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CHLOROPHYLLS c_1 AND c_2*

HAROLD H. STRAIN, BENJAMIN T. COPE, JR., GERALDINE N. MCDONALD, WALTER A. SVEC and JOSEPH J. KATZ

Chemistry Division, Argonne National Laboratory, Argonne, Illinois 60439, U.S.A.

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Abstract—Chlorophyll *c* has been separated into its components by chromatography on polyethylene, and the structures of the components established by proton magnetic resonance. The less sorbed chlorophyll c_1 is magnesium tetradehydropheoporphyrin a_5 monomethyl ester, and the more sorbed chlorophyll c_2 is magnesium hexadehydropheoporphyrin a_5 monomethyl ester. The relative amounts of the two components in preparations of *c* from diatoms and from several species of brown algae have been determined by chromatography and from the nuclear magnetic resonance spectra. The ratio, c_1/c_2, is about 0·6 in the various chlorophyll *c* preparations.

RECENT investigations[1–3] have led to the conclusion that chlorophyll *c*, as isolated by the usual chromatographic procedures based upon adsorption on mild adsorbents,[4] is a mixture of magnesium tetradehydro- and hexadehydropheoporphyrin a_5 monomethyl ester (Fig. 1). With a remarkably selective chromatographic method, utilizing specially-prepared, sorptive polyethylene power, Jeffrey[5,6] was able to separate chlorophyll *c* into two components, c_1 and c_2. We have repeated and confirmed these chromatographic results, and by means of NMR spectroscopy, we have found that c_1 is the tetradehydro compound and that c_2 is the hexadehydro compound.

In the course of our work, we have acquired considerable new NMR data on chlorophyll *c* and on its components prepared from several species of algae. All the previously reported NMR spectra for chlorophyll *c* were determined in trifluoracetic acid solution. In this solvent, unfortunately, the low-field methine proton resonances are obscured; consequently, we have now determined spectra for *c* in tetrahydrofurane-d_8 and pyridine-d_5, wherein the important methine proton resonances are readily detectable. With the aid of a curve resolver, it is possible to use spectra recorded in these solvents to deduce the relative amounts of the components in a chlorophyll *c* mixture.

The new NMR data clearly show that one component, c_1, has one vinyl group, whereas the other component, c_2, has two (Table 1). Primarily, for reasons of consistency with the prior literature, we shall continue to use the chlorophyll c_1 and c_2 designations originally employed by Jeffrey.[5,6]

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[1] R. C. DOUGHERTY, H. H. STRAIN, W. A. SVEC, R. A. UPHAUS and J. J. KATZ, *J. Am. Chem. Soc.* **88**, 5037 (1966).
[2] R. C. DOUGHERTY, H. H. STRAIN, W. A. SVEC, R. A. UPHAUS and J. J. KATZ, *J. Am. Chem. Soc.* **92**, 2826 (1970).
[3] J. W. F. WASLEY, W. T. SCOTT and A. S. HOLT, *Can. J. Biochem.* **48**, 376 (1970).
[4] H. H. STRAIN and W. A. SVEC, in *The Chlorophylls* (edited by L. P. VERNON and G. R. SEELY), p. 57, Academic Press, New York, New York (1966).
[5] S. W. JEFFREY, *Biochim. Biophys. Acta* **162**, 271 (1968).
[6] S. W. JEFFREY, *Biochim. Biophys. Acta* **177**, 456 (1969).

