* which has a fluorescence excitation maximum at this wavelength [23]. The absence of a Chl(ide) *b* emission shoulder at about 660 nm indicated the complete conversion of Chl *b* into Chl *b* oxime. (cf., Fig. 5Aa, b and Fig. 5Ac, d). The double Soret excitation maxima at 435 and 444 nm which were observed in the putative Chlide *b* pool after reaction with hydroxylamine (Fig, 5Bd) are due to the short-wavelength and long-wavelength Chlide *a* species which are usually observed at 77 K when excitation spectra are recorded at an emission wavelength of 665 and 668 nm [13,25]. This was expected, since Chl(ide) *a* does not react with hydroxylamine [19].

The above results indicated that the putative Chlide *b* pool did indeed possess a formyl group which reacted quantitatively with hydroxylamine to form Chlide *b* oximes which were spectrally identical to Chl *b* oximes.

Possible contribution of chlorophyllase to the generation of the Chlide b pool

Chlorophyllase (chlorophyll chlorophyllidohydrolase, EC 3.1.1.14) catalyzes the hydrolysis of the phytol ester linkage at position 7 of the Chl *a* and *b* macrocycles (Fig. 1) and is known to be active in both aqueous and organic solvents [26]. Since the concentration of Chlide *b* detected in the monocarboxylic tetrapyrrole pool of greening tissue was quite small (see below), it was of interest to know whether it had arisen as a product of Chl *b* hydrolysis in vitro.

In order to minimize chlorophyllase activity, we routinely prechilled all solvents and glassware to 0–4°C. Furthermore, tissues were frozen in liquid N_2 and were rapidly extracted while still in the frozen state. Under these conditions, the tissue was quickly pulverized and the pigment was readily extracted in acetone. The possible involvement of chlorophyllase in the generation of the Chlide *b* pool was, nevertheless, evaluated as described below. A measured amount of authentic [^{14}C]Chl *b* prepared as described in Materials and Methods was added to the ammoniacal acetone in which the cucumber cotyledons were homogenized. After homogenization, the monocarboxylic phorbin pool was extracted into diethyl ether (see Materials and Methods) and an aliquot removed for liquid scintillation analysis. The remaining extract was applied to a thin layer of silica gel and a control chromatogram was prepared with an equivalent aliquot of [^{14}C]Chl *b* plus carrier Chl *a* + *b* and Chlide *a*. The chromatograms were then developed simultaneously in toluene/ethyl acetate/ethanol (8 : 2 : 1, v/v) to separate the Chlide *a* + *b* pools from

TABLE I

ESTIMATION OF THE CONTRIBUTION OF CHLOROPHYLLASE ACTIVITY TO THE Chlide *b* POOL EXTRACTED FROM GREENING CUCUMBER COTYLEDONS

Procedural details are given in the text.

Tissue	Expt.	[^{14}C]Chl *b* added (dpm)	Net [^{14}C]Chlide *b* recovered [a] (dpm)	*A*: Hydrolysis of added [^{14}C]Chl *b* (%)	Ch(ide) *b* content of tissue (nmol/g fresh wt.)		*B*: Percent Chlide *b* content: $\frac{\text{Chlide } b}{\text{Chl } b} \times 100$	Percent chlorophyllase contribution to Chlide b pool $(\frac{A}{B} \times 100)$
					Chl *b*	Chlide *b*		
Etiolated + 5 h continuous illumination	1	145 270	310	0.21	14.46 [b] ± 4.5	0.154 [b] ± 0.043	1.06	13.2
	2	145 260	209	0.14				
	3	150 510	133	0.08				
	Average	–	–	0.14				
4-day-old photoperiodic	1	153 675	154	0.10	228.1 [b] ± 20.5	1.85 [b] ± 0.67	0.81	9.9
	2	456 000	264	0.06				
	Average	–	–	0.08				

[a] dpm Chlid *b* recovered in treatment – dpm Chlide *b* recovered in control.

[b] Average of three determinations ± S.D.

the Chl *a* + *b* pools. The silica gel of the Chlide *a* + *b* bands of the tissue extract and of the control were exhaustively extracted with methanol/acetone (4 : 1, v/v) as described in Materials and Methods. The pooled methanolic acetone extract was transferred to a 20 ml scintillation vial and evaporated to dryness under N_2. Methanolic acetone (0.8 ml) was added to the vials to redissolve the chlorophyllide residue, and 15 ml toluene-based scintillation fluid was also added prior to counting. The results of some representative experiments with both photoperiodically-grown and greening etiolated tissues (Table I) show that the net quantities of label recovered from the Chlide *a* + *b* band of etiolated cotyledons extracted after 5 h of continuous illumination or from the Chlide *a* + *b* band of photoperiodically-grown cotyledons indicated that Chl *b* hydrolysis in vitro may have contributed only about 13.2 and 9.9%, respectively, to the Chlide *b* pool detected in these tissues.

