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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC STUDY OF THE CHLOROPHYLL ALLOMERIZATION REACTION*

PETER M. SCHABER*
Department of Chemistry, Canisius College, 2001 Main Street, Buffalo, NY 14208 (U.S.A.)
JERRY E. HUNT
Chemistry Division, Argonne National Laboratory, 9700 S. Cass Avenue, Argonne, IL 60439 (U.S.A.)
RICHARD FRIES
Northern Petrochemical Company, Technical Center, Morris, IL 60450 (U.S.A.)
and
JOSEPH J. KATZ
Chemistry Division, Argonne National Laboratory, 9700 S. Cass Avenue, Argonne, IL 60439 (U.S.A.)

SUMMARY

The allomerization of chlorophyll has been investigated by reversed-phase HPLC. Four major product peaks (two sets of doublets) are observed in the HPLC chromatogram. These are shown to be pairs of stereoisomers of two major components. Similar results have been obtained from the allomerization of fully deuterated chlorophyll *a*. Attempts to characterize the reaction products and to delineate the reaction mechanism were initially studied by the oxidation of chlorophyll in the absence of extraneous nucleophiles. The structure of the reaction products of chlorophyll allomerization were conclusively identified as 10-hydroxychlorophyll and the 10-methoxylactone by co-chromatography, NMR, and ^{252}Cf plasma-desorption mass spectroscopy. Exploratory studies on the allomerization products of bacteriochlorophyll have also been carried out.

INTRODUCTION

The chlorophylls are a small group of closely related compounds (Fig. 1) which act as the primary light receptors in photosynthesis, as energy transfer agents, and as the central participants in the photoreaction center where light energy is converted to chemical oxidizing and reducing potential. Until quite recently, no convenient methods for determining the purity of chlorophyll preparations were available. Practical analytical procedures are a necessity because the chlorophylls, under relatively mild conditions, are subject to many kinds of alterations. These include, among

* Work carried out at: Chemistry Division, Argonne National Laboratory, 9700 S. Cass Avenue, Argonne, IL 60439, U.S.A.

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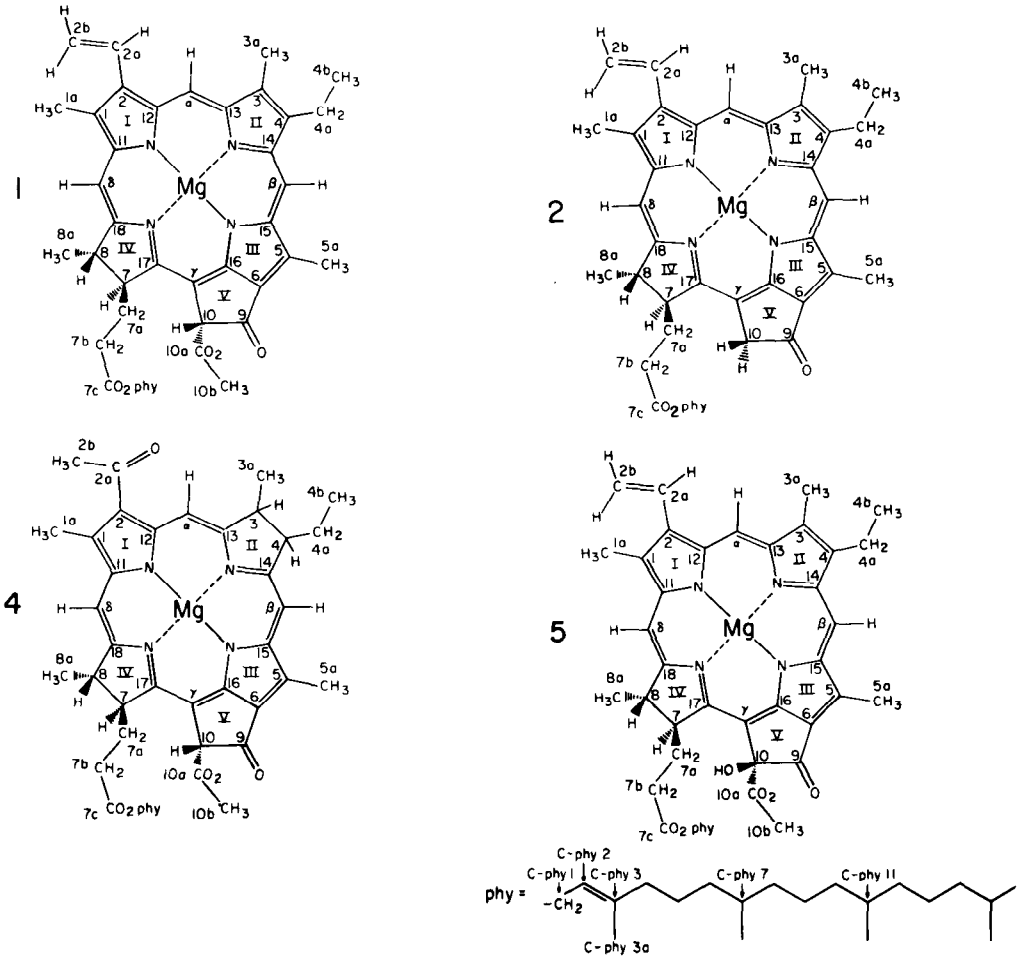