TABLE 1. CHEMICAL SHIFTS (δ, ppm)* IN CHLOROPHYLLS c_1 AND c_2 IN TETRAHYDROFURANE-d_8†

Proton	c_1‡ (0·038 M)	c_1§ (0·02 M)	c_1¶ (0·031 M)	c_2‡ (0·060 M)	c_2§ (0·02 M)	c_2¶ (0·051 M)
Methines	9·93	9·97	9·95	10·08	10·07	10·16
	9·89	9·91	9·90	9·98	9·98	10·03
	9·76	9·85	9·80	9·89	9·88	9·98
Acrylate 7a, 7b	8·87	8·98	8·83	9·00	8·93	9·04
	6·58	6·64	6·60	6·66	6·66	6·69
Vinyl I‖	8·29	8·31	8·25	8·31	8·38	8·31
	6·34	6·35	6·34	6·35	6·35	6·36
	6·04	6·05	6·04	6·05	6·06	6·06
Vinyl II**						
2				8·31	8·38	8·31
2′				6·31	6·32	6·33
2″				6·03	6·04	6·05
C-10	6·68	6·80	6·72	6·82	6·80	6·89

* Relative to hexamethyldisiloxane as internal standard.
† Only the protons at fields lower than 5·00 ppm are assigned.
‡ Ex. *Nereocystis luetkeana.*
§ Ex. *Nitzschia closterium.*
¶ Ex. *Fucus furcatus.*
‖ In chlorophyll c_1: $|J_{2',\,2''}|$, 2·0 Hz; $|J_{2,\,2'}|$, 11·5 Hz; $|J_{2,\,2''}|$, 18·7 Hz.
** In chlorophyll c_2: Vinyl I, $|J_{2,\,2''}|$, 2·0 Hz; $|J_{2,\,2'}|$, 11·8 Hz; $|J_{2,\,2''}|$, 17·8 Hz.
Vinyl II, $|J_{2',\,2''}|$, 1·8 Hz; $|J_{2,\,2'}|$, 11·7 Hz; $|J_{2,\,2''}|$, 17·7 Hz.

Chlorophylls c_2 *and* c_2

NMR chemical shift data for highly purified chlorophylls c_1 and c_2 are given in Table 1. The less sorbed (more rapidly moving fraction on polyethylene) designated chlorophyll c_1, has only one vinyl group, whereas the more sorbed c_2 has two. Both components contain the acrylate moiety, thus excluding the possibility that the mass difference of 2 hydrogen

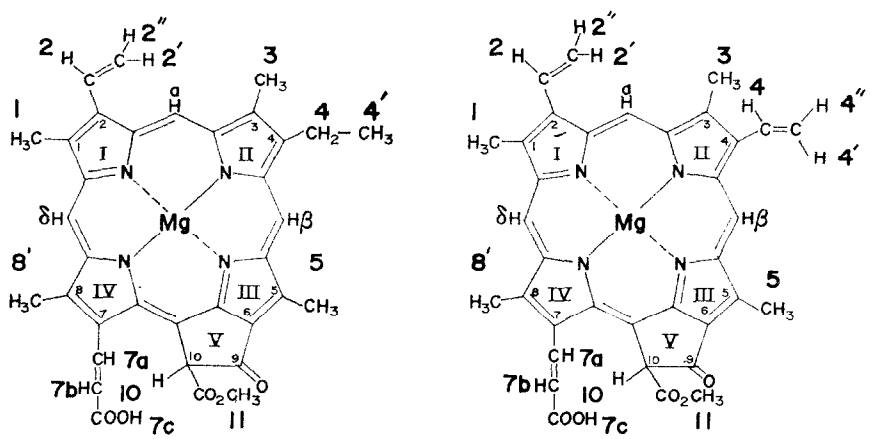

FIG. 1. STRUCTURAL FORMULAE AND PROTON DESIGNATIONS FOR CHLOROPHYLLS c_1 AND c_2. IN THESE FORMULAE, THE SIDE CHAINS ARE LOCATED ENTIRELY BY ANALOGY WITH CHLOROPHYLL *a*.

atoms between c_1 and c_2 is the result of the presence in chlorophyll c_1 of two vinyl groups and a propionic acid side-chain rather than an acrylic acid side-chain. The chemical shifts and coupling constants of the vinyl protons (Table 1, Footnotes || and **) in chlorophylls c_1 and c_2 are entirely consistent with chemical shifts and coupling constants observed for the vinyl group in methyl pheophorbide *a*.[7]

