The chemical identification of chlorophyllide (E458F674) (Belanger, F. C., and Rebeiz, C. A. (1980) Plant Sci. Lett. 18, 343-350) has been confirmed by chemical derivatization coupled to spectrofluorometric, spectrophotometric, and chromatographic analysis. Chlorophyllide (E458F674) and its demetallated analog were converted by catalytic hydrogenation into mesochlorophyllide a and mesoporphorbid a. Furthermore, methyl chlorophyllide (E458F674) was converted by partial hydrogenation into a mixture of monovinyl chlorophyllide a isomers and the latter into mesochlorophyllide a by further hydrogenation. On the other hand, chemical oxidation of methyl chlorophyllide (E458F674) converted it into methyl divinyl protochlorophyllide. Chlorophyllide (E458F674) was detected in several plant species and is proposed to be an important intermediate of the chlorophyll a biosynthetic pathway.

It has been recently proposed that the chlorophyll a pool of green plants is formed via four parallel biosynthetic branches, namely fully esterified and acidic branches, each of which is further split into monovinyl and divinyl sub-branches (1). The two fully esterified branches convert monovinyl protochlorophyllide ester (E437F624) and divinyl protochlorophyllide ester (E443F624) into monovinyl chlorophyll a (E447F674) and divinyl chlorophyll a (E458F674), respectively. The two acidic branches convert monovinyl protochlorophyllide (E437F625) and divinyl protochlorophyllide (E443F625) into monovinyl chlorophyllide a (E447F674) and divinyl chlorophyllide a (E458F674) (Fig. 1). In this context, E and F refer to the Soret excitation maximum and fluorescence emission maximum, respectively, in ether at 77 K. The monovinyl and divinyl chlorophyllide a are then converted into other chlorophyllide species (1).

Although the chemical structure of monovinyl chlorophyllide a has been well established (for a review, see Ref 4), the assignment of a divinyl nature to chlorophyllide a (E458F674) is still tentative and rests on the following experimental evidence. (a) The demonstration that the protochlorophyllide(ide) pool of plants is made up of monovinyl and divinyl components (3, 4). (b) The demonstration that the monovinyl/divinyl protochlorophyllide(ide) pool of etiolated plants is converted by a brief (47 ms) light treatment into monovinyl chlorophyllide a (E447F674) and the putative divinyl chlorophyllide(ide) a (E458F674) (5). (c) The demonstration that by successive 2.5-ms light treatments followed by 60-min dark incubations of etiolated tissues, the latter are induced to accumulate only divinyl protochlorophyllide (E443F625), which is photoconvertible by an additional 2.5-ms light pulse, at room temperature or at -10°C, into putative divinyl chlorophyllide a (E458F674) (6).

In this work, the presence of two peripheral vinyl groups in conjugation with the phorbin macrocycle of chlorophyllide a (E458F674) is ascertained by chemical derivatization.

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. Barley (Hordeum vulgare, var. Beacon Spring) was donated by the Department of Agronomy, University of Illinois at Urbana. Hybrid sweet corn (Zea mays L.) was purchased from Rogers Brothers Co., Idaho Falls, ID.

Etiolated seedlings were grown in moist vermiculite at 28°C in total darkness. All manipulations of etiolated plant material were performed under a green safelight. For growing photoperiodic tissue, the seeds were germinated under a light (14 h) and dark (10 h) photoperiodic regime at 28°C as previously described (7). Preparation of a Monovinyl-enriched Chlorophyll a Standard—Three g of 5-10-day-old photoperiodically grown cucumber cotyledons were homogenized in 20 ml of acetone. 0.1 N NH₄OH (9:1, v/v) at 0-4°C for 2 min in a Sorvall Omni-Mixer. The 80% acetone extract was centrifuged at 39,000 × g for 10 min. The chlorophylls were transferrred to hexane by extraction first with an equal volume of hexane, then with a ⅓ volume of hexane.

Chlorophyll a was separated from chlorophyll b by chromatography of the hexane extract on thin layers of cellulose MN 300 developed in isopropanol (60-80°C): n-propyl alcohol (5:3:1, v/v) at room temperature (5). Chlorophyll a (Rf = 0.34) was eluted in peroxide-free ether, dried under N₂ gas, and then was redissolved in ether for spectroscopic measurements. The absorption spectrum in ether at room temperature of this monovinyl chlorophyll a corresponded, within an instrumental accuracy of ±1 nm, to that reported by Strain et al. (9). It should be pointed out, however, that although the chlorophyll a thus prepared consisted mainly of monovinyl chlorophyll a, it still contained small amounts (about 10% or less) of a short wavelength chlorophyll a (E436F664) component of unknown structure that exhibited, at 77 K in ether, a Soret excitation maximum at 436 nm when the excitation spectrum was recorded at an emission wavelength of 664 nm (10). It is likely that pentacoordinated monovinyl chlorophyll a also contributes to the excitation maximum observed in these samples at 436 nm (11). For the purposes of this work, this slight
contamination of the monovinyl chlorophyll a preparation is, however, inconsequential.

Preparation of Mesoclorophyll a—Mesoclorophyll a was prepared by hydrogenation of chlorophyll a. Hydrogenation was carried out for 45 min to 1 h as described under catalytic hydrogenation. The visible absorption spectrum of the reaction product, in 1% pyridine in ether, was identical with that reported for methyl monovinyl chlorophyllide a by Fischer and Spielberger (12) with peaks in the visible region at 649, 606, 551, and 528 nm. The Soret maximum was at 425 nm.

Preparation of Mesopheophytin a—Mesopheophytin a was prepared by hydrogenation of phophénin a. Phophénin a was prepared by demetallation of chlorophyllide a as described under demetallation. Hydrogenation was performed for 45 min as described under catalytic hydrogenation. The visible absorption spectrum of the reaction product in dioxane was nearly identical with that reported for meso-methylphosphorohphoride a by Stern and Molvig (13) with peaks in the visible region at 666, 600, 551, and 530 nm. The Soret maximum was at 407 nm.