Fig. 1. Structures of the chlorophylls. 1 = Chlorophyll *a* (Chl*a*); 2 = pyrochlorophyll *a* (Pyrochl*a*); 3 = chlorophyll *b* (Chl*b*); 4 = bacteriochlorophyll *a* (Bchl*a*); 5 = 10-hydroxychlorophyll *a* (10-OH Chl*a*); 6 = 10-Methoxylactone of chlorophyll *a* (10-MeO lactone Chl*a*). Phy indicates a phytyl alcohol chain. Replacement of the Mg atom by 2H forms the corresponding pheophytin. Replacement of the phytyl group by a methyl group forms the Mg-containing methyl chlorophyllides or the Mg-free methyl pheophorbides. Chls *a* and *b* and Bchl *a* occur naturally in green plants and purple photosynthetic bacteria, respectively. 10-OH Chl*a* and the 10-MeO lactone of Chl*a* are the most common observed allomerization products (see text). Pyrochl*a* is a synthetic derivative of Chl*a* in which the carbomethoxy group normally present at position 10 is replaced by H.

others, loss of magnesium, photochemical degradation, and oxidation by molecular oxygen (allomerization).

The allomerization of chlorophylls[1] (the replacement of a hydrogen atom at the C-10 position with an oxygen-containing moiety) was first observed by Willstätter and co-workers almost a hundred years ago[2–4]. This oxidation reaction occurs both enzymatically and chemically[5] and poses serious problems in chlorophyll chemistry because, although allomerized products are chemically and structurally different from intact chlorophyll, they are nearly indistinguishable by visible absorption spectroscopy. They are also very troublesome to separate by conventional open column chromatography on sugar. Thus, it has been extremely difficult to determine to what extent chlorophyll samples have been contaminated by such alteration products. This difficulty has proved to be the single most important source of uncertainty in the interpretation of the physical and chemical data on chlorophyll systems to date.

Nuclear magnetic resonance (NMR), infrared (IR) and classical mass spectrometry (MS) data can be useful in the detection of allomerization products, but none are readily applicable to routine, rapid analysis of chlorophyll preparations. However, recent advances in reversed-phase liquid chromatography[6–8] and californium-252 plasma-desorption mass spectroscopy (^{252}Cf-PDMS)[9,10] provide new and rapid methods for the analysis and characterization of the chlorophylls and their derivatives. We have undertaken a thorough investigation of the chlorophyll allomerization reaction by both reversed-phase thin-layer chromatography (TLC) and modern closed-column reversed-phase high-performance liquid chromatography (HPLC). The focus of these studies is on the isolation and conclusive identification of the allomerization product of chlorophylls in both the presence and absence of polar (nucleophilic) solvents. Major reaction products were initially identified via co-chromatography with authentic sample (HPLC). Structures were confirmed by NMR and ^{252}Cf-PDMS data.

EXPERIMENTAL

Reversed-phase thin-layer chromatography

A variation of the methods developed by Daley *et al.*[8] for reversed-phase systems was employed. Reversed-phase TLC plates were prepared by dipping precut Eastman cellulose TLC sheets (without fluorescent indicator) into an 8% solution of Nujol in dichloromethane. The sheets were allowed to dry on the air for at least 15 min before use. The solvent systems employed were the following (composition by volume):

Solvent system	*Methanol*	*Acetone*	*2-Propanol*	*Water*	*Benzene*
A	35	50	10	2	–
B	60	20	10	10	2
C	60	20	–	15	10
D	60	5	5	10	10

Over a period of more than 5 h, no atmospheric allomerization of chlorophyll was observed in any of the above solvent systems. Since the Nujol contained the antioxidant tocopherol (a radical scavenger), no chlorophyll oxidation is believed to occur during chromatography (see text and Table I for R_F values).

Analytical reversed-phase liquid chromatography

The liquid chromatograph used was a modification of Beckman's Model 334 gradient liquid chromatograph (Beckman, Berkeley, CA, U.S.A.). This system is equipped with dual constant-flow pumps (Model 110A), a microprocessor–controller (Model 421), a sample injector (Model 210) fitted with a 20-μl loop, and a mixing chamber. The detector was a variable-wavelength ultraviolet–visible (UV–vis) detector (Model 155-10; Beckman). Wavelengths were set at 663 nm to detect chlorophyll and its derivatives and at 773 nm to detect bacteriochlorophyll and its derivatives. Chromatograms were recorded on a dual pen X-Y recorder (Model BD-41; Kipp & Zonen).

Stainless-steel columns (25 × 0.46 cm I.D.), packed with 5-μm Ultrasphere-ODS (Beckman) were used. Mobile phases consisted of mixtures of HPLC-grade methanol, tetrahydrofuran (THF), acetonitrile and water. The columns were operated at ambient temperature with flow-rates of 1 ml/min. Experiments designed to optimize the eluent composition were conducted. The eluent composition was varied and capacity ratios were determined.