Chlorophyll c_1 contains a $-CH_2CH_3$ group and should, therefore, possess a high field resonance arising from the methyl protons of this group. In a c_1 sample (in pyridine-d_5), high field resonances were observed at 1·79, 1·75, 1·67, and 1·60 ppm (relative to HMS as internal standard). In a c_2 sample only the resonance at 1·78 ppm can be seen. Thus, the triplet observed in c_1 centered at 1·78 ppm is assigned to the methyl protons of the ethyl group, which is present as expected in c_1 but absent in c_2. We have previously reported the chemical shift of these protons to be 1·85 ppm in chlorophyll *c* in trifluoracetic acid.[2] This difference in chemical shift is attributed to concentration and solvent effects, and to possible interactions between c_1 and c_2 in chlorophyll *c* mixtures.

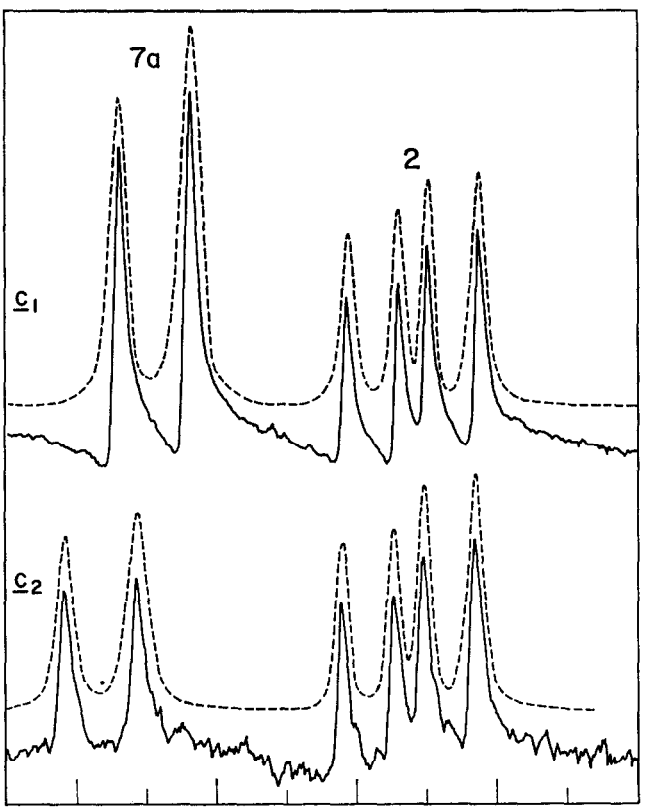

FIG. 2. COMPARISON OF ACRYLATE PROTON (PROTON 7a) TO VINYL PROTONS (2) IN CHLOROPHYLL c_1 AND c_2.
(———) NMR spectra; (– – – –) deconvolution on DuPont 310 curve resolver. The ratio obtained from curve analysis is 2:1 for proton 2:proton 7a in chlorophyll c_2 and the ratio is 1:1 in chlorophyll c_1. The NMR spectra were not collected at equal gain. Chemical shifts are given in Table 1.

[7] J. J. KATZ, R. C. DOUGHERTY and L. J. BOUCHER, in *The Chlorophylls* (edited by L. P. VERNON and G. R. SEELY), Chapter 7, pp. 224–228, Academic Press, New York, New York (1966).

We have compared the area of the high-field acrylate resonances (1 proton, 8·87 ppm) with the area of the 2 proton of the vinyl group (Fig. 2). For chlorophyll c_1, the ratio is very close to 1, confirming the presence of only one vinyl group in this component, whereas in c_2, the ratio is 2, indicative of the presence of two vinyl groups in this substance.