Preparation of Monovinyl and Divinyl Protochlorophyllide—The extraction and purification of monovinyl and divinyl protochlorophyllide was essentially as described previously by Belanger and Rebeiz (9). The monovinyl derivative of divinyl protochlorophyllide, extracted from Rhodopseudomonas spheroides, has been reported earlier by Jones (14).

Five-day-old etiolated cucumber cotyledons were excised and the hypocotyl hooks were removed. Photoconversion of the protochlorophyllides into chlorophyllides was achieved by exposing the cotyledons, at room temperature, to a 2.5-ms pulse of white actine light. The light flash was generated by a Sunpack Model Auto 611 photographic flash unit (Berkeley Marketing Co., Woodside, NY) powered by two 1600-W bulbs. The excised tissue in 9-cm Petri dishes and a mirror was placed 4 cm below the sample in order to reflect the incident light back onto the tissue. The protochlorophyllide pool was regenerated by returning the tissue to darkness for 60 min. This light/dark treatment was repeated two more times. At the end of the third dark period, the tissue contained only almost exclusively monovinyl divinyl protochlorophyllide (6). It was transferred to a tea strainer and was subjected to a fourth 2.5-ms light pulse which converted the divinyl protochlorophyllide a into putative divinyl chlorophyllide a (E458F674) (6). After this 4th light treatment, the tissue was immediately frozen by immersing the strainer in liquid Nz.

Extraction of Putative Divinyl Chlorophyllide a (E458F674) accumulation—This was done essentially as described by Duggan and Rebeiz (6). Four-day-old etiolated cucumber cotyledons containing a substantial pool of monovinyl + divinyl protochlorophyllide (5) were excited and the hypocotyl hooks were removed. Photoconversion of the protochlorophyllides into chlorophyllides was achieved by exposing the cotyledons, at room temperature, to a 2.5-ms pulse of white actine light. The light flash was generated by a Sunpack Model Auto 611 photographic flash unit (Berkeley Marketing Co., Woodside, NY) powered by two 1600-W bulbs. The excised tissue in 9-cm Petri dishes and a mirror was placed 4 cm below the sample in order to reflect the incident light back onto the tissue. The protochlorophyllide pool was regenerated by returning the tissue to darkness for 60 min. This light/dark treatment was repeated two more times. At the end of the third dark period, the tissue contained only almost exclusively monovinyl divinyl protochlorophyllide (6). It was transferred to a tea strainer and was subjected to a fourth 2.5-ms light pulse which converted the divinyl protochlorophyllide a into putative divinyl chlorophyllide a (E458F674) (6). After this 4th light treatment, the tissue was immediately frozen by immersing the strainer in liquid Nz.

Extraction of Putative Divinyl Chlorophyllide a (E458F674)—While still frozen, 3 g of cucumber cotyledons that had been induced to accumulate chlorophyllide a (E458F674) (see above) were homogenized in 20 ml of acetone, 0.1% NH₄OH (0.1, v/v) at 0-4 °C. Homogenization was accomplished either by grinding the tissue in a mortar with a small amount of white sand or by homogenizing the tissue for 1.5 min in a Waring Blender equipped with a 350-ml metal cup. After centrifugation at 38,000 × g for 10 min, the fully esterified pigments were removed by extracting the 80% acetone extract with hexane, first with an equal volume of hexane followed by ½ that volume of hexane (15). The chlorophylls and the unpolytransformed protochlorophyllides remained in the hexane-extracted acetone fraction and were transferred to ether as follows. To the hexane-extracted acetone fraction was added ½ volume of peroxide-free dichloromethane and the organic layer was evaporated to ½ volume of 0.5 M KClO₃. The ether epiphase was collected and was concentrated under Nz gas until the solution became cloudy and the pigments began to precipitate. This was re-extracted with 1 to 2 volumes of ether. The emulsion was broken by centrifugation and ether phase was removed. The aqueous phase was re-extracted as above until the ether extract was no longer fluorescent. The combined ether extracts containing the pigments were dried under Nz and were used for further chromatographic purification. Removal of water from the pigment extract, as just described, greatly improved subsequent chromatographic separations.

Purification of the Chlorophyllide Pool—Chlorophyllide (E458F674), induced and extracted as described above, was always accompanied by some fully esterified tetrapyroles which had not been transferred to hexane, by some xanthophylls, and by small amounts of divinyl protochlorophyllide that had not been photoconverted into chlorophyllide a by the fourth light pulse. The two methods described below were used to partially purify the chlorophyllide (E458F674), prior to chemical derivatization.

In one method, the concentrated ether extract, prepared as described above, was methylated with diazomethane. The latter was prepared from N-nitrosomethyl urea (16). The methylated pigments were chromatographed on thin layers of Silica Gel H, developed in toluene-ethyl acetate-ethanol (8:2:2, v/v/v) at 4 °C. Protochlorophyllide ester and chlorophyll ran with Rₚ values of about 0.90 and 0.86, respectively, while methyl monovinyl chlorophyllide (E458F674) migrated with Rₚ (0.80) ran behind methyl divinyl protochlorophyllide (Rₚ = 0.75) and was eluted in ether. Methyl chlorophyllide a (E458F674), thus prepared, was usually contaminated with very small amounts of methyl divinyl protochlorophyllide. The latter did not interfere, however, with recording the chlorophyllide a (E458F674) spectra because of the small amounts of protochlorophyllide contamination and because of the insignificant contribution of its vibrational band at 673-675 nm to the insignificant emission band of chlorophyllide a (E458F674) (6). When highly pure preparations of chlorophyllide a (E458F674) were desired, this last chromatographic step was repeated one more time.

