Chlorophyll Chemistry. I. Oxidation of the Vinyl Group of Ethyl Chlorophyllide *a*

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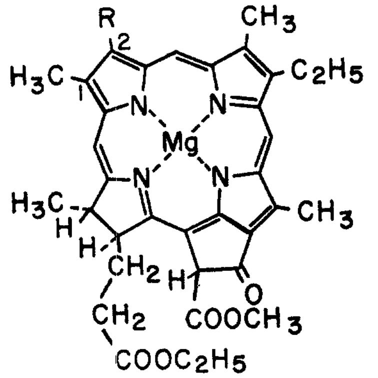
Ethyl 2-desvinyl-2-(1,2-dihydroxyethyl)chlorophyllide *a* has been prepared by hydroxylation of the vinyl group of ethyl chlorophyllide *a* with osmium tetroxide. Visible and infrared spectra and oxidation to ethyl 2-desvinyl-2-formylchlorophyllide *a* by sodium periodate indicates that the assigned structure is correct.

In attempting to identify intermediates and products from the photooxidation of chlorophyll,[1] we discovered that not many pure chlorophyll derivatives have been unequivocally characterized. We then undertook to prepare oxidation products of chlorophyll under carefully controlled conditions so that pure compounds could be isolated and characterized. With more complete and substantiated spectral records it is hoped that photooxidation processes may be more fully interpreted.

In the present paper we describe the preparation and properties of ethyl 2-desvinyl-2-(1,2-dihydroxyethyl)-chlorophyllide *a* (dihydroxy-Ia, Chart I), which has not been previously described,[2] and ethyl 2-desvinyl-2-formylchlorophyllide *a* (ethyl chlorophyllide *d*, Id) which has been isolated from natural sources[3] and also prepared by a nonspecific oxidation.[4]

Chart I

Nomenclature



Compound	R	Mg present
Ethyl chlorophyllide *a* (Ia)	$CH{=}CH_2$	+
Ethyl pheophorbide *a* (IIa)	$CH{=}CH_2$	−
Ethyl *meso*-chlorophyllide *a* (*meso*-Ia)	CH_2CH_3	+
Ethyl *meso*-pheophorbide *a* (*meso*-IIa)	CH_2CH_3	−
Ethyl 2-desvinyl-2-(1,2-dihydroxyethyl)chlorophyllide *a* (dihydroxy-Ia)	$CHOHCH_2OH$	+
Ethyl 2-desvinyl-2-(1,2-dihydroxyethyl)pheophorbide *a* (dihydroxy-IIa)	$CHOHCH_2OH$	−
Ethyl 2-desvinyl-2-formylchlorophyllide *a* (ethyl chlorophyllide *d*, Id)	CHO	+
Ethyl 2-desvinyl-2-formylpheophorbide *a* (ethyl pheophorbide *d*, IId)	CHO	−

Results

Oxidation.—Ethyl chlorophyllide *a* was smoothly hydroxylated with osmium tetroxide in ethyl ether at room temperature and the osmate was decomposed with an aqueous solution of sodium sulfite. The dihydroxy compound was purified by several chromatographs on polyethylene resin followed by sugar chromatography. The analysis, visible spectrum (Figure 1), and the infrared spectra indicate the expected hydroxylation of the vinyl group. Further evidence for the structure of dihydroxychlorophyllide was its smooth conversion to ethyl 2-desvinyl-2-formylchlorophyllide *a* (chlorophyllide *d*, Id) with sodium metaperiodate.

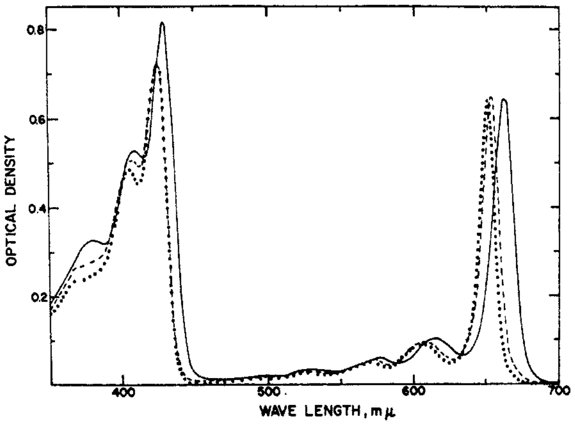


Figure 1.—Visible absorption spectra of ———, ethyl chlorophyllide *a* (Ia) in ether; – – – –, ethyl dihydroxychlorophyllide *a* (dihydroxy-Ia) in ether; and · · · ·, ethyl *meso*-chlorophyllide *a* (*meso*-Ia) in ether.