Chlorophyll c *Mixture*

The NMR spectra of the chlorophyll *c* mixture extracted from seven species of algae[2,4] provided the results recorded in Table 2. All the chlorophyll *c* preparations show the expected six methine resonances, three for each component.

We have used integration to estimate the relative amount of c_1 and c_2 in chlorophyll *c* mixtures. Because the methine proton resonances overlap to some extent, we have resolved the methine proton resonances into Lorentzian components and estimated the amounts of the two components from the areas of the deconvoluted curves. The results (Table 2) are in reasonable agreement with yields of c_1 and c_2, obtained from chlorophyll *c* by chromatography. The resolving and integrating procedure is illustrated in Fig. 3.

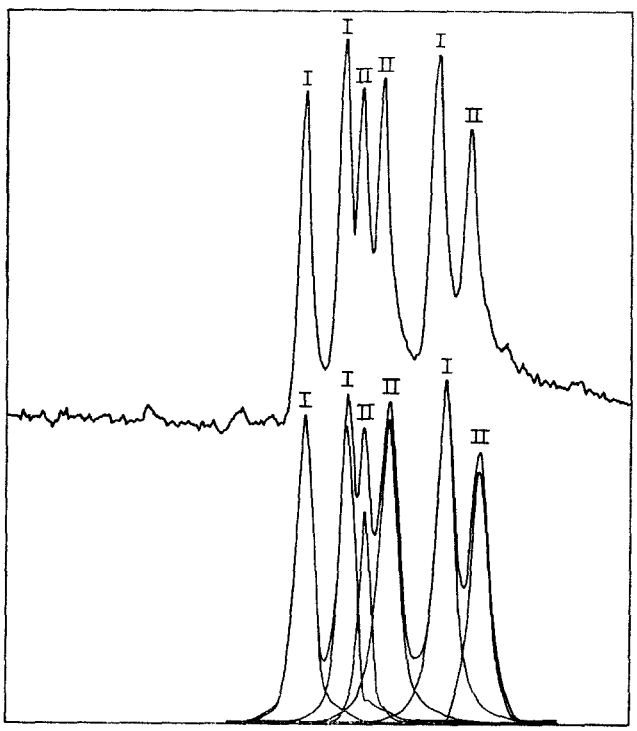

FIG. 3. DETERMINATION OF c_1/c_2 RATIO IN CHLOROPHYLL *c* FROM *Nereocystis luetkeana* METHINE RESONANCES BY DUPONT 310 DECONVOLUTION.
(I), c_2; (II), c_1. Ratio given in Table 2. Chemical shifts are given in Table 1.

The *c* in all the algae that we examined proved to be a mixture of c_1 and c_2 in the ratio of c_1/c_2 of about 0·6. In other remotely related species, however, c_2 has been found with little or no c_1.[5,6]

All the algae that contain chlorophyll *c* contain *a* as the principal pigment. Those with c_1 and c_2 thus contain three green pigments. The spectral absorption properties of c_1 and

TABLE 2. CHEMICAL SHIFTS (δ, ppm) IN CHLOROPHYLLS *c* FROM DIFFERENT SOURCES (TETRAHYDROFURANE-d_8, HMS INTERNAL STANDARD)