Alternatively, the concentrated ether extract containing chlorophyllide (E458F674) as well as other pigments (see above) was directly chromatographed on thin layers of Silica Gel H which were developed in toluene-ethyl acetate-ethanol (8:2:2, v/v/v) at 4 °C. The chlorophyllide (E458F674) and the divinyl protochlorophyllide pools were migrated together, away from other pigments, with an average Rₚ of about 0.44 and were eluted in methanol-acetone (4:1, v/v) (17). The methanol-acetone eluate was mixed with an equal volume of ether and this mixture was passed through 4 to 5 times its volume of 0.37 M potassium phosphate buffer, pH 7.0. The ether epiphase containing the chlorophyllide + protochlorophyllide pigments was removed and dried under Nz gas. During the drying process, an aqueous hypophase formed which was removed prior to further evaporation of the ether to dryness. The spectra of the methylated and free acid forms of chlorophyllide (E458F674) were essentially identical.

Catalytic Hydrogenation—Catalytic hydrogenation of vinyl groups was carried out through a 2.5-ms light pulse. The pigments in ether at 0-4 °C in the presence of 1-2 mg of catalyst made up of asbestos containing 10% palladium. The extent of the reaction was monitored spec trofluorometrically at 15-min intervals. Although the time required for complete hydrogenation varied from sample to sample, in most cases it took about 1.26 h for the reaction to proceed to completion. At the end of the reaction, the catalyst was removed by centrifugation.

Demetallation—Metallated pigments were dissolved in 0.1 ml of ether which was then mixed with 2 ml of 2 N HCl. Two ml of fresh ether were added immediately and the acicodylic hypophase layer was removed with solid NaHCO₃. The demetallated tetrapyroles passed into the ether epiphase which was collected and washed once with an equal volume of H₂O. This ether solution was dried under Nz gas and the pigment residue was redissolved in ether for spectrosopic determinations.

Conversion of Chlorophyllides into Protochlorophyllides by Chemical Oxidation—Conversion of the single bond at position 7-8 of chlorophyllide into a double bond was achieved by treatment with 2,3-dichloro-5,6-dicyanobenzoquinone, a known oxidant of chlorines (18), in ether at room temperature. The reaction of chlorophyllide a or methyl chlorophyllide a (E458F674) and of 100 nmol of 2,3-dichloro-5,6-dicyanobenzoquinone (Eastman Chemical Co., practical grade) in a total ether volume of 0.5 to 1.0 ml. The reaction was initiated by addition of the 2,3-dichloro-5,6-dicyanobenzoquinone and was terminated by freezing in liquid Nz. Under the above conditions, the reaction proceeded nearly to completion without the side reactions and substrate degradation...
which were observed when higher temperatures and longer reaction periods were employed. Furthermore, conducting the oxidation in diethyl ether made it possible to monitor the reaction by direct spectrophotometric examination of the reaction mixture without the heavy losses or artifacts which may be associated with the further purification of the reaction products. Although 2,3-dichloro-5,6-dicyanobenzoquinone is fluorescent and exhibits emission and excitation maxima at 422 and 363 nm, respectively, at 77 K in ether, its fluorescence was too far removed from that of chlorophyllide \( (E458F674) \) or of divinyl protochlorophyllide \( (E443F625) \) to interfere with their spectrophotometric properties.

Spectrophotometry—Absorption spectra were recorded with an Aminaco dual wavelength spectrophotometer model DW-2, operated in the split beam mode, at a slit width of 2-3 nm. Under these conditions, the spectral accuracy of the reported maxima is about ±1 nm.

Spectrophotometry—Fluorescence spectra were recorded on a fully corrected, photon counting spectrophotometer Model SLM 8000 DS, equipped with two red-sensitive, extended S20 photomultipliers (EMI 9658) and interfaced with a Hewlett-Packard microcomputer system Model 9825 S. Pigment solutions were monitored either at room temperature in cylindrical microcells 3 mm in diameter or were transferred to cylindrical sample tubes and were monitored at 77 K as described elsewhere (19). Emission spectra were recorded at an excitation band width of 4 nm and an emission band width of 2 nm. Excitation spectra were recorded at an emission band width of 4 nm and an excitation band width of 2 nm. The photon count was integrated for either 0.5 or 1 s at each 1 nm increment. Under these conditions, the spectral accuracy of the reported maxima is about ±1 nm.

The spectra reported in Fig. 6 were recorded on a Perkin-Elmer spectrophotometer, Model MPF-3, equipped with a corrected spectra accessory (17).

RESULTS

Comparison of the Electronic Spectroscopic Properties of Monovinyl Chlorophyll \( a \) and Chlorophyllide \( (E458F674) \)—It was reported earlier that in comparison to monovinyl chlorophyllide \( a \), chlorophyllide \( a \) \( (E458F674) \) exhibited red-shifted Soret excitation/absorption properties at 77 K and at room temperature (6). It was therefore desirable to reconfirm and to extend these earlier observations before undertaking more detailed investigations of the chemical structure of chlorophyllide \( a \) \( (E458F674) \).

Since esterification of the carboxyl groups at positions 6 and 7 of the macrocycle has been shown to have no significant effect on the electronic absorption and fluorescence properties of tetrapyroles (3, 4, 9, 20, 21), the optical spectroscopic properties of methyl chlorophyllide \( (E458F674) \) were compared to those of the more readily available monovinyl chlorophyll \( a \) instead of monovinyl chlorophyllide \( a \). As is well known, monovinyl chlorophyll \( a \) is esterified with phytol at position 7 of the macrocycle, while chlorophyllide \( a \) has a free propionic acid residue at that same position (Fig. 1, Ba and Bb).