Discussion

Oxidation of the vinyl double bond of ethyl chlorophyllide *a* by osmium tetroxide appeared to be specific. The phase test[5] remained positive indicating that the cyclopentanone ring was intact. There was no base-soluble material present, indicating the absence of any free acid group. That hydroxylation of the double bond had occurred was further substantiated by the close similarity of the visible absorption spectrum to that of ethyl *meso*-chlorophyllide *a* (Figure 1). All details of the spectra are similar except for a 3-mμ shift of the "red" absorption maxima.

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[1] G. Sherman and H. Linschitz, in preparation.

[2] A. S. Holt [*Can. J. Botany*, **39**, 327 (1961)] mentions that 2-desvinyl-2-(1,2-dihydroxyethyl)chlorophyll *a* is the first product of the stepwise potassium permanganate oxidation of chlorophyll *a* and has an absorption spectrum almost identical with that of *meso*-chlorophyll *a*. However, no mention is made of isolation, purification, or characterization of the material.

[3] Reference 2, and references therein.

[4] A. S. Holt and H. V. Morley, *Can. J. Chem.*, **37**, 507 (1959); A. Jensen and O. Aasmundrud, *Acta Chem. Scand.*, **17**, 907 (1963).

[5] H. Molisch, *Ber. Deut. Botan. Ges.*, **14**, 16 (1896); A. S. Holt, *Can. J. Biochem. Physiol.*, **36**, 439 (1958).

TABLE I

VISIBLE ABSORPTION PROPERTIES

Compd	Mol wt	Solvent	λ_{max}, mμ Red	Blue	Ratio of blue/red	Ratio of blue/shoulder	Molar absorption coefficient at "red" max
			Magnesium Chelates				
Ia	643.03	E-Py[a]	661	429	1.27	429/409 1.59	80,000[b]
		Ether	661	429	1.28	429/410 1.60	
meso-Ia	645.05	E-Py	650	426	1.12	426/406 1.49	98,300
		Ether	650	426	1.13	426/405 1.48	
Dihydroxy-Ia	677.05	E-Py	653	426	1.12	426/408 1.47	96,800
		Ether	653	426	1.11	426/406 1.43	
Id	645.00	E-Py	687	445	0.85	445/391 1.75	70,000
		Ether	685	445	0.86	445/390 1.70	
			Magnesium-Free				
IIa		Ether	666	408	1.97		
meso-IIa		Ether	656	404	2.01		
Dihydroxy-IIa		Ether	659	404	1.89		
IId		Ether	681	421	1.08	421/383 1.12	

[a] 0.1% pyridine in ether. [b] Obtained on a Beckman DU spectrophotometer; all other spectra recorded on a Cary 14.

TABLE II

INFRARED ABSORPTION MAXIMA

(cm^{-1})

Compd	Solvent	Ester carbonyls	Free ketone carbonyl	Assoc ketone carbonyl	Aldehyde carbonyl
Ethyl chlorophyllide *a* (Ia)	Mull	1718	...	1661	
	$CHCl_3$[a]	1727	1675	...	
Chlorophyll *a*	CCl_4	1736	1695	1653	
Pheophytin *a*	CCl_4	1748	1715	...	
Ethyl *meso*-chlorophyllide *a*	CCl_4	1730	1686	1650	
(*meso*-Ia)	$CHCl_3$	1730	1678	...	
Ethyl dihydroxychlorophyllide *a* (dihydroxy-Ia)	Mull	1733	1681	1645	
	$CHCl_3$	1733	1681	...	
Ethyl chlorophyllide *d* (Id)	CCl_4	1724	1692		1664
	$CHCl_3$	1724	1684		1664
Ethyl pheophorbide *d* (IId)	CCl_4	1748	1718		1689

[a] Chloroform stabilized with alcohol.

