The Structure, Properties, and Distribution of Chlorophyll c

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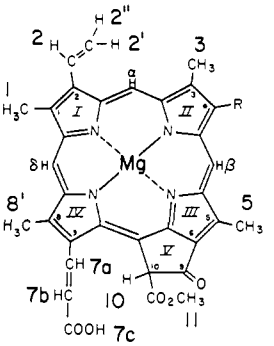
Abstract: All available evidence suggests that chlorophyll c is a mixture of magnesium tetradehydro- and hexadehydropheoporphyrin a_5 monomethyl ester (Ia, Ib). On treatment of chlorophyll c with dilute acid the pheoporphyrins c_5 monomethyl esters (IIa, IIb) are formed. Mineral acids, in methanol, convert the pheoporphyrins c_5 into esters of compounds derived from an isochloroporphyrin ring structure (IIIa, IIIb, IV). The visible, infrared, nmr, and mass spectra of chlorophyll c and its derivatives are reported along with observations on the relative abundance of Ia and Ib in *Nitzschia closterium*. Crystalline chlorophyll c has now been isolated from brown algae of three major taxonomic classes. It has been separated into c_1 (Ia) and c_2 (Ib) by chromatography.

Marine diatoms, dinoflagellates, and brown algae constitute a major portion of the world's CO_2 fixation capacity. All these organisms contain chlorophyll c in addition to the predominant chlorophyll a. The photosynthetic processes and the chlorophylls of these organisms are, therefore, of major economic and fundamental interest.

Maximum photosynthetic efficiency of autotrophic plants is usually obtained with red light; however, plants growing in an extensive aqueous environment, as in lakes and the sea, receive relatively more yellow than red light, due to the preferential absorption of the latter by water. With diatoms, which are often greatly dispersed in water, Dutton and Manning[1] found an equivalent photosynthetic efficiency in yellow (500–600 nm) and red (600–690 nm) light. From the absorption of chlorophyll c in the 500–600 nm region, and the weaker absorption of chlorophyll a, it is clear that chlorophyll c must participate effectively in photosynthesis as an accessory pigment similar, in its functional activity, to chlorophyll b of higher plants.[2]

Chlorophyll c is common in widely distributed plants including the most abundant organisms of the open seas. It has been identified in the extracts of various diatoms (Bacillariophyceae), dinoflagellates (Dinophyceae), brown algae or kelps (Phaeophyceae) which include the only large, free-floating sea plant, *Sargassum*, and some species of Chrysophyceae as *Phaeodactylum* sp. and *Amphidiniam* sp.[3-9]

We recently presented a preliminary report,[10] which indicated that chlorophyll c, isolated in crystalline form as the bistetrahydrofuranate from the diatom *Nitzschia closterium*,[11] is a mixture of similar porphyrin acrylic acids, namely, magnesium tetradehydropheoporphyrin a_5 (Ia) and magnesium hexadehydropheoporphyrin a_5 (Ib) monomethyl esters.



Ia R = $-CH_2-CH_3$

Ib R = $-CH=CH_2$

IIa = Ia $-Mg+2H$

IIb = Ib $-Mg+2H$

Compounds which are closely related to chlorophyll c have been isolated from flagellates,[12] photosynthetic bacteria,[13,14] bacterial and algal mutants,[15,16] and the seed coats of Cucurbitaceae.[17] Most of these chlorophyll c-like compounds have not been obtained in sufficient quantity and purity for a detailed study of their various spectroscopic properties. With the exception of the studies by Ellsworth and Aronoff,[16] structural assignments have been made only on the basis of their reaction products. It is known that the absorption spectra of porphyrins, and particularly chlorophyll c,[18] are extremely sensitive to the conditions under which the spectra are recorded. The electronic basis for this spectral variability has been the subject of recent reports.[19]

(1) H. J. Dutton and W. M. Manning, *Am. J. Botany*, **28**, 516 (1941).
(2) H. H. Strain, *Science*, **112**, 161 (1950).
(3) H. H. Strain and W. M. Manning, *J. Biol. Chem.*, **144**, 625 (1942).
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(5) F. T. Haxo and D. C. Fork, *Nature*, **184**, 1047 (1959).
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(7) S. W. Jeffrey, *Nature*, **194**, 600 (1962).
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(11) H. H. Strain and W. A. Svec, "The Chlorophylls," L. P. Vernon and G. R. Seely, Ed., Academic Press, New York, N. Y., 1966, p 57 ff.
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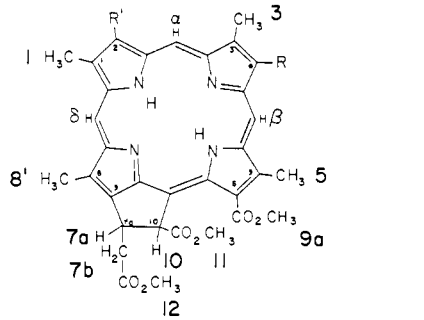
(13) O. T. G. Jones, *Biochem. J.*, **86**, 429 (1963).
(14) O. T. G. Jones, *ibid.*, **89**, 182 (1963).
(15) M. Griffiths, *J. Gen. Microbiol.*, **27**, 427 (1962).
(16) R. K. Ellsworth and S. Aronoff, *Arch. Biochem. Biophys.*, **125**, 269 (1968).
(17) O. T. G. Jones, *Biochem. J.*, **96**, 60 (1965).
(18) S. W. Jeffrey, *ibid.*, **86**, 313 (1963).
(19) A. H. Corwin, A. B. Chivvis, R. W. Poor, D. G. Whitten, and E. W. Baker, *J. Amer. Chem. Soc.*, **90**, 6577 (1968).