Comparison of the amount of Chlide b to the amounts of other monocarboxylic tetrapyrroles present in greening tissues

In the past we detected several monocarboxylic tetrapyrrole intermediates of chlorophyll biosynthesis in greening tissue which were not detectable by conventional spectrophotometric techniques [10,11, 16], derived the appropriate equations for their assay [15,16,27] and determined their pool sizes. It was deemed desirable, therefore, to extend these observations by determining the pool size of Chlide *b* in greening tissues for comparison with the pool sizes of Mg-protoporphyrin momoester, Pchlide *a*, and Chlide *a* – see Table II. Also reported in Table II are results for photoperiodically-grown cucumber cotyledons. The reported values for Chlide *a* and Chlide *b* were determined spectrofluorometrically and have been corrected for contamination by small amounts of Chl *a* and *b* that were not extracted by the hexane treatment, as described in Materials and Methods. It is apparent that for each type of tissue the Chlide *b* concentration was generally in the same range as that of the other monocarboxylic tetrapyrrole pools. The concentration of the Mg-protoporphyrin monoester pool of the photoperiodic tissue was much lower, however, than those of the Pchlide *a*, Chlide *a* or Chlide *b* pools in that tissue.

Discussion

To our knowledge, this is the first report of the detection and assay of a Chlide *b* in greening plant tissues, under conditions where its formation by chlorophyllase would have been severely limited (Table I). The occurrence of Chlide *b* in greening tissues has probably been overlooked in the past for two reasons: (a) the common use of low resolution spectrophotometric techniques for monitoring the Chl(ide) pools of green plants, (b) the direct determination of pigment concentrations in crude extracts such as 80% acetone extracts, which does not permit the distinction between fully-esterified and monocarboxylic tetrapyrroles [4,28,29].

The assignment of a Chlide *b* structure to this spectroscopic component of the monocarboxylic tetrapyrrole pools of greening cucumber cotyledons is based not only on the formation of chemical derivatives but also on solubility, spectrofluorometric and

TABLE II

CONCENTRATIONS OF MONOCARBOXYLIC Mg-PORPHYRINS AND PHORBINS IN GREENING CUCUMBER COTYLEDONS

Details are described in the text. Values are pigment concn. (pmol/g fresh wt.) and represent the mean of three determinations ± S.D. Chlide concentrations were corrected for chlorophyll contamination (see Materials and Methods). MPE, Mg-protoporphyrin monoester.

Tissue	Pigment concentration (pmol/g fresh weight)			
	Chlide *a*	Chlide *b*	Pchlide	MPE
4-day-old photoperiodically grown	3 690 ± 640	1 850 ± 670	3 030 ± 250	77 ± 7
4-day-old etiolated + 5 h continuous illumination	193 ± 81	154 ± 25	456 ± 55	236 ± 20

chromatographic evidence. Putative Chlide *b* exhibited fluorescence emission and excitation properties that were nearly identical to those of standard Chl *b* (Figs. 3 and 4) but differed from Chl *b* by its solubility and chromatographic properties. These properties indicated that the putative Chlide *b* was more polar than Chl *b* and this was consistent with the absence of a phytol (or other long-chain alcohol) ester linkage. It is indeed well known that esterification of tetrapyrroles affects their polarity without affecting their optical spectroscopic properties. This is best exemplified by the nearly identical spectrofluorometric properties of monovinyl Pchlide *a* [9] and monovinyl Pchlide ester *a* [24] and those of Chlide *a* and Chl *a* [13].

This assignment of Chlide *b* was further confirmed by esterification with diazomethane which converted the putative Chlide *b* into a compound that retained its original spectrofluorometric properties but exhibited the chromatographic mobility of a fully esterified tetrapyrrole, and also by treatment with hydroxylamine which confirmed the presence of a formyl group and formed a compound that exhibited identical spectrofluorometric properties to those of standard Chl *b*-oxime (Fig. 5). The small amounts of this compound that can be presently prepared preclude at this stage its further structural characterization by NMR, infrared and mass spectroscopy.

Chlide *b* may arise in any of three ways: catabolically (a) by in vitro or (b) in vivo hydrolysis of Chl *b* with or without the aid of chlorophyllase; or (c) it can be formed anobolically from an unknown precursor. The results presented in Table I suggest that Chlide *b* does not arise from in vitro chlorophyllase-catalyzed hydrolysis of Chl *b*. Furthermore, the fact that the size of the Chlide *b* pool of the tissues examined in this work was similar to the pool sizes of other well-known intermediates of the chlorophyll biosynthetic pathway suggests that this phorbin may represent a metabolically important endogenous pool in greening tissues. Finally, although these studies do not preclude the possibility that at least part of the detected Chlide *b* pool might have been derived catabolically by the in vivo hydrolysis of Chl *b*, preliminary results (in preparation) suggest that at least part of the Chlide *b* pool of greening tissues may be formed from a rapidly metabolized pool of endogenous divinyl Chlide *a* [13,30]. This in turn is fully compatible with the recently reported conversion of exogenous Chlide *b* into Chl *b* in vitro [7].

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