Semi-preparative reversed-phase liquid chromatography

The liquid chromatograph used was a modified Beckman Model 320 liquid chromatograph. This model is equipped with a dual-piston constant-flow pump (Model 100A) and a sample injector (Model 210) fitted with a 2000-μl loop. The detection source was a refractive index detector (Altex, Model 156). Chromatograms were recorded with a dual-pen X-Y recorder (Model BD-41, Kipp & Zonen).

Stainless-steel semi-preparative columns (25 × 2.1 cm I.D.), filled with 7-μm Zorbax-ODS (DuPont, Wilmington, DE, U.S.A.) were used. Mobile phases consisted of mixtures of HPLC-grade methanol, acetonitrile, THF, and water. Columns were operated at ambient temperatures with a flow-rate of 10 ml/min.

Nuclear magnetic resonance spectra

Solution spectra were measured on a Nicolet NTC spectrophotometer, operating at 200 MHz. Proton chemical shifts are reported in ppm from hexamethyldisiloxane (HMS). High purity deuterated solvents were obtained from Norell (Landisville, NJ, U.S.A.).

Californium-252 plasma desorption mass spectroscopy

The instrument used is a modification of Macfarlane's basic design, which has been described in detail elsewhere[9,10]. A ^{252}Cf fission source is employed with a time-of-flight mass analyzer. The intensity of the ^{252}Cf source is approximately 1000 fissions/sec. Spectra, on the average, take about 2 h to accumulate.

Analyses were conducted on solid samples by evaporating or electrospraying organic solutions of the chlorophylls onto the sample plate under an inert atmosphere. Typically, the amount of sample deposited is approximately 20 μg, distributed over an area of 100 mm^2.

Isolation of chlorophyll a (Chla)

Chl*a* was obtained via procedures developed by Strain and Svec[11,12]. Purity was established using standard spectroscopic techniques and the reversed-phase HPLC techniques developed in these studies. All Chl*a* samples were stored under vacuum in a nitrogen dry-box (absence of light), prior to use.

*Isolation of deuterochlorophyll a (^{2}H-Chl*a*)*

^{2}H-Chl*a* is the major green pigment in blue-green algae (*Chlorella vulgaris* and *Scenedesmus obliquus*) grown in 99.6% deuterium oxide. Isolation procedures developed by Katz and co-workers[13–15] were employed. Standard spectroscopic[12] and reversed-phase HPLC techniques developed in these studies were used to establish purity. Samples of ^{2}H-Chl*a* were stored under vacuum in a nitrogen dry-box prior to use.

Isolation of methyl-chlorophyllide a (Me-Chla)

Me-Chl*a* was prepared via a three-step synthetic procedure. Initially, Chl*a* was converted to pheophytin *a* (Pheo*a*) by treatment with acid. Typically, 10 mg of Chl*a* were dissolved in 20 ml of oxygen-free dichloromethane. The dichloromethane solution was shaken with an equal volume of 3 *M* hydrochloric acid for about 2 min and washed three times with 20-ml portions of water. The organic layer was then separated, dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure (rotary evaporator), at 30°C. The resultant Pheo*a* was thoroughly dried and stored under vacuum.

Preparation of methyl-pheophorbide *a* (Me-Pheo*a*) requires treatment of Pheo*a* with refluxing 10% sulfuric acid in methanol. The crude product is subsequently purified by chromatography on silica gel columns, dried and stored under high vacuum[12].

Me-Chl*a* was prepared by the reinsertion of Mg^{2+} into Me-Pheo*a*, followed by chromatography on sugar columns[12]. The purified product was thoroughly dried and stored under high vacuum in a nitrogen dry-box. Degree of purity was established by using standard spectroscopic[12] and HPLC techniques developed in these studies.

Isolation of bacteriochlorophyll a (Bchla)

Crude Bchl*a* was obtained from *Rhodospirillum rubrum* cells as described elsewhere[16]. Isolation of the Bchl*a*–dioxane adduct followed procedures described by Omata and Murata[17].

A 1.00-g sample of the Bchl*a*–dioxane adduct was dissolved in a minimal amount of acetone and applied to a DEAE-Sepharose CL-6B column (15 × 1.5 cm I.D.). This column was continuously developed with acetone, at a flow-rate of approximately 2 ml/min, until the eluate appeared clear. Developing solvent was then immediately changed to an acetone–methanol mixture (10:3, v/v) to elute adsorbed Bchl*a*. The eluate containing Bchl*a* was collected and the solvent was removed under reduced pressure at 30°C.