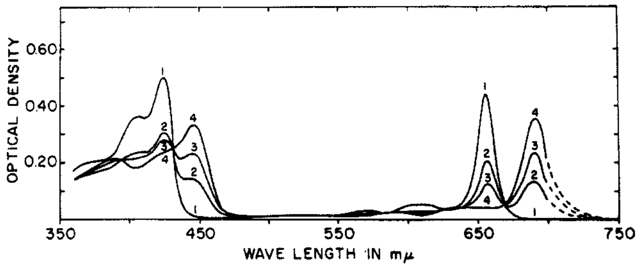

Figure 2.—Reaction of ethyl dihydroxychlorophyllide *a* (dihydroxy-Ia) with sodium metaperiodate, buffered at pH 7.25: curve 1, starting material; curve 2, 3-hr reaction time; curve 3, 5-hr reaction time; and curve 4, 10-hr reaction time. (Recorded on a Beckman DB spectrophotometer.)

Cleavage of the *vic*-glycol by periodate also proceeded without complications. Spectra (Figure 2) indicate complete conversion to the aldehyde, although several minor bands appeared during chromatography and recovery of pure material was small. These contaminants may arise during the purification procedures since both the dihydroxy compound and the aldehyde are less stable than chlorophyll itself. Once the vinyl side chain is partially oxidized further changes readily occur. Holt's concern[6] that chlorophyll *d* may be an artifact formed during isolation procedures may be well founded.[7]

The visible (Table I) and infrared spectra (Table II) are in agreement with those reported by Holt[2] for 2-desvinyl-2-formylchlorophyll *a* prepared by permanganate oxidation. Molar extinction coefficients (Figures 3 and 4) were determined in 0.1% pyridine in ether to ensure complete dissolution of the material. Although some compounds surely contain water, the molar extinction coefficients are calculated without water for the sake of uniformity since the exact amount of water is not known. The specific absorption coefficients found for ethyl chlorophyllide *d* are 92.2 and 109. These are in fair agreement with those calculated from magnesium content, 97.8 and 100.4, for chlorophyll *d* in ether, isolated from *Gigartina agardhii* by Smith and Benitez.[8]

The infrared spectrum of ethyl dihydroxychlorophyllide *a* exhibited an intense, broad band in the O–H stretching region (3390 cm^{-1}). However, this is not unequivocal evidence for the presence of hydroxyl groups since ethyl chlorophyllide *a* itself often shows peaks in this region owing to bound water. The carbonyl region of dihydroxy-Ia in chloroform was very

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(6) A. S. Holt, *Can. J. Botany*, **39**, 327 (1961).

(7) A photooxidation product[1] isolated from chlorophyll will slowly change to chlorophyll *d* on standing. R. L. Livingston and K. E. Owen [*J. Am. Chem. Soc.*, **78**, 3305 (1956)] have also observed the formation of material resembling chlorophyll *d* on illumination of chlorophyll at room temperature in the presence of oxygen.

(8) J. H. C. Smith and A. Benitez, "Modern Methods of Plant Analysis," Vol. 4, Springer-Verlag, Berlin, 1955, p 169.

similar to *meso*-Ia. A Nujol mull of the dihydroxy compound exhibited, in addition to the ester band, two carbonyl bands of about equal intensity, suggesting its existence as a dimer in the crystalline state.[9]

Ethyl chlorophyllide *d* shows three bands in the carbonyl region in carbon tetrachloride. If chlorophyllide *d* is aggregated in carbon tetrachloride solution the associated carbonyl must absorb at the same wavelength as the aldehyde carbonyl. In chloroform stabilized with alcohol, which does not permit aggregation,[9] the aldehyde band of chlorophyllide *d* is at 1664 cm.$^{-1}$, identical with the position of the aldehyde band observed with carbon tetrachloride as the solvent.