In anticipation of the isolation of chlorophyll and bacteriochlorophyll intermediates and accessory pigments in sufficient quantity for detailed studies using infrared, nmr, and mass spectra, in addition to electronic absorption spectroscopy, we herein report the details of our observations on chlorophyll c and its derivatives. We also present additional observations on the occurrence and distribution of chlorophyll c, which supplement earlier reports.

A word about nomenclature appears in order. For various reasons, it seems desirable to retain the term chlorophyll c for the compounds we have isolated from natural sources. However, unlike the chlorophylls a and b, these substances are not esterified at the 7 position, and like protochlorophyll, they are in a higher oxidation state, lacking the two H atoms at positions 7 and 8 as found in most chlorophylls. They do contain an isocyclic ring V, as in the other chlorophylls, and for this, as well as historical reasons, it seems justifiable to retain the name chlorophylls c. It must be clearly understood that these substances are comparable to chlorophyllides (as they lack an ester function at position 7). The Mg-free derivatives are, however, not true pheophytins but resemble pheophorbides. More accurately, because the chlorophylls c and chlorophyllides c are porphins and not dihydroporphins, we shall designate them as pheoporphyrins c_5 monomethyl esters. The individual pheoporphyrins c_5 may be designated relative to the pheoporphyrin a_5 of chlorophyll a. Thus, IIa may be designated as tetradehydropheoporphyrin a_5 monomethyl ester, and IIb must then be hexadehydropheoporphyrin a_5 monomethyl ester.[10] Compounds IIIa and IIIb are isomeric with what would be a chloroporphyrin c_6. As the true chloroporphyrins do not contain an isocyclic ring, we designate our compounds IIIa and IIIb as isochloroporphyrins c_6 trimethyl esters and those compounds with reduced vinyl groups as *meso*-isochloroporphyrin c_6 trimethyl ester (IV).



Experimental Section

Preparation of Chlorophylls c (Chlorophyllides c) (Ia plus Ib). Freshly centrifuged cells of *Nitzschia closterium* f_1 *minutissima* (358 g, from 40 l. of culture) were suspended in a small amount of the culture medium and added to 1 l. of vigorously boiling medium buffered with 20 g of ammonium acetate. The boiling was continued 2 min to inhibit enzymatic alteration of the chlorophylls during extraction. After cooling with ice, the cells were collected by centrifugation and extracted twice with 1.6 l. of a mixture of methanol, diethyl ether, and petroleum ether (5:2:1). A third extraction was made with methanol (300 ml), diethyl ether (200 ml), and petroleum ether (100 ml). The extracts were shaken against aqueous salt solution to remove the methanol and combined. The aqueous methanol layer was extracted in portions with a total of 1 l. of carbon tetrachloride. The combined organic extracts were evaporated *in vacuo*, and the residue was dried under high vacuum. The dried residue was dissolved in ether (80 ml), diluted with petroleum ether (320 ml), and added in equal portions to eight columns of powdered sugar (8 × 36 cm). The columns were washed with petroleum ether with a concentration gradient of 0–7.5% 1-propanol. The final washing carried the yellow-green zone of chlorophyll c, the most sorbed zone, nearly to the bottom of the columns. The chlorophyll c zone was cut from each column and eluted with ethanol followed by ether. The combined elutriates were washed with water and evaporated with a flash evaporator. The primary residue was taken up in roughly 15 ml of methanol and diluted with water (1.5–2.5 ml). After refrigeration overnight, the mixture was cooled for several hours with carbon dioxide. The supernatant was decanted, and the viscous residue washed with methanol–petroleum ether (6 ml, 6 ml). The residual crystals were collected by centrifugation and washed twice with methanol–petroleum ether. After drying, the material was taken up in 6 ml of tetrahydrofuran, clarified by centrifugation, and reprecipitated by addition of 40 ml of petroleum ether followed by Dry Ice cooling. At this stage, the yield of vacuum-dried chlorophyll c bistetrahydrofuranate was *ca.* 16 mg. *Anal.* Calcd for $C_{35}H_{30}N_4O_5Mg\cdot(C_4H_8O)_2$: C, 68.34; H, 6.09. Found: C, 68.60; H, 6.16; one CH_3O group per mole (by Crobaugh Lab.).

Chlorophyll c, free of external ligands (as shown by nmr), was prepared from the bistetrahydrofuranate either by heating at 150° under high vacuum or by dissolving the material in ethyl acetate followed by vacuum evaporation and repeated washing with carbon tetrachloride, each washing followed by solvent evaporation on a vacuum line (Ia plus Ib). There was no spectral, chromatographic, or nmr indication of decarboxylation or isomerization, as at C-10.

Pheoporphyrins c_5 Monomethyl Esters (Tetra- plus Hexadehydropheoporphyrins a_5 Monomethyl Esters) (Pheophorbides c) (IIa plus IIb). Chlorophyll c dissolved in tetrahydrofuran was treated with aqueous hydrochloric acid (10%). Chloroform was added to the mixture, and the solution was repeatedly shaken against water. The organic extracts were dried ($MgSO_4$) and evaporated *in vacuo*. The pheoporphyrins c_5 ester residue was recrystallized from methylene chloride–methanol or methylene chloride–petroleum ether mixtures.