Final purification was achieved by isocratic elution of concentrated Bchl*a*/THF solutions (*ca.* 200 mg Bchl*a*/mL THF) from Zorbax-ODS semi-preparative columns (see above). The eluent system consisted of methanol–THF–water (73.1:24.4:2.5, v/v/v). Eluate containing Bchl*a* was collected and partitioned between equal volumes (10 ml) of dichloromethane and water, saturated with sodium chloride. The organic layer was successively washed three times with water, dried over anhydrous magnesium sulfate, and filtered. Solvent was removed under reduced pressure at 30°C. The dried Bchl*a* was stored under vacuum in a nitrogen dry-box. Purity was established by using standard spectroscopic techniques[12] and the HPLC techniques developed in these studies.

Preliminary procedures for allomerization

Solvents were of analytical (HPLC) grade, provided by Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). THF was freed of peroxides by passing it through aluminium oxide. Prior to use all solvents were dried over 3Å Molecular Sieves and deoxygenated by freeze–thaw cycles under high vacuum.

Samples of chlorophylls were azeotropically distilled several times with carbon tetrachloride or benzene and thoroughly dried under high vacuum. Lithium bromide was dried under high vacuum at 95°C before use.

General allomerization procedures in the absence of extraneous nucleophile (THF)

Typically, 1–2 mg of azeotropically dried sample (Chl*a*, ^{2}H-Chl*a*, Me-Chl*a*, etc.) was dissolved in 1–2 ml of oxygen-free, peroxide-free THF and filtered in a nitrogen dry-box. To this was added 1–2 ml of THF, saturated with lithium bromide. The entire solution was periodically exposed to the atmosphere to facilitate allomerization, and the course of the reaction was followed by isocratic, analytical HPLC. Results from an HPLC study of the Chl*a* allomerization reaction *vs.* time are given in Fig. 2.

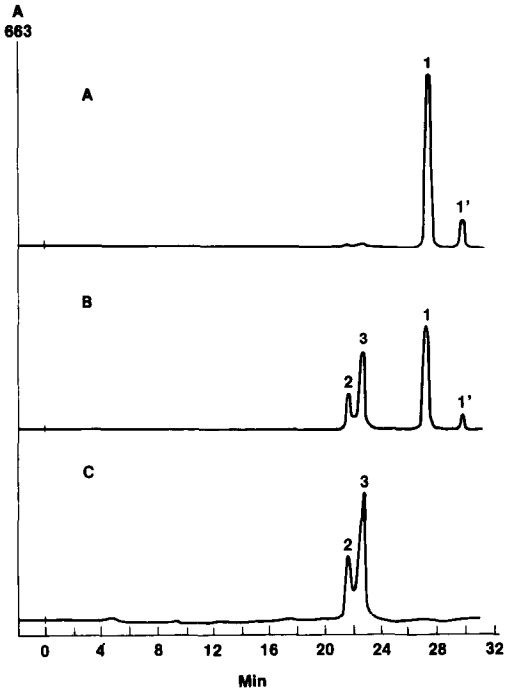


Fig. 2. Course of the Chl*a* allomerization reaction in the absence of extraneous nucleophile (THF) *vs.* time, followed by HPLC on Ultrasphere-ODS (25 × 0.46 cm I.D.) columns. The mobile phase consisted of THF–methanol–water (36:54:10, by vol.). Flow-rate was 1 ml/min and detection was at 663 nm. (A) Chromatogram recorded before exposure to atmospheric oxygen. (B) Chromatogram recorded 3 h after exposure to atmospheric oxygen. (C) Chromatogram recorded 6 h after exposure to atmospheric oxygen. Peaks: 1 = Chl*a*; 1′ = Chl*a*′; 2 and 3 = 10-OH Chl*a* (stereoisomers, see text).

General allomerization procedures in the presence of extraneous nucleophile (methanol)

Typically, 1–2 mg of azeotropically dried sample (Chl*a*, ^{2}H-Chl*a*, Me-Chl*a*, etc.) was dissolved in 1–2 ml of oxygen-free methanol and filtered in a nitrogen dry-box. The entire solution was exposed to the atmosphere and the course of the allomerization reaction was followed by the procedures described in the previous section (Fig. 3).

Isolation of allomerization products (HPLC)

Products of allomerization were routinely isolated by isocratic elution from Zorbax-ODS semi-preparative columns (see above).

Reaction mixtures containing allomerized species were partitioned between equal volumes (10 ml) of dichloromethane and water saturated with sodium chloride. The organic layer was washed three times with water, dried over anhydrous magnesium sulfate and filtered. Solvent was removed under reduced pressure at 30°C. The dried extracts were dissolved in 1 ml of THF and eluted with solvents of the following composition (by volume):