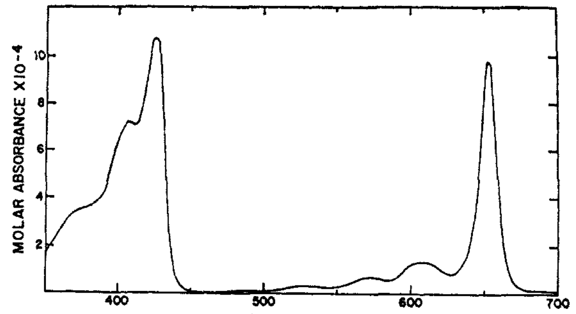

Figure 3.—Absorption spectrum of ethyl dihydroxychlorophyllide *a* (dihydroxy-Ia) in 0.1% pyridine–ether.

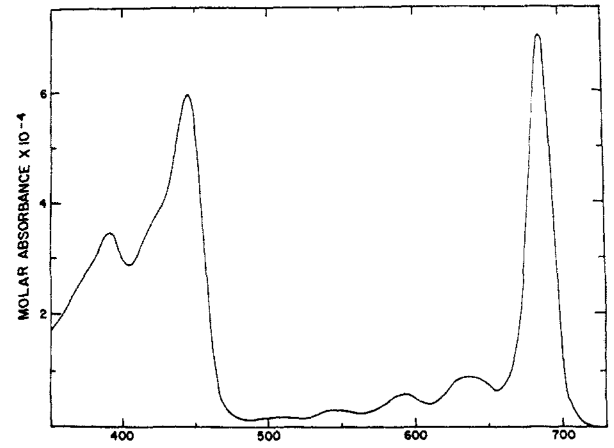

Figure 4.—Absorption spectrum of ethyl chlorophyllide *d* (Id) in 0.1% pyridine–ether.

Experimental Section

Visible spectra were recorded on a Cary Model 14 spectrophotometer unless noted otherwise. Infrared spectra were recorded on a Perkin-Elmer Model 237 spectrophotometer. Solvents and chemicals were used as received except pyridine which was distilled from barium oxide.

Isolation of Ethyl Chlorophyllides.—Isolations[10] were carried out in dim light, in an air-conditioned room. Leaves from *Ailanthus altissima* were used and special effort was made to select dark green, tender leaves.[11] Fresh leaves (2 kg) were macerated in a water-cooled Waring Blendor with 6 l. of 95% ethanol. This mixture (pH 6.6) was allowed to incubate at room temperature for 3 hr at which time the green pigments could be extracted from an ether solution with 22% HCl. The mixture was filtered under suction; the meal was pressed dry with a rubber dam and rinsed with 1 l. of acetone. The filtrate was divided among large beakers containing a total of 100 g of Celite (Johns Manville, L-665-A). A volume of water equal to the volume of the filtrate was added dropwise to each beaker during about a 40-min period while cooling in an ice bath and stirring mechanically. After standing for 0.5 hr the Celite containing the precipitated pigments was collected by suction filtration and washed about ten times alternately with 100-ml portions of acetone–water (1:1) and petroleum ether (bp 30–60°). The material was extracted from the Celite by stirring the Celite with about 600 ml of absolute ethanol, filtering, and washing the Celite with another 600 ml of ethanol. After this process the Celite was white and would be reused. The optical density of the filtrate was usually between 150 and 200 at 660 mμ. This ethanol filtrate was diluted slowly, while stirring in an ice bath, with an amount of water equal to 60% of the volume of the absolute ethanol. After standing in the cold for 0.5 hr, the crystals were collected on Whatman No. 50 filter paper and washed alternately with portions of acetone–water (1:1) and petroleum ether until the washings were colorless or a faint yellow.

Two recrystallizations were carried out by dissolving the chlorophyllides in absolute ethanol to give an optical density of 150–180 at 660 mμ and diluting with water as above.

From 2 kg of good leaves, 1.0–2.0 g of highly crystalline material were obtained, which was deep purple-blue with a high metallic luster. The ratio of the *a* to *b* Soret peak is 2.3–2.5 and only traces of ethyl phenophorbides and base-soluble material were present. The *a* and *b* mixture is obtained in a highly crystalline state more easily than pure ethyl chlorophyllide *a*. This may suggest that there is some highly ordered arrangement of *a* and *b* in the solid state. If good leaves are used and the procedure is carried out carefully, several grams of a highly pure mixture of ethyl chlorophyllide *a* and *b* can be obtained in 1 week.