Proton	*Fucus furcatus* (0·097 M)	*Pelvetia fastigiata* (0·088 M)	*Leathesia diformis* (0·122 M)	*Macrocystis integrifolia* (0·076 M)	*Nereocystis luetkeana* (0·091 M)	*Postelsia palmaeformis* (0·050 M)	*Nitzschia closterium* (0·068 M)	*Nitzschia closterium* (0·083 M)
Methines	10·06	10·03	10·04	10·07	10·00	10·04	10·05	10·05
	9·97	9·95	9·96	9·97	9·95	9·96	9·97	9·97
	9·95	9·93	9·93	9·95	9·92	9·94	9·95	9·95
	9·89	9·89	9·89	9·88	9·89	9·90	9·91	9·91
	9·86	9·83	9·84	9·87	9·80	9·85	9·85	9·86
	9·82	9·78	9·80	9·83	9·75	9·80	9·81	9·81
Acrylate 7a, 7b	8·96	8·90	8·95	8·98	8·94	8·95	8·93	8·93
	6·64	6·61	6·65	6·65	6·61	6·63	6·66	6·63
	6·62	6·58	6·63	6·63	6·59	6·61	6·63	6·58
Vinyl								
2	8·30	8·29	8·30	8·32	8·30	8·29	8·29	8·31
2′, 2″	6·18	6·19	6·19	6·20	6·20	6·19	6·19	6·19
C-10	6·80	6·74	6·78	6·79	6·72	6·76	6·76	6·78
c_1/c_2	0·55*			0·30*	0·60*			0·41*
		0·67†	0·64†	0·59†	0·63†	0·63†	0·72†	0·63†

* By chromatography.
† By integration of the methine proton resonances.

c_2 in the visible spectrum are remarkably similar. In deep water, which absorbs blue and red light, the absorption by the *c* components supplements that of the *a* in all these species.[2] The production, maintenance, and function of these pigments in the various algae pose important new problems in photosynthesis and ecology.

EXPERIMENTAL

NMR spectra were recorded on a Varian HA-100 spectrometer fitted with a C-1024 time averaging device for signal–noise enhancement. Chemical shifts are given in δ, parts per million (ppm) relative to hexamethyldisiloxane (HMS) as an internal standard. Deconvolution of the spectra was carried out with a Du Pont 310 curve resolver, and the relative areas of the Lorenztian components read from the curve resolver integrator.

Crystalline chlorophyll *c* mixture was prepared from the algal extracts as previously described. About 0·5 kg of diatoms and 2 kg of the brown algae were employed for each preparation. The crystalline chlorophyll *c* mixture was obtained as the *bis*-tetrahydrofuranate after separation from tetrahydrofurane upon the addition of light petroleum.[1,2,4] The yields varied from *ca.* 20 to 50 mg.

For preparation of c_1 and c_2, the *c* mixture, about 30 mg, was dissolved in 2 ml warm tetrahydrofurane, which was then diluted with warm acetone, 60 ml. This solution was sorbed in two columns, about 5 × 35 cm of the specially-prepared polyethylene,[2] and the sorbed pigments, in a zone about 2 cm deep, were washed with acetone. Traces of a very weakly-sorbed pigment with the spectral properties of c_1 were carried through and discarded. This was followed by the c_1, also washed through. The upper portions of the adsorbent, about 17 cm, were removed and washed with tetrahydrofurane providing traces of pigment also with the spectral properties of the c_1.

The c_2 in the lower half of the column was eluted with tetrahydrofurane. The solutions of c_1 and c_2 were evaporated to dryness in a rotary evaporator. The green residues were dissolved in tetrahydrofurane, about 0·5 ml, leaving a white, water-soluble residue. The green solution was centrifuged, decanted and diluted with light petroleum. It was cooled overnight with solid CO_2, and the crystals that separated were collected, dried in vacuum and weighed. The sum of the c_1 plus the c_2 was 60–80% of the original mixture. The spectral absorption maxima and the absorption ratios were very close to the average of the values reported by Jeffrey.[6]

The polyethylene employed for the c_1–c_2 separation was recovered by extraction with tetrahydrofurane followed by washing with acetone and drying in air. Even after several uses, the sorption capacity and the selectivity of the polyethylene were unaltered.

The plurality of chlorophyll *c* was indicated by our experiments with sugar columns over 30 years ago. With extracts of diatoms and brown algae and with lightly loaded columns, the *c* usually separated into two principal, contiguous yellow-green zones and several very minor zones. Readsorption of the eluted *c*, using heavily loaded sugar columns, provided less effective separations than adsorption of the extracts themselves; hence, this procedure was not suitable for the isolation of the two principal components, now readily separable with the special polyethylene.