Isochloroporphyrins c_6 Trimethyl Esters (IIIa plus IIIb). Chlorophyll c (20 mg) was dissolved in dry methanolic hydrochloric acid (3% w/v, 60 ml) and heated under reflux in a nitrogen atmosphere for 18 hr. The reaction was quenched by addition of chloroform and saturated aqueous sodium bicarbonate. After washing with water and drying ($MgSO_4$), the product was crystallized from chloroform–petroleum ether. The crystallized material was homogeneous on silica tlc and sucrose column chromatography (molecular formulas by mass spectrometry—see Table I). When the reaction was terminated at 8 hr, two major products were obtained. Since the intermediate, more sorbed product was smoothly converted to the less sorbed final product, its structure was not investigated. This intermediate may be the pheoporphyrin c_5 partial

Table I. Selected High-Resolution Mass Spectal Data

Compd	Formula	Ion	m/e Measured	m/e Theoretical
IIa	$C_{33}H_{28}N_4O_2$	M − $C_2H_4O_3$	512.2230	512.2212
IIb	$C_{33}H_{28}N_4O_2$	M − $C_2H_4O_3$	510.2034	510.2056
IIIa	$C_{37}H_{38}N_4O_6$	M	634.2807	634.2791
	$C_{36}H_{34}N_4O_5$	M − CH_4O	602.2504	602.2529
	$C_{35}H_{35}N_4O_4$	M − $C_2H_3O_2$	575.2639	575.2658
IIIb	$C_{37}H_{36}N_4O_6$	M	632.2649	632.2635
	$C_{36}H_{32}N_4O_5$	M − CH_4O	600.2350	600.2373
	$C_{35}H_{33}N_4O_4$	M − $C_2H_3O_2$	573.2485	573.2502
IV	$C_{37}H_{40}N_4O_6$	M	636.2948	636.2947
	$C_{36}H_{37}N_4O_5$	M − CH_3O	605.2732	605.2764
	$C_{35}H_{38}N_4O_4$	M − $C_2H_2O_2$	578.2873	578.2893

Table II

Compd	Formula	Mg	Group at C-2	Group at C-4
Chlorophylls c	Ia	Present	$-CH=CH_2$	$-CH_2-CH_3$
	Ib	Present	$-CH=CH_2$	$-CH=CH_2$
Pheoporphyrins c_5	IIa	Absent + 2 H	$-CH=CH_2$	$-CH_2-CH_3$
monomethyl esters	IIb	Absent + 2 H	$-CH=CH_2$	$-CH=CH_2$
Isochloroporphyrins c_6	IIIa	Absent + 2 H	$-CH=CH_2$	$-CH_2-CH_3$
trimethyl esters	IIIb	Absent + 2 H	$-CH=CH_2$	$-CH=CH_2$
meso-Isochloroporphyrin c_6 trimethyl ester	IV	Absent + 2 H	$-CH_2-CH_3$	$-CH_2-CH_3$

esters or, more probably, the esters of chloroporphyrin c_6 (with ring V open), which isomerizes to the isochloroporphyrins IIIa and IIIb.

***meso*-Isochloroporphyrin c_6 Trimethyl Ester (IV).** The natural mixture of pheoporphyrins c_5 monomethyl esters (10 mg) was dissolved in trifluoroacetic acid (9 ml). Palladium on carbon (10%) 6 mg) was added, and hydrogen was bubbled through the suspension. After 4 hr, there was no further change in the absorption spectrum. The solution was then filtered (to remove the catalyst) and evaporated *in vacuo*. The residual reduction product was refluxed with methanolic hydrochloric acid, as with III, and crystallized from chloroform–petroleum ether.

Pheoporphyrin a_5 Dimethyl Ester. Chlorophyll c (20 mg) suspended in 9 ml of acetic acid was treated with aqueous HI (density 1.95 g/ml, 1.0 ml) and heated (55°) for 6 min. At that time, the initial green color had become distinctly red. The reaction was quenched by pouring into 70 ml of ice water. The centrifuged precipitate was dissolved in chloroform. The solution was washed with aqueous sodium acetate and water and then dried ($MgSO_4$), and the solvents were evaporated *in vacuo*. The residual pheoporphyrin a_5 monomethyl ester was esterified with diazomethane and subjected to thin layer chromatography on silica (chloroform, petroleum ether). The material obtained after chromatography was chromatographically and spectroscopically (electronic, infrared, nmr, and mass spectra) identical with an authentic sample of pheoporphyrin a_5 dimethyl ester, which was prepared by Fischer's method[20] from pheophorbide a.

Spectroscopic Techniques. Electronic spectra were determined in ether using samples dissolved in a drop or two of tetrahydrofuran, with a Cary Model 14 recording spectrophotometer. Infrared spectra were obtained using a Perkin-Elmer ir spectrophotometer. The spectra of solutions were recorded in tetrahydrofuran, but because of the limited solubility of chlorophyll c and its derivatives, most of the spectra were obtained with the solid c using KBr pellets. Nmr spectra were recorded with a Varian HA-100 nmr spectrometer, equipped with a time-averaging device and internal frequency controls. All the spectra discussed in this paper were observed in trifluoroacetic acid or its deuterated analog. The spectra recorded for other solvents were entirely consistent with the TFA spectra; however, spectra of porphyrins in solvents other than TFA show stronger concentration dependence. Mass spectra were determined by use of the direct insertion system on an AEI MS-902 mass spectrometer. Accurate mass measurement was obtained by using the Nier peak matching system and the peaks from perfluorotri-*n*-butylamine as an internal standard.

Results

Our original conclusions concerning the structure of chlorophyll c[10] were based on spectroscopic data obtained on chlorophyll c and four of its derivatives, namely II, III, IV, and the HI reduction product. This reduction product, pheoporphyrin a_5 dimethyl ester, described above, unequivocally relates chlorophyll c to chlorophyll a and establishes a firm basis for discussion of the spectra and structure of the other derivatives. It has been pointed out by Holt[21] that the final methylation product of chlorophyll c, regarded before as mono- and divinylpheoporphyrin a_5 dimethyl ester 7′-methyl ether,[10] is most likely an isomeric chloroporphyrin c_6 trimethyl ester (III).[20] The spectroscopic evidence which follows clearly indicates that these compounds have the chloroporphyrin composition. As our compound appears to contain an isocyclic ring, we have designated it as an isochloroporphyrin, as noted above. For convenience, we have summarized the proposed structures for I–IV in Table II. We have presented the spectra of each of the four substances as a group to facilitate cross comparison.