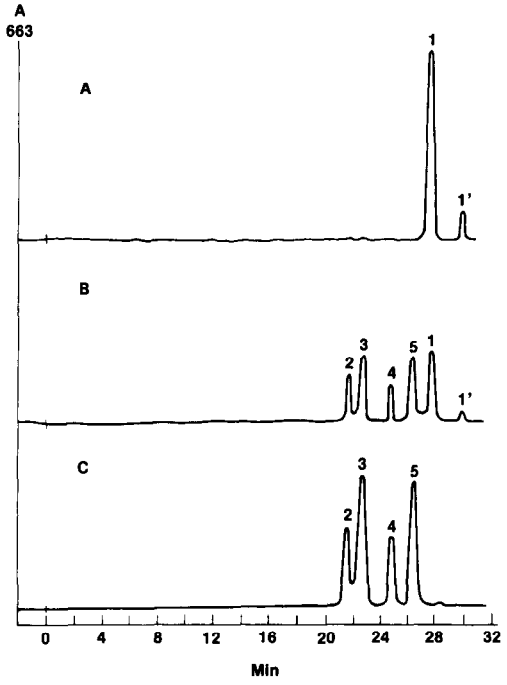


Fig. 3. Course of the Chl*a* allomerization reaction in the presence of extraneous nucleophile (methanol) *vs.* time; followed by HPLC on Ultrasphere-ODS (25 × 0.46 cm I.D.) columns. The mobile phase consisted of THF–methanol–water (36:54:10, by vol.). Flow-rate was 1 ml/min and detection was at 663 nm. (A) Chromatogram recorded before exposure to atmospheric oxygen. (B) Chromatogram recorded 2 h after exposure to atmospheric oxygen. (C) Chromatogram recorded 5 h after exposure to atmospheric oxygen. Peaks: 1 = Chl*a*; 1′ = Chl*a*′; 2 and 3 = 10-OH Chl*a* (stereoisomers); 4 and 5 = 10-MeO lactone Chl*a* (stereoisomers, see text).

Allomerization products from	*THF*	*Methanol*	*Water*	*Acetonitrile*
Chl*a*	36	54	10	–
^{2}H-Chl*a*	36	54	10	–
Me-Chl*a*	–	90	10	–
BChl*a*	23.3	28.5	5	43.2

Each fraction containing an allomerization product was individually collected and again partitioned between equal volumes (20 ml) of dichloromethane and water saturated with sodium chloride. The organic layer was worked up as described above. Dried allomerization products were stored under high vacuum in a nitrogen dry-box.

RESULTS

The allomerization of chlorophyll is generally considered to occur only in polar (nucleophilic) organic solvents; classically, it is a reaction associated with the hydroxylic solvent methanol. However, many manipulations and research involving physico-chemical studies of chlorophylls are carried out in other polar and non-polar solvents as well. Thus, studies of the chlorophyll allomerization reaction in both polar (hydroxylic) and relatively non-polar (non-hydroxylic) solvents were carried out, and reaction products were identified in each case.

Preliminary investigations with reversed-phase TLC plates indicate that polar solvents are necessary for the unpromoted allomerization of chlorophylls with molecular oxygen. Methanol solutions of Chl*a* (1 mg Chl*a*/ml methanol) were found to allomerize spontaneously when exposed to atmospheric oxygen and ambient light. The reaction was generally complete in less than 6 h and the appearance of two major reaction products, both with R_F values greater than that of intact Chl*a*, were observed. These products were tentatively identified as 10-hydroxychlorophyll *a* (10-OH Chl*a*) and the 10-methoxylactone of chlorophyll *a* (10-MeO lactone Chl*a*), based on TLC comparison with authentic samples[5]. Similar results were obtained in meth-

TABLE I

R_F VALUES FOR VARIOUS CHLOROPHYLLS AND DERIVATIVES ON CELULLOSE-NUJOL

For solvent systems A–D, see Experimental.

Compound	R_F	*Solvent system*
Chl*a*	0.90	A
	0.73	B
	0.56	C
10-OH Chl*a*	0.76	C
10-MeO lactone of Chl*a*	0.67	C
Pheo*a*	0.37	B
10-OH Pheo*a*	0.55	B
10-MeO lactone of Pheo*a*	0.50	B
Bchl*a*	0.90	A
	0.83	B
	0.74	C
	0.58	D
10-OH Bchl*a*	0.79	C
	0.69	D

anol solutions of the Mg-free derivative (Pheo*a*; Table I). The rate of formation of reaction products from the Mg-free derivative was, however, slower, as has been previously reported[18].

The effect of added metal ion on the rate of allomerization in methanol solution has been well documented[19]. Mono-, di- and trivalent metal ions have been shown to enhance greatly the allomerization rate, and similar results have been obtained here. However, addition of metal ions to chlorophylls dissolved in non-hydroxylic solvents (THF) also initiate allomerization. Reversed-phase TLC indicates that no appreciable allomerization of Chl*a* occurs in a peroxide-free THF solution exposed to atmospheric oxygen. When small amounts of lithium bromide or other metal ion salts are added (*ca.* 5 mg LiBr/mg Chl*a*), allomerization with molecular oxygen proceeds at an appreciable rate. That molecular oxygen is responsible for the oxidation process is evidenced by lack of significant reaction in THF solutions of metal ions and Chl*a* in an oxygen-free atmosphere. The exclusive product of Chl*a* allomerization in the absence of extraneous nucleophile (THF) was tentatively identified as 10-OH Chl*a*. Similar results were obtained from Pheo*a* under identical conditions (Table I).