The absorption spectra of monovinyl chlorophyll \( a \) and of methyl chlorophyllide \( a \) \( (E458F674) \), in ether at room temperature, are compared in Fig. 2. Both compounds exhibited identical red absorption maxima at 659-660 nm (Fig. 2). However, methyl chlorophyllide \( (E458F674) \) exhibited a Soret absorption maximum at 435 nm, which was red-shifted by 6 nm in comparison to that of monovinyl chlorophyll \( a \) which was observed at 429 nm (Fig. 2). Furthermore, chlorophyllide \( a \) \( (E458F674) \) exhibited a higher Soret/Red \( (S/R) \) absorption ratio \( (S/R = 1.50) \) than monovinyl chlorophyll \( a \) \( (S/R = 1.33) \). As was previously reported (6), the fluorescence properties of monovinyl chlorophyllide \( a \) \( (E458F674) \) differed from one another at room temperature and at 77 K. For example, while monovinyl chlorophyll \( a \) and methyl chlorophyllide \( (E458F674) \) exhibited nearly identical emission maxima at 666 and 665 nm, respectively, in ether at room temperature, they exhibited different Soret excitation maxima: at 430 nm for monovinyl chlorophyll \( a \) and at 436 nm for methyl chlorophyllide \( (E458F674) \). At 77 K, the fluorescence emission and excitation maxima of both monovinyl chlorophyll \( a \) and methyl chlorophyllide \( (E458F674) \) underwent a red-shift of 9 nm (emission) and 17 and 22 nm (Soret excitation), respectively. This was probably due to the formation of hexacoordinated complexes with the solvent molecules at that low temperature (11, 22). In this case too, monovinyl chlorophyll \( a \) and chlorophyllide \( (E458F674) \) exhibited identical emission maxima at 674-675 nm, but differed in the position of their Soret excitation maxima which were found at 447 nm for monovinyl chlorophyll \( a \) and at 458 nm for chlorophyllide \( a \) \( (E458F674) \).

Altogether the above results reconfirmed and extended the earlier observations of Duggan and Rebeiz (6) and prompted us to further investigate the possible structural differences between chlorophyllide \( (E458F674) \) and monovinyl chlorophyll \( a \).

Conversion of Chlorophyllide \( (E458F674) \) into Mesochlorophyllide \( a \) by Catalytic Hydrogenation—It is well documented that peripheral vinyl group substituents in tetrapyroles lead to red-shifted electronic transition spectra (23). For example, by substituting a vinyl group for an ethyl group in Mg-mesoporphyrin, a 6 nm red-shift is observed in the position of the Soret absorption/excitation maximum (24). By substituting a second vinyl group for the remaining ethyl group, an additional Soret absorption/excitation red-shift of 7 nm is observed (24). Also, divinyl protochlorophyllide, with two vinyl groups, has a Soret excitation peak which is 6 nm red-shifted in comparison to monovinyl protochlorophyllide which has only one vinyl group (3). Since chlorophyllide...
The latter were numbered alphabetically. Spectra which were recorded of the spectra. In order to facilitate the location of individual spectra, wavelengths were assigned the same letter, but were distinguished by a prime symbol. Arrows point to wavelengths of interest. Hydrog. after catalytic hydrogenation.

(E458F674) exhibited a red-shifted Soret absorption/excitation maximum in comparison to monovinyl chlorophyll \( a \) (Fig. 2), and since it was shown to be formed from divinyl protochlorophyllide by a 2.5-ns light treatment at room as well as at subzero temperatures (6), it was logical to propose that chlorophyllide \( a \) (E458F674) may be a divinyl chlorophyllide \( a \) (6). It was therefore conjectured that if the above structural assignment is correct, it should be possible to convert chlorophyllide (E458F674) into 2,4-diethyl (i.e. meso) chlorophyllide \( a \) by catalytic hydrogenation. The successful completion of this reaction can then be assessed by comparing the spectral properties of hydrogenated chlorophyllide (E458F674) to those of authentic mesochlorophyll \( a \).

The room temperature absorption and fluorescence properties of chlorophyllide (E458F674), before and after catalytic hydrogenation, are compared to those of authentic mesochlorophyll \( a \) and to those of other chlorophyll \( a \) derivatives in Tables I and II. After catalytic hydrogenation, chlorophyllide (E458F674) was converted into a compound which, within our instrumental accuracy of \( \pm 1 \) nm, exhibited the same electronic spectroscopic properties as those of authentic mesochlorophyll \( a \). They both exhibited absorption maxima at 424 and 648 nm and Soret excitation and emission maxima at 424-425 and 652-653 nm, respectively (Tables I and II). These values corresponded to hydrogenation-induced emission and Soret absorption/excitation blue-shifts of 12 and 11-12 nm, respectively. The magnitude of these shifts were similar to those observed after catalytic hydrogenation of 2,4-divinyl Proto, 2,4-divinyl-Mg-Proto, and 2,4-divinyl protochlorophyllide (23, 24).

The fluorescence properties at 77 K of chlorophyllide (E458F674) after catalytic hydrogenation are compared to those of authentic mesochlorophyll \( a \) in Fig. 3. Both standard mesochlorophyll \( a \) and hydrogenated chlorophyllide (E458F674) exhibited identical spectral profiles with an emission maximum at 659-660 nm and a Soret excitation maximum at 438-439 nm. Small amounts of unreacted pigment became apparent when the sample was excited at 450 nm (data not shown). The small emission peak at 616 nm (Fig. 3A, b) is caused by small amounts of mesoprotoporphyrin (E458F674). Altogether the above results suggested rather strongly that chlorophyllide (E458F674) is a divinyl chlorophyllide \( a \).