Mass Spectra. Figure 1 presents the high-mass portions of low-resolution mass spectra of I–IV. The mass spectrum of pheoporphyrin a_5 dimethyl ester has been reported in a comprehensive survey of porphyrin mass spectra.[22] Chlorophyll c gives no appreciable mass spectrum at temperatures below 350°. At that temperature, the high-mass portion of the spectrum is virtually identical with that given by the mixture of pheoporphyrins c_5 esters (II). The mass spectrum of the pheoporphyrin mixture from chlorophyll c is not what one would expect from a porphyrin mixture[22] in that the molecule ions are not present in the spectrum. The group of high-mass peaks that resembles the molecule ion groups of porphyrins was centered at *m/e* 512. The masses of these ions corresponded to those of the anticipated parent ions, M, less the elements of methyl carbonate (CO_2 + CH_3OH) (see Table I). This extrusion reaction is eliminated when the acid side chain is modified (Figure 1, III and IV); however, it is not clear that the reaction is initiated by electron impact. The requirement of the acrylic acid function suggests structures like V for the *m/e* 510 and 512 ions.

Temperature dependence in the mass spectra is not restricted to chlorophyll c in this series. None of the compounds in this series are very volatile, and the relative abundance of ions in the singly and doubly ionized portions of the spectra appears to be critically dependent on temperature. The relative abundance of ions within either the singly or doubly ionized portions of the spectrum was, however, reasonably reproducible.

Neither chlorophyll c nor its pheoporphyrins gives a molecule ion even at low emission voltages. The nmr spectrum of II (see below) gave evidence of one more methyl group than allowed by an ion at *m/e* 512. Quantitative analysis for magnesium[23] in addition to changes in the imine proton resonances in the nmr required that the molecular weight of chlorophyll c be yet higher than that of the pheoporphyrins.

In our hands, attempted esterification of chlorophyll c with diazomethane resulted in formation of a complex mixture of products. Mass spectra of the mixture suggested that it contained the diazomethane adduct of the presumed acrylic ester side chain in addition to other products. The limited supplies of materials

(20) H. Fischer and A. Stern, "Die Chemie des Pyrrols, Band II, 2. Hälfte," Akademische Verlagsges, Leipzig, 1940, p 166.
(21) A. S. Holt, personal communication.
(22) A. H. Jackson, G. W. Kenner, K. M. Smith, R. T. Alpin, H. Budzikiewicz, and C. Djerassi, *Tetrahedron*, **21**, 2913 (1965).
(23) S. Granick, *J. Biol. Chem.*, **179**, 505 (1949).

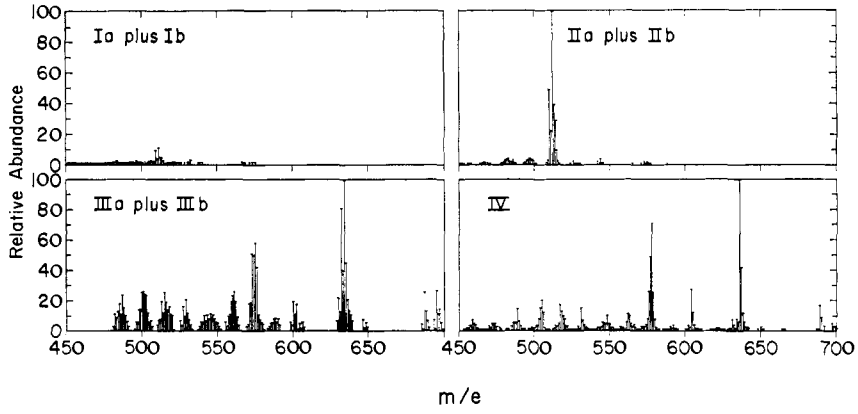


Figure 1. Low-resolution mass spectra of chlorophyll c and derivatives.

and the complexity of this mixture prompted us to explore other methylation procedures.

Treatment of the pheoporphyrins c_5 monomethyl esters (IIa and IIb) with dry methanolic hydrochloric acid resulted in formation of two easily separated products (X and III). The first (product X) was the more sorbed on sucrose columns and was difficult to characterize. Mass spectra of this product were highly variable. In one spectrum a low intensity "parent group" appeared at *m/e* 628–630. This group could correspond to the molecule ions of the dimethyl esters of IIIa and b or derivatives of pheoporphyrin a_5 monomethyl ether. Product X was converted into III on continued reflux in dry methanolic HCl. After 18 hr, the only major product of the reaction was III. The mass spectrum of this mixture is shown in Figure 1,III. The metal chelate impurity peaks in the *m/e* 680–700 region are common to all porphyrins and chlorins that have been handled with commercial solvents,[22] and we have, thus far, failed in attempts to obtain completely metal-free porphyrins after extensive handling. The chlorophyll c pheoporphyrins were substantially free of metal impurities (Figure 1,II) but all subsequent handling introduces metals, notably manganese, copper, nickel, and iron.

The parent group of III, like the *m/e* 510, 512 group in the chlorophyll c pheoporphyrins, showed two major peaks that persisted at low voltages (*m/e* 632, 634). The ratio of the abundance of these two molecule ions, when corrected for isotope peaks, was not constant from sample to sample, but varied from 1.1 to 1.9 with *m/e* 634 being consistently the larger of the two peaks.

The doubly ionized portion of the spectrum of III was generally somewhat lower in intensity than in most porphyrin spectra.[22] There were no ^{13}C isotope peaks at *m/e* 295.5 and 296.5, which would have corresponded to the isotope peaks for loss of CH_2CO from the doubly ionized molecule. It has been suggested that loss of CH_2CO from doubly ionized porphyrins is characteristic of propionic ester side chains.[22] The absence of these peaks provided a clear indication that there was no propionic ester side chain in III.

Although chlorophyll c, and its derivatives II and III, were homogeneous on sucrose chromatography, the evidence above clearly indicated that these substances were mixtures of two compounds that differed in their state of reduction. The following evidence suggests that these compounds differ only in the oxidation state of their side chains.