With the initial successes in detecting the products of allomerization obtained by reversed-phase TLC we decided to undertake a thorough investigation of the chlorophyll allomerization reaction by modern analytical HPLC.

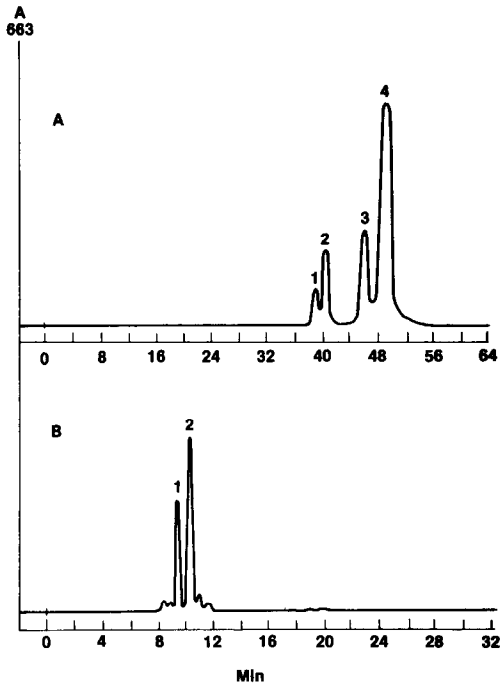


Fig. 4. HPLC chromatograms of the allomerization products of ^{2}H-Chl*a* and Me-Chl*a* in the presence of extraneous nucleophile (methanol) on Ultrasphere-ODS (25 × 0.46 cm I.D.) columns. (A) ^{2}H-Chl*a*; the mobile phase consisted of THF–methanol–water (35:53:12, by vol.). Flow-rate was 1 ml/min and detection was at 663 nm. Peaks: 1 and 2 = 10-OH ^{2}H-Chl*a* (stereoisomers); 3 and 4 = 10-MeO lactone of ^{2}H-Chl*a* (stereoisomers). (B) Me-Chl*a*; the mobile phase consisted of methahol–water (90:10, v/v). Flow-rate and detection as in A. Peaks: 1 = 10-OH Me-Chl*a*; 2 = 10-MeO lactone of Me-Chl*a* (see text).

The allomerization of Chl*a* in the presence of extraneous nucleophile (methanol) was followed as a function of time by isocratic, reversed-phase HPLC. The mobile phase consisted of THF–methanol–water (36:54:10, by vol.) (Fig. 3). At the expense of Chl*a*, the growth of four major product peaks (two sets of doublets), all more polar than intact Chl*a*, were observed. Reaction was complete in less than 5 h as judged by the disappearance of the Chl*a* signal. Under identical reaction conditions, similar results have been obtained with the fully deuterated derivative of Chl*a* (^{2}H-Chl*a*) and the closely related Me-Chl*a*, as well (Fig. 4).

The allomerization of Chl*a* in the absence of extraneous nucleophile (THF) was also followed by isocratic HPLC (Fig. 2). Reaction was judged complete in 6 h. The sole product under these conditions splits into a doublet in the HPLC chromatogram. The identity of this component was firmly established as 10-OH Chl*a* by co-chromatography (HPLC) with an authentic sample[5]. In similar manner the more polar component pair of Chl*a* allomerization in the presence of extraneous nucleophile (methanol) was confirmed as 10-OH Chl*a* (see Fig. 5 and Table II). It should be noted that, whereas synthetically produced (authentic) 10-OH Chl*a* consists almost exclusively of a single stereoisomer, allomerization of Chl*a* results in production of the two possible isomeric forms about C-10. HPLC was easily able to separate these stereoisomers whose identity was consequently confirmed by NMR and ^{252}Cf-PDMS data (see below). That 10-OH Chl*a* is a major product of the allomerization of Chl*a* is evidenced by the fact that it is produced in both the presence and absence of extraneous nucleophile.

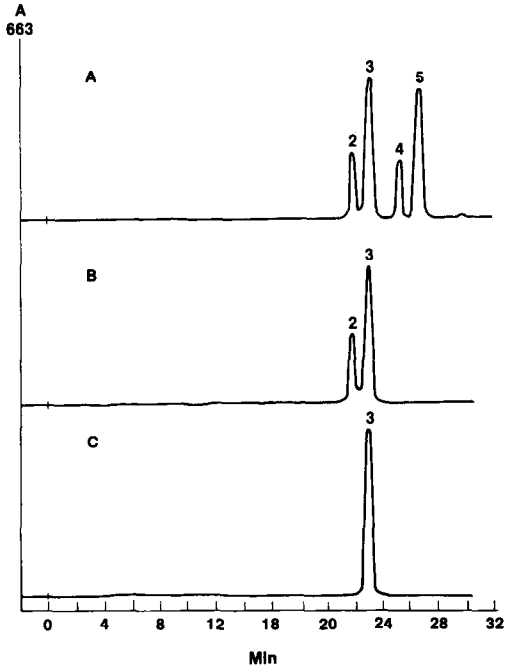


Fig. 5. Identification of allomerization products by HPLC co-chromatography. Chromatographic parameters and peak identification as in Fig. 3. (A) Chromatogram of Chl*a* allomerization products in the presence of extraneous nucleophile (methanol). (B) Chromatogram of Chl*a* allomerization products in the absence of extraneous nucleophile (THF). (C) HPLC of authentic 10-OH Chl*a*.