Conversion of Demetallated Chlorophyllide \( a \) (E458F674) into Mesoporphyrin \( a \)---Duggan and Rebeiz (6) have reported that demetallation of putative chlorophyllide (E458F674) resulted in the formation of a pheophorbide-type tetrapyrrole that exhibited fluorescence properties in ether, at 77 K, which differed from standard monovinyl pheophorbide \( a \) only by its Soret excitation maximum which was red-shifted by about 9 nm in comparison to the Soret excitation maximum of standard monovinyl pheophorbide \( a \). This indicated that: (a) the spectroscopic differences between chlorophyllide \( a \) (E458F674) and monovinyl chlorophyll \( a \) were essentially preserved upon removal of the central Mg atom and (b) the Soret excitation red-shift (9 nm) between the demetallated chlorophyllide (E458F674) and standard monovinyl pheophorbide \( a \) suggested that their spectroscopic differences may be due to an additional electron-withdrawing peripheral group which is present in demetallated chlorophyllide (E458F674), but which is absent in authentic monovinyl pheophorbide \( a \).

It was therefore conjectured that if that electron-withdrawing group is a vinyl group, it should be possible to convert deme-

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1 F. C. Belanger and C. A. Rebeiz, manuscript in preparation.
tallated chlorophyllide (E458F674) into blue-shifted meso-
phosphorbid a by catalytic hydrogenation.

The absorption and fluorescence properties in ether at room
temperature of demetallated chlorophyllide a (E458F674) be-
fore and after catalytic hydrogenation are compared with
those of authentic monovinyl pheophytin a and mesopheo-
phytin a in Tables I and II. It is obvious that although
demetallated chlorophyllide a (E458F674) and monovinyl
pheophytin a exhibited similar red emission maxima at 671 to
672 nm in ether, at room temperature, the Soret absorption/
excitation maximum of demetallated chlorophyllide a
(E458F674) was red-shifted by 8 nm in comparison to that of
monovinyl pheophytin a (Tables I and II). Catalytic hy-
genation of demetallated chlorophyllide (E458F674) con-
verted it into a compound that exhibited the spectral prop-
erties of authentic mesopheophytin a in ether, at room tem-
erature, and caused a blue-shift of about 9, 12, and 11 nm,
respectively, in the red absorption, red emission, and Soret
excitation maxima in ether at room temperature (Tables I and
II).

The fluorescence properties in ether at 77 K of demetallated
chlorophyllide (E458F674) before and after catalytic hydrogenation are compared to those of standard monovinyl pheo-
phytin a and mesophytin a in Fig. 4. As reported else-
where by Duggan and Rebeiz (6), demetallated chlorophyllide
(E458F674) differed from standard pheophytin a only by its
Soret excitation maximum which was observed at 424 nm in
ether at 77 K and was red-shifted by 10 nm in comparison to
the Soret of standard monovinyl pheophytin a (Fig. 4, a and
b). After catalytic hydrogenation, demetallated chlorophyllide
a (E458F674) was converted into a compound that was iden-
tical in its spectral properties at 77 K, with authentic meso-
phophytin a (Fig. 4, c and d). It exhibited blue-shifted
emission and Soret excitation maxima at 653 and 408 nm,
respectively. The shoulder observed at 666 nm in the fluores-
cence emission profiles of standard mesophytin a and in
the demetallated, hydrogenated chlorophyllide a (E458F674)
are those of the unreacted demetallated bases (Fig. 4A, c and
d).

Altogether, the above results suggested very strongly that
demetallated chlorophyllide a (E458F674) differs from mon-
ovinyl pheophytin a by the presence of an additional periph-
eral vinyl group.

Conversion of Chlorophyllide (E458F674) into Monovinyl
Chlorophyllide a and Conversion of the Latter into Meso-
chlorophyllide a—If the assignment of a divinyl structure to
chlorophyllide (E458F674) is correct, it should be possible to
convert it by partial hydrogenation into a mixture of mono-
vinyl chlorophyllide a isomers, namely 2-vinyl, 4-ethyl chlo-
rophyllide a (i.e. conventional monovinyl chlorophyllide a)
and its 2-ethyl, 4-vinyl isomer. Due to the asymmetry of the
chlorophyllide macrocycle, these two isomers may be expected
not to exhibit different spectroscopic properties, as was recently
found for various isomeric diacetyleuroporphyrins and di-
formyldeuteroporphyrins by Clezy and Fookes (23, 26). Simi-
larly, it has been proposed (24) that the two N-H tautomeric
forms of monovinyl protoporphyrin have different fluores-
cence characteristics. Furthermore, it should be possible to
convert the two putative isomers, by further hydrogenation,
to mesochlorophyll a.

In order to test the above hypothesis, chlorophyllide
(E458F674) was purified by chromatography on thin layers of
silica and then was methylated with diazomethane. The
pigment was then subjected to partial catalytic hydrogenation
until there were about equal amounts of the fully reacted
meso form and unreacted methyl chlorophyllide (E458F674);
this was achieved in about 1 h and 25 min. The reaction
products were separated by chromatography on thin layers of
cellulose, developed in ligoine:acetone:n-propyl alcohol
(90:10:1, v/v/v) (27). Three main fluorescent bands were ob-
served; they were eluted in ether, dried under N2, and then
were redissolved in ether for spectrofluorometric determina-
tions at 77 K.

The fastest moving band (band 1) (Rf = 0.46) exhibited
fluorescence properties identical with those of authentic meso-
chlorophyll a (E439F659). The slowest moving band (band 3)
(Rf = 0.37) consisted of unreacted methyl chlorophyllide
(E458F674). The middle band (band 2) (Rf = 0.41) exhibited
split emission maxima at 675 and 662 nm (Fig. 5A, 5). How-
ever, both emissions exhibited identical monovinyl chlo-
rophyll a Soret excitation maxima at 447-448 nm (Fig. 5B, b and
b') which suggested that band 2 (Rf = 0.41) consisted of
putative methyl monovinyl chlorophyllide a (E447F674) and
of an unknown reaction product that will be referred to as
compound (E447F662). Repeated chromatography on cellu-
lose did not separate the two fluorescent species indicating that
they were very similar in structure. On the other hand, room
temperature fluorescence spectra of band 2 also exhibited a
split emission at 655 and 667 nm and a single Soret excitation
maximum at 491 nm which suggested that the spectral het-
erogeneity of band 2 was not attributable to a low tempera-
ture artifact.