The pheoporphyrin c_5 mixture was reduced with hydrogen–palladium on carbon in TFA solution at 1 atm, because these conditions smoothly reduce vinyl side chains but do not reduce porphyrin rings or substituents that require more vigorous conditions than a vinyl group for reduction.[24] Subsequent methylation with methanolic hydrochloric acid produced IV, which appeared to be one compound by both mass spectra (Figure 1,IV) and chromatography. The change in mass for the molecule ion to *m/e* 636 corresponded to addition of 2 or 4 hydrogen atoms to the components of III.

The mass spectral fragments that indicate the loss of CH_3O (605) and $C_2H_2O_2$ (578) correspond to the analogous fragmentation of III (see Table I). These cleavage reactions would be expected if III and IV had structures similar to the esters of pheoporphyrin a_5.

The doubly charged spectrum of IV (Figure 2) was substantially different from that of pheoporphyrin a_5. This fact alone strongly suggests that the carbon skeletons are different in the two compounds. For a given accuracy of mass measurement, the uncertainty in assignment of empirical formulae is twice as large for doubly charged ions as their singly charged counterparts. For this reason, we have simply listed the most probable neutral fragments for each major doubly charged ion. Most of the doubly charged ionic compositions can be rationalized by single or successive β-cleavage reactions or single rearrangements. This is not true for the ion at *m/e* 272, which corresponded to loss of 2 mol of formic acid from the doubly charged molecule ion. Given the formulation of IV, the sequence leading to this ion (*m/e* 272) can be rationalized by sequential rearrangements that ultimately lead to a doubly charged porphyrin with an annelated methyl tropillium between the 7 and δ positions.

None of the compounds mentioned thus far have provided a firm basis for establishing the molecular weight of the chlorophyll c nucleus. It was hoped vigorous reduction of chlorophyll c with high density HI would reduce both the presumed acrylic acid and vinyl side chains of this compound, and provide the basis for examining the porphyrin nucleus unadorned, save extra hydrogen atoms. After reduction, methyla-

(24) R. C. Dougherty, unpublished results.

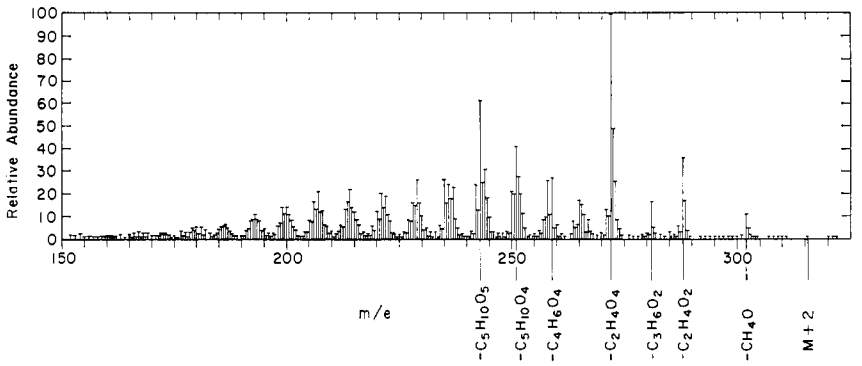


Figure 2. Doubly charged region of the low-resolution mass spectrum of *meso*-isochloroporphyrin c_6 trimethyl ester (IV).

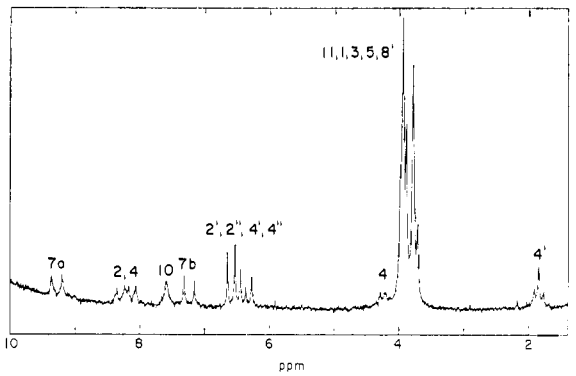


Figure 3. Nmr spectrum (~0.1 *M* in TFA) of chlorophyll c (Ia + Ib).

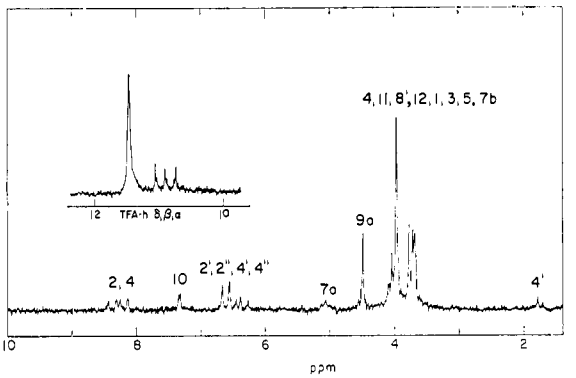


Figure 5. Nmr spectrum (~0.1 *M* in TFA-*d*) of isochloroporphyrins c_6 trimethyl esters (IIIa + IIIb).

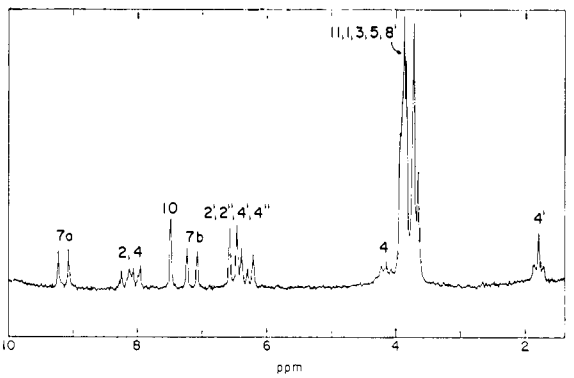


Figure 4. Nmr spectrum (~0.1 *M* in TFA) of pheoporphyrins c_5 monomethyl esters (IIa + IIb).