Separation and Purification of Ethyl Chlorophyllide *a* and *b*.—The method of Holt and Jacobs[10] was used for the separation of ethyl chlorophyllides *a* and *b* on sugar columns. A glass column (50 × 10 cm) was packed with 5 lb of dry, sifted 10X confectioner's sugar by tamping with a plunger while under suction. A mixture of ethyl chlorophyllide *a* and *b*, 250 mg, was dissolved in 50 ml of pyridine and diluted with 1950 ml of petroleum ether (Fisher, bp 30–60°, used as received) and poured onto the top of the column. After adsorption, the column was developed with a solution of 0.5% pyridine and 0.5% isopropyl alcohol in petroleum ether. A trace of ethyl pheophorbide *a* was eluted first, followed by ethyl chlorophyllide *a*. The ethyl chlorophyllide *a* eluate was evaporated to dryness at room temperature with a Büchler flash evaporator. When the ethyl chlorophyllide *b* reached the bottom of the column all the sugar above it was removed and discarded and the *b* was eluted with ethyl ether. The ethyl ether was evaporated at room temperature. The dry ethyl chlorophyllide *a* obtained as described above was dissolved in 40 ml of acetone, filtered through fine-porosity sintered glass, and diluted with an equal volume of water added dropwise while stirring. After standing in the cold for 30 min, the crystals were collected by suction on Whatman No. 50 filter paper and dried in a vacuum desiccator over Drierite: yield 120 mg.

In one purification procedure the ethyl chlorophyllide *a* was further purified by three additional chromatographs on sugar essentially as described above. This material was highly crystalline, a definite melting point was not observed but contraction and softening occurred between 175 and 195°.

Anal. Calcd for $C_{37}H_{38}MgN_4O_5 \cdot 2H_2O$: C, 65.44; H, 6.23; Mg, 3.58; N, 8.25. Found:[12] C, 66.17; H, 6.16; Mg, 3.48; N, 7.86. Calcd for $C_{37}H_{38}MgN_4O_5$: C, 69.11; H, 5.96; Mg, 3.78; N, 8.71. Found (after drying in a high vacuum at 100°): C, 69.64; H, 6.20; Mg, 4.33; N, 8.87. Wt loss on drying: 5.55%. Calcd for $2H_2O$: 5.31%.

ϵ values were 116,000 (429 mμ) and 90,900 (661 mμ) in ether, assuming a molecular weight of 679.03. Infrared[9,13] and nmr spectra[14] are in good agreement with those published.

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(9) J. J. Katz, G. L. Closs, F. C. Pennington, M. R. Thomas, and H. H. Strain, *J. Am. Chem. Soc.*, **85**, 3801 (1963).

(10) A. S. Holt and E. E. Jacobs, *Am. J. Botany*, **41**, 710 (1954).

(11) Rather serious drought conditions have existed in the Poughkeepsie area for the past few summers. We have found that the leaves in exposed areas, such as the edge of fields, change very rapidly under unusually dry conditions. The yield of chlorophyllides from these leaves gradually decreases and the material isolated is gummy and highly contaminated with pigments without magnesium until finally it is not possible to isolate any magnesium-containing material at all.

(12) Alfred Bernhardt, Microanalytisches Laboratorium im Max-Planck-Institut für Kohlenforschung, Mülheim (Ruhr), Germany.

(13) A. S. Holt and E. E. Jacobs, *Plant Physiol.*, **30**, 553 (1955).

(14) G. L. Closs, J. J. Katz, F. C. Pennington, M. M. Thomas, and H. H. Strain, *J. Am. Chem. Soc.*, **85**, 3809 (1953).