In order to determine whether compound (E447F662) could
be a 2-ethyl, 4-vinyl methyl chlorophyllide a isomer, band 2
was subjected to further catalytic hydrogenation for 30 min.
Following this reduction, the spectral heterogeneity of band
2 disappeared and it was converted into a single product that
exhibited the spectral properties of mesochlorophyll a

![Fig. 4. Comparison of the fluorescence emission (A) and excitation (B) spectra in ether at 77 K of authentic pheophytin a and mesopheophytin a with those of demetallated chlorophyllide (Chlde) (E458F674), before and after catalytic hydrogenation. Hydrog, after catalytic hydrogenation; Demet, after demetallation; Pheo, pheophytin. All other symbols are as in Fig. 3.](http://www.jbc.org/Downloaded from)
Conversion of Methyl Chlorophyllide a (E458F674) into Methyl Divinyl Protochlorophyllide by Chemical Oxidation—Chlorins such as chlorophyll(ides) differ from protochlorophyll(ides) by the presence of a single covalent bond instead of a double bond at the 7-8 position of the macrocycle (Fig. 1 Aa, and Bb). The conversion of the single bond of chlorophyll derivatives into a double bond by chemical oxidation with 2,3-dichloro-5,6-dicyanobenzoquinone has already been reported (18). It was therefore conjectured that if chlorophyllide a (E458F674) is indeed a divinyl chlorophyllide a, as suggested by the results so far obtained, the oxidation of methyl chlorophyllide a (E458F674) with 2,3-dichloro-5,6-dicyanobenzoquinone should convert it into methyl divinyl protochlorophyllide. Conversely, a similar oxidation of standard monovinyl chlorophyll a should yield monovinyl protochlorophyllide-7-phytyl ester. The absorption and fluorescence properties of divinyl and monovinyl protochlorophyll(ides) have been well described (3, 28, 29) and the outcome of the 2,3-dichloro-5,6-dicyanobenzoquinone oxidation of chlorophyllide (E458F674) and of authentic monovinyl chlorophyll a can therefore be readily monitored by comparing the spectra of the oxidation products with the known spectra of the monovinyl and divinyl protochlorophyllides.

Oxidation of methyl chlorophyllide a (E458F674) with 2,3-dichloro-5,6-dicyanobenzoquinone converted it into a compound that exhibited the same spectral properties as authentic divinyl protochlorophyllide (Fig. 6, e and f). Following the oxidation, the fluorescence spectrum of chlorophyllide a (E458F674), with an emission maximum of 674 nm, changed...
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from that of a chlorophyllide spectrum to that of a protochlorophyllide spectrum having an emission maximum at 624 nm (Fig. 6A, d and e). On the other hand, the Soret excitation profile changed from that of a chlorophyllide with a single excitation maximum at 458 nm into a divinyl protochlorophyllide spectrum with a split Soret having maxima at 443 nm and 451 nm (Fig. 6B, d and e). In the past, the Soret excitation maximum at 451 nm, which was invariably observed in purified divinyl protochlorophyllide preparations, was attributed to a distinct protochlorophyllide spectral species (3). It now appears (6) that the doublet at 443 and 451 nm which is observed in the Soret excitation spectra of pure divinyl protochlorophyllide preparations most probably represents the well resolved By (O−O), Bx (O−O) splitting of the divinyl protochlorophyllide blue electronic transition as suggested by Houssier and Sauer (29) and may therefore be considered collectively as a marker of the divinyl protochlorophyllide pool. In this context, Bx – By splitting refers to the removal of the degeneracy of the X and Y Soret transition energy levels, as would be expected for an asymmetric porphyrin such as protochlorophyllide.

Likewise, oxidation of standard monovinyl chlorophyll a converted it into a compound with the spectral properties of standard monovinyl protochlorophyllide (Fig. 6, b and c). In this case, too, the split Soret observed in the purified monovinyl protochlorophyllide profile at 437 and 443 is now attributed to the well resolved By (O−O), Bx (O−O) splitting of the blue electronic transition of monovinyl protochlorophyllide as suggested by Houssier and Sauer (29).

Altogether, the above results constituted a reasonable data base for proposing that chlorophyllide a (E458F674) is a divinyl chlorophyllide a.

Accumulation of Divinyl Chlorophyllide a (E458F674) in Other Plant Species—In order to determine if the induction of divinyl chlorophyllide a (E458F674) accumulation by intermittent dark-light treatments is a general phenomenon in higher plants, the formation of this pool in some representative dicotyledonous and monocotyledonous plant species was examined. As reported by Duggan and Rebeiz (6), the protochlorophyllide pool regenerated in the dark, after three 2.5 ms light, 60-min dark treatments, consisted almost exclusively of divinyl protochlorophyllide. This divinyl protochlorophyllide could then be converted by a fourth 2.5-ms light flash into chlorophyllide a (E458F674) which was reasonably identified in this work with divinyl chlorophyllide a. Thus, in order to investigate the formation of divinyl chlorophyllide a in other plant species, etiolated bean leaves (a dicotyledonous species) and etiolated corn and barley leaves (monocotyledonous species) were subjected to 3 light-dark treatments, then the acidic phorbin pool was extracted and examined before and after a fourth 2.5-ms phototransforming light flash.