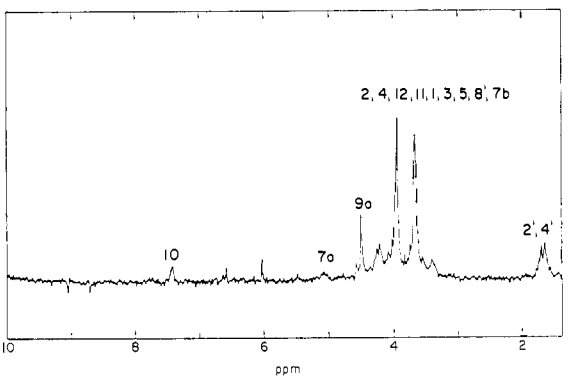


Figure 6. Nmr spectrum (~0.004 *M* in TFA) of *meso*-isochloroporphyrin c_6 trimethyl ester (IV).

tion with diazomethane, and separation, pheoporphyrins c_5 yielded a mixture of porphyrins, which contained a compound identical in every respect with pheoporphyrin a_5 dimethyl ester. The spectroscopic and chromatographic identity of the two samples of pheoporphyrin a_5, one obtained from chlorophyll a_5[20] and one obtained from chlorophyll c, clearly established the relationship between the two substances.

Nmr Spectra. The nmr spectra of I–IV are illustrated in Figures 3–6. With the exception of the spectrum of III (Figure 5), which was recorded in TFA-*d*, all spectra were recorded in TFA. Under these conditions, the porphyrin methine hydrogens (α,β,δ) are obscured by the TFA resonance; however, the exchangeable hydrogens, notably the C-10 hydrogen, are visible. Recording porphyrin spectra in TFA has a number of advantages, which were first pointed out by Abraham, *et al.*[25] The most important of these is the fact that porphyrin spectra in TFA are virtually concentration independent. Thus, the spectra of chlorophylls c, the chlorophyll c pheoporphyrins, and their isochloroporphyrin derivatives (Figures 3–5), which were taken at 0.10 *M* concentrations, could be directly compared with spectra of the much less readily available samples of IV (Figure 6), which was recorded at concentrations of 0.04 *M*.

Imine proton resonances were observed as two peaks in the region +4.3 to +4.5 ppm from internal TMS for II–IV, and not for chlorophyll c itself. Other than confirming the metal-free porphyrin nature of these

(25) R. J. Abraham, A. H. Jackson, and G. W. Kenner, *J. Chem. Soc.*, 3468 (1961).

substances, the imine resonances offer little structural information. For these reasons, we have not included the imine resonances in Figures 3–6.

The low-field methyl region[26,27] of chlorophylls and pheoporphyrins c (Figures 3 and 4) is very narrow in TFA (relative area 15), indicating the absence of chlorin hydrogens or oxygenated side chains such as $-CHORCH_3$, $-CH_2CO_2R$, or $-CH_2CH_2CO_2R$. The chemical shifts of the macrocyclic methine protons of I in TFA-*d* (11.01, 11.03, 11.16 ppm) also exclude chlorin- or formyl-substituted porphyrin structures for chlorophyll c. The narrowness of the low-field methyl region of I virtually excluded definitive assignments of those resonances; however, the remaining resonances in the spectrum may be easily assigned on the basis of the large volume of previously published work in this field.[26] The low and somewhat variable intensity triplet at 1.85 ppm (**4'**) [one can visibly discern intensity differences in the spectra of I and III (Figures 3 and 5), which were prepared from different diatom extracts] has the proper chemical shift, but not the proper intensity (relative area 1.6) for the methyl resonances of a porphyrin ethyl side chain. The partially distinct low-intensity quartet at 4.26 ppm (**4**), which double resonance showed to be coupled with the triplet, corresponds to the ethyl methylene. The relative intensity of both of these groups is consistent with the mass spectral data, which indicated that chlorophyll c was a mixture of two compounds, differing by two hydrogen atoms, the reduced form being 1–2 times as abundant as its unsaturated counterpart. These nmr data clearly suggest that the two compounds differ in the reduction of a porphyrin vinyl side chain.

In the low-field portions of the nmr spectra, chlorophyll c and its derivatives showed three distinct methine resonances. For spectra taken in TFA-*d* there were small satellites on the methine peaks (10.7, 10.9, 11.1 ppm, Figure 5) with up to half the intensity of the major peak. Similar satellites have previously been observed in chlorophyll a nmr spectra and assigned to diastereoisomerism involving position 10.[28] Diastereoisomerism is clearly possible in III and IV, but chlorophyll c is clearly a planar porphyrin and not a chlorin, which suggests that either the existence of other asymmetric centers in the molecule is not essential to the effect of the ring V carbomethoxy group on chemical shifts around the ring, or the satellites arise from the low abundance component of the mixture. The latter explanation seems the more likely inasmuch as chlorophyll c is almost certainly racemic.

The doublet (J = 16.8 Hz) at 9.30 ppm (**7a**) (Figures 3 and 4) is coupled to the doublet at 7.28 ppm (**7b**). Each of these peaks had a relative area of 1, when compared with one of the methine resonances. This coupling constant is consistent with that required for a *trans*-substituted vinyl group. The chemical shifts, roughly 1 ppm below the normal vinyl region,[26] require that the nonporphyrin substituent be highly electronegative. Biosynthetic considerations,[29] as well as information about the reactivity of this group, also require that it be a *trans*-acrylate residue. Moreover, this set of two resonances does not appear in the spectrum of III (Figure 5), compounds for which the mass spectral data suggest that this unsaturation has been removed.