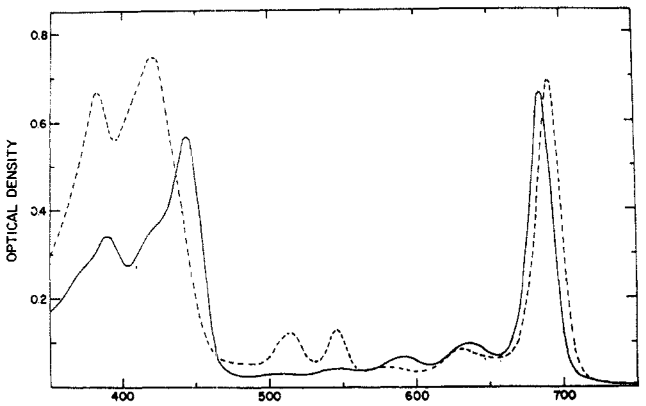

Figure 5.—Absorption spectra of ———, ethyl chlorophyllide *d* (Id) in ether; and - - - -, ethyl pheophorbide *d* (Id) in ether.

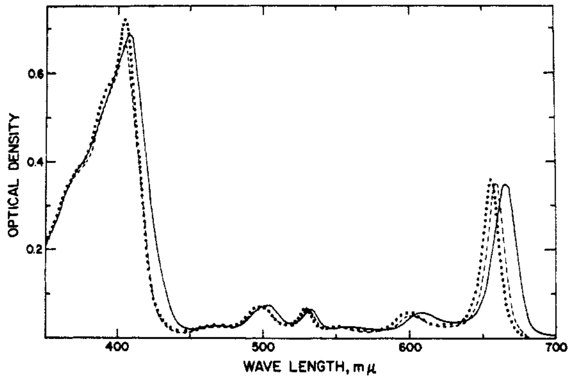

Figure 6.—Absorption spectra of ———, ethyl pheophorbide *a* (IIa) in ether; - - - -, ethyl dihydroxypheophorbide *a* (dihydroxy-IIa) in ether; and . . . ., ethyl *meso*-pheophorbide *a* (*meso*-IIa) in ether.

Molar extinction coefficients and ratios are reported in Table I. We consistently observed a blue/red ratio in diethyl ether of 1.28–1.29 which is not in agreement with Anderson and Calvin[15] but is in agreement with Perkins and Roberts,[16] Holt,[10] Pennington,[17] Strain,[18] and others. We also find that a decrease in the ratio 429/410 mμ (1.60) is a good indication of contamination by absorbing pigments. Some fairly satisfactory elementary analyses have been obtained, but we are in perfect agreement with Pennington[17] that difficulties are often encountered. It is found that if combustion is complete, as judged by the amount of residual ash, that fair agreement with calculated values can be obtained if the amount of water present is known. The analysis reported seems to indicate 2 moles of water/mole of chlorophyll. However, other analyses have indicated less water content. As Kutyurin and Knyazev[19] pointed out, the water content will vary with conditions.

Osmium Tetroxide Oxidation.—Ethyl chlorophyllide *a* (400 mg, 0.6 mmole) was dissolved in 80 ml of ether. Osmium tetroxide (500 mg, 2 mmoles) was dissolved in 40 ml of ether and added to the ethereal *a* solution in two portions at a 30-min interval. (The anhydrous ether was passed through an alumina column to remove peroxides.) The reaction mixture was incubated at 26° with constant stirring for 1 hr at which time 50 ml of saturated Na_2SO_3 solution was added. Spectral examination (peak at 661 mμ completely shifted to 653 mμ) indicated decomposition to be complete in about 2 hr. The aqueous layer was separated and extracted with fresh portions of ether until no more pigment could be extracted. The combined ether layers were washed several times with water and once with saturated NaCl solution, dried over anhydrous Na_2SO_4, and evaporated. The pigment was dissolved in 300 ml of methanol and diluted to 500 ml by addition of water slowly in an ice bath. After adsorbing the solution on a polyethylene resin[20] column (35 × 8 cm), 2 l. of 60% methanol–water was used to elute the dihydroxy compound while the unchanged ethyl chlorophyllide *a* remained on top of the column. The dihydroxy compound was recovered as described above in 25% yield.

A second chromatography on polyethylene resin was carried out as described above. Two minor bands appeared below the major band, one of which was identified as allomerized dihydroxy material since the spectrum was identical with the main band but the phase test was negative. Following a third chromatography on polyethylene, the pigment was dissolved in 1% pyridine and 2% propanol in CCl_4 and chromatographed on sugar. Development with 2% propanol in CCl_4 revealed a homogeneous band except for some brown residual material at the top of the column. After elution and evaporation of the solvents, the residue was dissolved in $CHCl_3$, filtered through sintered glass, and crystallized by addition of petroleum ether.