Etiolated bean leaves behaved exactly like etiolated cucumber cotyledons. After three light-dark cycles, the regenerated protochlorophyllide pool consisted almost entirely of divinyl protochlorophyllide (Fig. 7a). Following photoreduction by a fourth light pulse, the divinyl protochlorophyllide was converted into divinyl chlorophyllide a (E458F674) (Fig. 7b).

On the other hand, 6-day-old etiolated barley leaves did not respond to the light-dark treatments as did cucumber and bean. After three light-dark cycles, the regenerated protochlorophyllide pool consisted mainly of monovinyl protochlorophyllide (Fig. 7c). The small shoulder at about 451 nm in the excitation spectrum indicated however that some divinyl protochlorophyllide was also present as reported previously (3). After phototransformation by a fourth light flash, the monovinyl protochlorophyllide pool was converted into monovinyl chlorophyllide a (E447F675) (Fig. 7d). Small amounts of divinyl chlorophyllide a were formed, however, probably from the small amounts of divinyl protochlorophyllide present in their tissues, as evidenced by the weak Soret excitation shoulder at 459 nm (Fig. 7b, d).

Finally, etiolated corn leaves fell in between the dicotyledonous seedlings and barley in their response to the intermittent light-dark treatment. After three light-dark cycles, the regenerated protochlorophyllide pool consisted of both monovinyl and divinyl protochlorophyllide as evidenced by the pronounced divinyl protochlorophyllide Soret excitation max-
imum at 443 nm and by the monovinyl protochlorophyllide excitation shoulder at 437 nm (Fig. 7c). Following phototransformation by a fourth light flash, the hybrid monovinyl/divinyl protochlorophyllide pool was converted into a hybrid monovinyl/divinyl chlorophyllide a pool as evidenced by the monovinyl and divinyl chlorophyllide a Soret excitation maxima at 448 nm and 458 nm, respectively (Fig. 7B, f). Because the quantum yield of fluorescence of divinyl chlorophyllide a is still unknown, it is not yet possible to determine exactly the proportion of divinyl chlorophyllide a in the corn extract. It does appear, however, from preliminary titrations of monovinyl/divinyl chlorophyllide a mixtures containing different amounts of these two species that as much as 50% of the chlorophyllide a pool depicted in Fig. 7B, f, may consist of divinyl chlorophyllide a.

Altogether, the above results indicated that divinyl chlorophyllide a is of general occurrence in higher plants, although the relative proportions of monovinyl and divinyl chlorophyllide differ among different plant species which are subjected to intermittent dark-light treatments.

**Discussion**

A synthetic-degradative chemical approach coupled to spectrofluorometric analysis was used in order to assign a divinyl structure to chlorophyllide a (E458F674). This approach consisted essentially in: (a) converting chlorophyllide a (E458F674) into monovinyl chlorophyllide a and into diethyl chlorophyllide a (mesochlorophyllide a) by catalytic hydrogenation and (b) in converting methylated chlorophyllide a (E458F674) into methylated divinyl protochlorophyllide by chemical oxidation (See Tables I and II and Figs. 3 to 6).

Catalytic hydrogenation of peripheral tetrapyrrole vinyl groups is a well established technique that has been used extensively in the past by porphyrin chemists (30). Reduction of a peripheral tetrapyrrole vinyl group to an ethyl group usually results either in a blue-shift of the Soret absorption/excitation maximum or in a blue-shift of both of the Soret maximum and the red absorption/emission maxima, depending on the tetrapyrrole species and the number of vinyl groups involved (21, 31). Soret absorption/excitation maxima of tetrapyrroles are usually very sensitive to the reduction of peripheral vinyl groups and in Table III we have attempted to compare the blue-shifts induced by the hydrogenation of chlorophyllide a (E458F674) to the Soret blue-shifts undergone by other tetrapyrroles following catalytic hydrogenation. It is apparent from this table that the extent of the Soret excitation/absorption blue-shift calculated from room temperature spectra depends upon the tetrapyrrole species and upon whether it is the first vinyl group (i.e. divinyl to monovinyl), the second vinyl group (i.e. monovinyl to meso), or both vinyl groups (i.e. divinyl to meso) which are being reduced. In general, one can conclude that reduction of one vinyl group usually results in a 3-6 nm blue-shift, while reduction of two vinyl groups results in a Soret blue-shift of about 9-11 nm, depending on the tetrapyrrole species. In this, conversion of chlorophyllide a (E458F674) into monovinyl chlorophyllide a (E447F675) resulted in a blue-shift of 5 nm. On the other hand, conversion of monovinyl chlorophyllide a into mesochlorophyllide a (E3458F674) resulted in an additional blue-shift of 5 nm (Table III). In both cases, the extents of the blue-shifts were compatible with the conversion of only one vinyl group to an ethyl group. On the other hand, conversion of chlorophyllide a (E458F674) or its demetallated analog into meso-chlorophyllide a or mesoephosphorhile a resulted in a Soret blue-shift of 11-12 nm, which is consistent with the conversion of two vinyl groups to two ethyl groups. Vinyl groups, however, can readily be converted into hydroxyethyl groups (21, p. 125) during experimental manipulations. Because of the spectral similarities between tetrapyrroles with one vinyl and one hydroxyethyl group and those with one vinyl and one ethyl group (24), it is possible that there may be small amounts of pigments with hydroxyethyl groups in our hydrogenated samples. For the purpose of monitoring spectral shifts, however, this would be irrelevant.