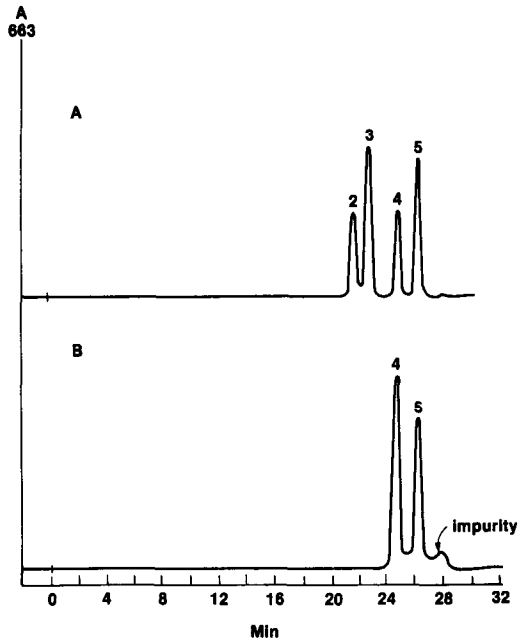


Fig. 6. Identification of allomerization products by HPLC co-chromatography. Chromatographic parameters and peak identification as in Fig. 3. (A) Chromatogram of Chl*a* allomerization products in the presence of extraneous nucleophile (methanol). (B) Chromatogram of authentic 10-MeO lactone Chl*a* (stereoisomers).

Me-Chl*a* in the presence of methanol was likewise additionally confirmed. ^{252}Cf-PDMS conclusively verifies the above assignments. Calculated and observed molecular mass data are given in Table IV.

TABLE III

^{1}H NMR DATA ON Chl*a* AND 10-OH Chl*a* AND 10-METHOXY LACTONE Chl*a* (ACETONE-d_6, HMS = 0 ppm)

	Chemical shift (ppm)		
	Chla	*10-OH Chla*	*10-Methoxy lactone Chla*
β	9.61	9.62	9.61
α	9.28	9.35	9.37
δ	8.35	8.41	8.44
10-H	6.06		
10-OH		6.21	
10-CH_3O			3.89
Methyls			
10b	3.76	3.62	3.70
5a	3.56	3.52	3.46
1a	3.26	3.28	3.27
3a	3.23	3.26	3.25

TABLE II

RETENTION TIMES AND CAPACITY FACTORS (k') FOR VARIOUS CHLOROPHYLLS AND ALLOMERIZATION DERIVATIVES ON ULTRASPHERE-ODS (25 × 0.46 cm I.D.) COLUMNS

	Retention time (min)	*k'*
*Chla and allomerization derivatives**		
Chl*a*	27.70 (29.84)**	8.50 (9.29)
10-OH Chl*a*	22.56 (21.75)	6.92 (6.63)
10-MeO lactone Chl*a*	26.31 (25.08)	8.23 (7.80)
*Me-Chla and allomerization derivatives****		
Me-Chl*a*	12.43	3.01
10-OH Me-Chl*a*	9.54	2.35
10-MeO lactone Me-Chl*a*	10.36	2.64
Bchla and allomerization derivatives§*		
Bchl*a*	11.05	4.26
10-OH Bchl*a*	9.86	2.68

* Mobile phase as in Fig. 3.
** Values in parentheses are for the less abundant stereoisomer about C-10, where observed.
*** Mobile phase as in Fig. 4.
§ Mobile phase as in Fig. 7.

The identity of the less polar compartment pair of Chl*a* allomerization in methanol was also confirmed via co-chromatography (HPLC) as the 10-MeO lactone of Chl*a* (Fig. 6; Table II). A third product, the 10-methoxy derivative (10-MeO Chl*a*), reported in the old literature[20] could not be detected by HPLC.

Isolation of allomerization products on Zorbax-ODS semi-preparative columns is described in the experimental section. NMR and ^{252}Cf-PDMS were used to identify conclusively the products of allomerization. The NMR chemical shift data of intact Chl*a* and authentic 10-OH Chl*a*, recorded in acetone-d_6, are listed in Table III. In comparison to the Chl*a* spectrum, the main feature of interest is the absence of the 10-H resonance line and appearance of a 10-OH line in the 10-OH Chl*a* spectrum. These data indicate substitution of hydrogen at the C-10 position of Chl*a* by an –OH moiety. NMR data collected on the more abundant allomerization product of Chl*a* formed in the absence of extraneous ligand (Peak 3; Fig. 2) gave results identical to those obtained from authentic 10-OH Chl*a*. Data collected on the less abundant product (Peak 2; Fig. 2) revealed the presence of the related stereoisomer. Identical results were obtained for the more polar allomerization pair resulting from reaction of Chl*a* in the presence of extraneous nucleophile (Peaks 2 and 3; Fig. 3).