Anal. Calcd for $C_{37}H_{40}MgN_4O_7 \cdot 0.5H_2O$: C, 64.78; H, 6.02; Mg, 3.54; H_2O, 1.31. Found:[12] C, 65.12; H, 6.55; Mg, 3.90; H_2O, 2.09. Calcd for $C_{37}H_{40}MgN_4O_7$: C, 65.63; H, 5.96; Mg, 3.59. Found (after drying at 100° in a high vacuum): C, 66.22; H, 6.24; Mg, 3.26.

The spectral properties are reported in Tables I and II.

Sodium Periodate Oxidation.—Ethyl dihydroxychlorophyllide *a* (50 mg) was dissolved in 50 ml of 10% methanol–ether. To this solution was added 50 ml of a saturated solution of sodium metaperiodate buffered with 0.02 *M* sodium phosphate at pH 7.25. The reaction proceeded faster without a buffer but the Mg was lost. The reaction mixture was incubated at 30° for 10 hr with vigorous stirring. At this time the peak at 653 mμ had disappeared and a new peak at 687 mμ appeared (Figure 2). The product was isolated from the ether as described above, dissolved in 40 ml of $CHCl_3$ and 200 ml of petroleum ether, and adsorbed on a sugar column (45 × 5 cm). Five bands appeared on the column as follows: band 1 (on top of column), brown; 2, green, unchanged dihydroxy material; 3, pale olive green, allomerized *d;* 4, green, main zone of ethyl chlorophyllide *d;* and 5, pale brown, pheophorbide *d*.

The ethyl chlorophyllide *d* was eluted and the residue was dissolved in 5 ml of $CHCl_3$ and diluted with petroleum ether. About 10% of pure product was obtained. Spectral properties are reported in Tables I and II. The absorption spectra of the material with and without magnesium (Figure 5) and the infrared spectra are in perfect agreement with those obtained by Holt[2] on material obtained by oxidation of the vinyl group with potassium permanganate which he correlated with chlorophyll *d* isolated from natural sources.[6]

Preparation of Ethyl *meso*-Chlorophyllide *a*.—Ethyl chlorophyllide *a* (50 mg) was dissolved in 20 ml of acetone with a small amount of 10% palladium on charcoal. Hydrogenation under atmospheric pressure was carried out for 30 min with stirring. The solution changed from greenish blue to deep blue early in the reaction. The reaction mixture was filtered, diluted with water, and extracted with ether, which was washed, dried, and evaporated. On washing the residue with petroleum ether brilliant lustrous plates resulted. This material was chromatographed on sugar by the same method used for ethyl chlorophyllide *a* and crystallized from ether–petroleum ether. Brilliant purple crystals with a metallic luster were obtained. The material melted at about 170° followed by resolidification. The spectral properties are reported in Tables I and II. The maxima agree with that reported by Fischer.[21]

The ethyl pheophorbides (Figure 6) were prepared by shaking the phyllins in ether with 1 *N* HCl.

Acknowledgments.—We wish to thank Professor Henry Linschitz of Brandeis University and Dr. Frank Roberts of Texaco Research Laboratories for use of their Cary spectrophotometers. This research was supported by Public Health Service Grant GM-11529.

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[15] A. F. H. Anderson and M. Calvin, *Nature*, **194**, 285 (1962).

[16] H. J. Perkins and D. W. A. Roberts, *Biochim. Biophys. Acta*, **79**, 20 (1964).

[17] F. C. Pennington, H. H. Strain, W. A. Svec, and J. J. Katz, *J. Am. Chem. Soc.*, **86**, 1418 (1964).

[18] H. H. Strain, M. R. Thomas, and J. J. Katz, *Biochim. Biophys. Acta*, **75**, 306 (1963).

[19] V. M. Kutyurin and V. P. Knyazev, *Dokl. Akad, Nauk SSSR*, **149**, 456 (1963).

[20] Polyethylene Resin QX-2187 supplied by Dow Chemical Co., Midland, Mich.

[21] H. Fischer, *Ann.*, **515**, 142 (1935).