At 77 K, the magnitude of the Soret blue-shifts which are observed after the conversion of chlorophyllide (E458F674) to monovinyl chlorophyllide a (E447F675) and the latter into mesochlorophyllide a appeared to double (Table III). This was confirmed by a blue-shift of 20 nm following the direct conversion of chlorophyllide a (E458F674) to mesochlorophyllide a (Table III). The reason for the doubling of the observed blue-shifts at 77 K are not presently understood but may be related in some way to the fact that chlorophyll(ides) exist mainly as monosolvates (pentacoordinated Mg species) in ether at room temperature and as disolvates (hexacoordinated Mg species) in ether at 77 K (11, 22). Altogether, the titration of the Soret blue-shifts induced by catalytic hydrogenation of chlorophyllide a (E458F674) are fully compatible with the presence of two vinyl groups in this tetrapyrrole.

The presence of two peripheral vinyl groups in chlorophyllide a (E458F674) was further confirmed by its oxidation to divinyl protochlorophyllide with 2,3-dichloro-5,6-dicyanobenzoquinone. The room temperature absorption and fluorescence properties of monovinyl and divinyl protochlorophyllide have been well investigated and are well known (28, 29, 32). Oxidation of chlorophyllide (E458F674) and of authentic monovinyl chlorophyllide a yielded protochlorophyllide species that were indistinguishable from authentic divinyl and monovinyl protochlorophyllide, respectively. This in turn lent further credence to the assignment of a divinyl structure to chlorophyllide a (E458F674) and to chlorophyll a (E458F673) (5). Furthermore, since divinyl chlorophyllide a was formed from protochlorophyllide (E443F625) by a 2.5-ms light treatment at room temperature and at $-10^\circ C$ (6), this in turn further ascertained the divinyl structure of protochlorophyllide (E443F625).

Because of its resolution and high sensitivity, spectrofluorometry coupled to chemical derivatization proved very convenient in helping us to probe the chemical structure of chlorophyllide a (E458F674). It is nevertheless very desirable to further confirm the identity of divinyl chlorophyllide a (E458F674) by some independent means. The feasibility of preparing relatively large amounts of pure chlorophyllide a (E458F674) has now made it possible to further ascertain the identity of this tetrapyrrole by NMR spectroscopy. If chlorophyllide (E458F674) is indeed a divinyl chlorophyllide a as the present data indicate, then the proton chemical shift signals of the missing ethyl group, which is present at position 4 of the monovinyl chlorophyllide a macrocycle, should be missing from the spectral profile of chlorophyllide a (E458F674). The NMR profile of the latter should then be devoid of the quartet signal at about 3.75 ppm and of the triplet signal at about 1.72 ppm (33). Experiments aimed at assessing that situation are now in progress.

Finally, the induction of divinyl chlorophyllide a accumulation in several plant species by intermittent dark-light treatments indicates that this phenomenon is of general occurrence in nature. It is also important to note that divinyl protochlorophyllide is an important metabolic precursor which also accumulates in response to the light-dark treatments. Recently, a lethal maize mutant has been described which is devoid of monovinyl chlorophyll a, but which accumulates a pigment that appears to be identical with divinyl chlorophyll a (E458F674) (34). We therefore propose that (a) the biosyn-
The blue-shifts induced by reducing a peripheral vinyl group into an ethyl group in some of the tetrapyrroles were calculated from data published in the cited references.

| Experimental conditions | Tetrapyrroles | Blue-shifts in Soret absorption/excitation maxima for the following vinyl/ethyl pairs | Number of known or estimated vinyl groups involved in partial or full hydrogenation | Ref. |
|-------------------------|---------------|----------------------------------------------------------------------------------|---------------------------------------------------------------------------------|------|
|                         |               | DM → MV | MV → Meso | DV → Meso | Chlde a (E458F674) |                                      |                                      |      |
| From absorption spectra in ether at room temperature | Proto | 6 | 3 | 9 | 2 | 24 |
| | Mg-Proto | 6 | 5 | 11 | 2 | 24 |
| | DV Pchlide a | 5 | | 1 | 3 |
| | MV Chl a | 5 | | 1 | This work |
| | Chlde (E458F674) after complete hydrogenation | 11 | | | 2* | This work |
| | MV Pheophytin a | | 4 | | 1 | This work |
| | Demetallated Chlde (E458F674) after complete hydrogenation | | 12 | | 2* | This work |
| From fluorescence excitation spectra recorded in ether at room temperature | Proto | 4 | 5 | 9 | 2 | 24 |
| | Mg-Proto | 5 | 5 | 10 | 2 | 24 |
| | DV Pchlide a | 6 | | 3 | 3 |
| | MV Chl a | 5 | | 1 | This work |
| | Chlde a (E458F674) after partial hydrogenation | 5 | | | 1* | This work |
| | Chlde (E458F674) after complete hydrogenation | 12 | | | 2* | This work |
| | MV Pheophytin a | | 3 | | 1 | This work |
| | Demetallated Chlde a (E458F674) after complete hydrogenation | | 11 | | 2* | This work |
| From fluorescence excitation spectra recorded in ether at 77 K | Proto | 3 | 7 | 10 | 2 | 24 |
| | Mg-Proto | 7 | 6 | 13 | 2 | 24 |
| | DV Pchlide a | 6 | | 3 | 3 |
| | MV Chl a | 9 | | 1 | This work |
| | Chlde a (E458F674) after partial hydrogenation | 11 | | | 1* | This work |
| | Chlde a (E458F674) after partial to complete hydrogenation | 9 | | | 1* | This work |
| | Chlde a (E458F674) after complete hydrogenation | 20 | | | 2* | This work |
| | MV Pheophytin a | | 6 | | 1 | This work |
| | Demetallated Chlde a (E458F674) after complete hydrogenation | | 15 | | 2* | This work |

* The abbreviations used are: DV, divinyl; MV, monovinyl; Chlde, chlorophyllide; Chl, chlorophyll; Pchlide, protochlorophyllide.

The number of vinyl groups was estimated by reference to similar shifts undergone by the standard compounds reported in the table.

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