The pattern which centers at about 8.2 ppm and the larger one at 6.4 ppm coupled with it appear in the spectra of I–III but not in IV. The chemical shifts and double resonance experiments indicate that these patterns represent normal and porphyrin vinyl resonances.[26] The distortion from a standard ABX pattern[30] may be quantitatively accounted for by assuming the presence of only two nonequivalent vinyl groups in the ratio of 1:0.5. The coupling constants which satisfied all of the experimental data were A_1X_1 = 12.0 Hz; A_2X_2 = 11.7 Hz; B_1X_1 = 18.5 Hz; B_2X_2 = 18.5 Hz; $A_1B_1 \sim A_2B_2 < 1.8$ Hz.

The slightly broadened peak at 7.6 ppm in the spectrum of chlorophylls c was slightly shifted upfield in the spectrum of the pheoporphyrins. This peak was rapidly diminished when the hydrogen of these compounds was exchanged with TFA-*d*. This resonance has all the characteristics of a C-10 hydrogen.[26] The corresponding peak in the spectra of III and IV was shifted by approximately 0.3 ppm to higher field. This chemical shift and the fact that this resonance, in III and IV, is only slowly diminished in TFA-*d* suggest the occurrence of a fundamental change in the structure of ring V in the transformation of II to III or IV. The partially resolved doublet structure of the C-10 resonance in both III and IV (J < 2 Hz) is compatible[30] with either *cis* or *trans* orientation to another hydrogen atom in a five-membered ring fused to the porphyrin structure.

The two methylated derivatives of chlorophylls c (III and IV) show considerable expansion of the "low-field methyl region."[26] The sharp singlet at 4.49 ppm (Figure 5, relative area 3) has too low a chemical shift to be associated with a porphyrin propionic acid ester group. It seems unlikely that even a derivatized propionic acid ester would be shifted to this extent. The chemical shift is, however, quite reasonable for a porphyrin carboxylic acid ester. The chemical shift of the ester methylene at position 6 in 6-ethoxycarbonyl-2,3-diethyl-7-methoxycarbonylethyl-1,4,5,8-tetramethylporphyrin in TFA was 5.01 ppm.[31] The chemical shift of the methyl ester at position 6 in chlorin c_6 trimethyl ester is 4.28 ppm in a nonpolar solvent.[32] The assignment of the 4.5 ppm resonance to a porphyrin carboxylic acid methyl ester is thus reasonable, and any other assignment would be difficult to rationalize. The triplet, at 5.1 ppm (Figure 5), and the resonance coupled to it, which lies under the packet of methyl resonances between 3.6 and 4.0 ppm, may be easily assigned to the **7a** and **7b** resonances in the isocyclic ring in structure III.

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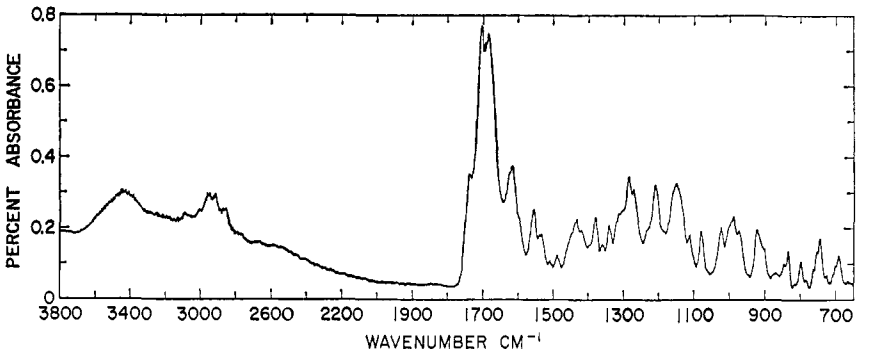


Figure 7. Infrared absorption spectrum of chlorophylls c (KBr disk).

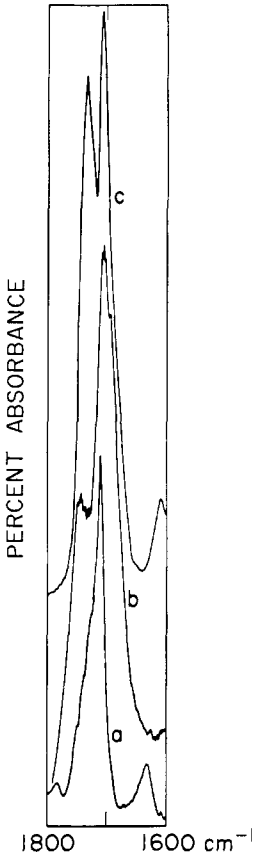


Figure 8. Infrared spectra in THF of (a) Ia + Ib; (b) IIIa + IIIb; (c) IV.

Infrared Spectra. The limited solubility of chlorophylls c and most of their derivatives has precluded examination of solvent dependence of their infrared spectra. Figure 7 presents the infrared spectrum of chlorophylls c in a KBr disk (1 mg/200 mg of KBr). The spectrum of the magnesium-free pheophorbides was very similar with the exception of minor variations in the fingerprint region and the addition of N–H absorption at 3260 cm^{-1}. In the solid state the carbonyl region is somewhat broadened;[26] however, the assignment of the absorption between 1700 and 1740 cm^{-1} to ketone and ester carbonyls is consistent with other data. The very broad absorption in the 2500–3000 cm^{-1} region is also consistent with the presence of a carboxylic acid function in the molecule.

Figure 8 illustrates the infrared absorption in the carbonyl region for Ia plus Ib, IIIa plus IIIb, and IV dissolved in THF. Even in a "disaggregating" solvent like THF, the chlorophyll c carbonyl peaks are indistinct and broadened. This is partially due to the free carboxylic acid residue; however, the major factor must be the presence of the central magnesium, because the spectrum of II is much sharper than that of I. The carbonyl absorption in III and IV is clearly split; however, the splitting cannot provide a substantial amount of structural information without a detailed analysis of aggregation behavior and other factors which critically affect porphyrin infrared absorption.[26]

Electronic Spectra. The electronic spectral values for I–IV are recorded in Table III. The absorption maxima are within 5 nm of reported values for I and II.[9,11] This is an acceptable error range in view of the solvent dependence of porphyrin and chlorin spectra. The extinction coefficients for I and II are obtained as averages for three separate preparations. The mixture of IIIa and IIIb gave a spectrum of mixed type, which indicates that electronic spectra by themselves are not very definitive. Compound IV had a clearly aetio type spectrum. This spectrum is compatible with a structure that is electronically related to chloroporphyrin c_4,[20] and it is definitely incompatible with a structure which is related to pheoporphyrin a_5.

Distribution of Chlorophylls c. In preface to an investigation of the state of hydrogenation of chlorophyll c in different organisms we have isolated crystalline chlorophyll c from a variety of organisms (see Table IV). These results establish the wide distribution of chlorophyll c in the brown algae, of various taxonomic classes, collected on the Monterey Peninsula, Calif.

Discussion

Jeffrey[9] separated two chlorophyll c components, c_1 and c_2, from algal extracts by chromatography in columns of cellulose followed by chromatography on thin layers of a special sorptive polyethylene, but additional supplies of this latter sorbent were unobtainable. Another selective, sorptive polyethylene was recently prepared by the Koppers Co., which provided us with samples a few weeks before proof for this report was received. With columns of this selective polyethylene, now marketed by Polysciences Inc., Warrington, Pa., and with acetone as the wash liquid, we have separated our crystalline c mixture obtaining the c_1 and c_2 described by Jeffrey. The nmr shows the less sorbed c_1 to be the monoethyl monovinyl compound Ia and the more sorbed c_2 to be the divinyl compound Ib.

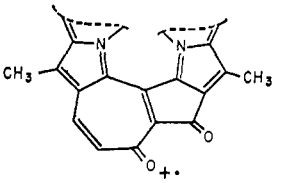
Table III. Electronic Spectra of Solvent-Free Chlorophylls c and Derivatives in Diethyl Ether

Chlorophylls c (Ia + Ib)		Pheoporphyrins c_5 monomethyl esters (IIa + IIb)		Isochloroporphyrins c_6 trimethyl esters (IIIa + IIIb)		*meso*-Isochloroporphyrin c_6 trimethyl ester (IV)	
λ_{max} (nm)	log ϵ	λ_{max} (nm)	log ϵ	λ_{max} (nm)	Order	λ_{max} (nm)	Order
627	4.563	592	4.24	636	2nd	627	4th
579	4.530	569	4.30	588	4th	579	3rd
		531	4.25	548	3rd	565	2nd
				512	1st	515	1st
447	5.511	430	5.34	411		413	

Table IV. Brown Algae (Phaeophyta) from Which Crystalline Chlorophyll c Has Been Isolated

Class	Subclass	Order	Family	Species
Cyclosporeae		Fucales	Fucaceae	*Fucus furcatus*
Cyclosporeae		Fucales	Fucaceae	*Pelvetia fastigiata*
Cyclosporeae		Fucales	Sargassaceae	*Cystoseira osmundaceae*
Heterogeneratae	Haplostichineae	Chordariales	Corynophloeaceae	*Leathesia difformis*
Heterogeneratae	Polystichineae	Laminariales	Alariaceae	*Egregia menziesii*
Heterogeneratae	Polystichineae	Laminariales	Laminariaceae	*Laminaria andersonii*
Heterogeneratae	Polystichineae	Laminariales	Lessoniaceae	*Macrocystis integrifolia*
Heterogeneratae	Polystichineae	Laminariales	Lessoniaceae	*Nereocystis luetkeana*
Heterogeneratae	Polystichineae	Laminariales	Lessoniaceae	*Postelsia palmaeformis*
Isogeneratae		Ectocarpales	Heterochordariaceae	*Heterochordaria abietina*

The mass spectrum of IV and the conversion of chlorophyll c to pheoporphyrin a_5 dimethyl ester along with nmr evidence provide a clear basis for establishing the molecular weight of chlorophyll c as that corresponding to the structure Ia and Ib. The existence of ring V in chlorophyll c is strongly supported by nmr evidence, as is the existence of the acrylic acid side chain.



The conversion of II to III by treatment with acid in a nucleophilic solvent (methanol) provides strong support for the positional isomer indicated in structure I. The occurrence of this reaction fixes the relationship of the acrylic acid side chain to ring V, and by analogy confirms the porphyrin type III structure of chlorophyll c. The placement of the other groups, whose existence has been indicated by nmr and mass spectroscopy, around the ring is based upon comparison of the pheoporphyrin a_5 dimethyl ester prepared from chlorophylls c and a and upon analogy with chlorophyll a and other porphyrin natural products that presumably share common biosynthetic pathways[29] with chlorophyll c.

The existence of an acrylic acid side chain in chlorophyll c coupled with the observation that the hydrogens at positions 7′ and 7′′ exchanged with the medium during the biosynthesis of bacteriochlorophyll in D_2O[33,34] supports the hypothesis[16] that chlorophyll c-like intermediates may be involved in the normal biosynthetic pathways of the other chlorophylls. The hydrogen exchange at 7′ and 7′′ in bacteriochlorophyll, obtained from organisms grown in D_2O with hydrogen substrates,[34] is also related to the question of isomer type of chlorophyll c. Chlorophyll c's acrylate side chain would be a very reasonable intermediate for the hydrogen exchange, and a corresponding unit at C-6 would provide for the formation of ring V.[35,36]

The problem of the structure of chlorophyll c and its derivatives could not have been solved with the application of only one or two spectroscopic techniques. Now that techniques for dealing with extremely small samples are available, it would be definitely worthwhile to obtain nmr and mass spectral data for other known chlorophyll or bacteriochlorophyll precursors, which are widely distributed in nature.

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