In similar fashion, the identity of the less polar component pair of Chl*a* allomerization in methanol (Peaks 4 and 5; Fig. 3) was further established as the 10-MeO lactone of Chl*a* (stereoisomers).

Similar NMR data collected on the allomerization products of Me-Chl*a* in the absence of extraneous nucleophile (THF), confirm the formation of 10-OH Me-Chl*a* as the sole product. Production of 10-OH Me-Chl*a* and the 10-MeO lactone of

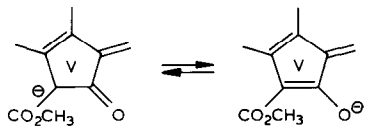
TABLE IV

^{252}Cf-PDMS ANALYSES

Compound	*Molecular weight*	
	Calculated	*Observed*
Chl*a*	892.5	892.5
10-OH Chl*a*	908.5	908.2
10-MeO lactone Chl*a*	938.5	938.6
Me-Chl*a*	629.0	629.0
10-OH Me-Chl*a*	645.0	645.2
10-MeO lactone Me-Chl*a*	675.0	674.4
Bchl*a*	904.5	904.5
10-OH Bchl*a*	920.5	920.3

DISCUSSION

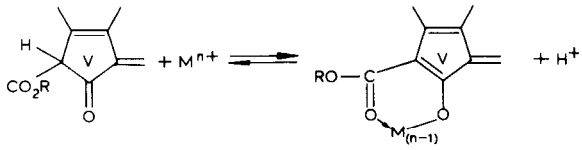
Recently several possible reaction mechanisms for the allomerization of Chl*a* have been entertained in the literature. Hynninen and co-workers[21,22] propose two possible routes; both involve as a preliminary step the formation of the chlorophyll enolate anion. The possibility of enol involvement in Chl*a* allomerization has been discussed elsewhere[23,24].



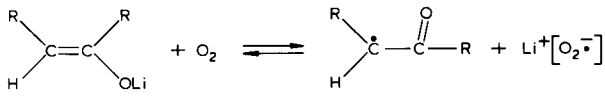
"enolate anion"

One mechanistic approach of Hynninen assumes direct addition of singlet oxygen ($^1O_2$) to the 9,10-double bond of the enolate anion. The other assumes a free-radical chain reaction, involving triplet oxygen ($^3O_2$), Chl C-10 radicals and peroxide radicals, and Chl C-10 hydroperoxide. The singlet oxygen mechanism is able to account for the formation of the 10-MeO lactone derivative observed in the present study, but not for 10-OH Chl*a*. Hynnenin suggests this latter reaction product may be formed by direct action of hydroxyl ion (OH^-) on the C-10 atom of Chl*a* without the preliminary enolization step[22]. Evidence supporting the view that allomerization of Chl*a* proceeds by a free-radical mechanism is based on Hynnenin's observation that formation of the 10-MeO lactone derivative is inhibited by a radical scavenger (β-carotene) in the dark. The proposed reaction sequence for the formation of this allomerization product involves nucleophilic attack by hydroxyl ion (OH^-) on the C-9 position of the Chl C-10 hydroperoxide, followed by solvation and elimination steps described elsewhere[21]. A chain-terminating reaction between hydroxyl radicals ($OH^\cdot$) and Chl C-10 radicals is believed to be responsible for the formation of the 10-OH Chl*a* derivative.

Our results in non-hydroxylic solvents (THF) can be explained by formation of a *metal* enol complex at ring V, which labilizes the proton at C-10.



Similar complexes with pheophytin have been described[25]. Since lithium ion would be expected to form a neutral enolate, it appears to be a most versatile promoter of allomerization. There is also a possibility that the lithium enolate itself catalyzes the radical reaction by molecular oxygen. Suggestions that lithium enolates react with molecular oxygen to provide an α-keto radical and the lithium salt of the peroxide radical anion have been reported[26].



However, it has become increasingly evident from our studies that, mechanistically, allomerization products are formed by nucleophilic attack on the chlorophyll radical cation ($Chl^{\ddagger}$)[10]. The $Chl^{\ddagger}$ radical cation is believed to be formed via extraction of an electron from the π-system of chlorophyll by molecular oxygen. In the classical allomerization reaction of Willstätter and Stroll, the $Chla^{\ddagger}$ radical cation is subsequently attacked by nucleophilic methanol:

$$Chla + O_2 \rightarrow Chla^{\ddagger} + O_2^{-} \qquad \text{(step 1)}$$

$$Chla^{\ddagger} + CH_3OH \rightarrow \text{allomerization products} \qquad \text{(step 2)}$$

The details of the reaction are as yet poorly understood. However, the radical nature of oxygen attack on the C-10 atom has been verified via inhibition of allomerization by β-carotene, hydroquinone, triphenylphosphine, and tocopherol, all of which are known radical scavengers. It also has been observed that reagents with high redox potentials, such as benzoquinone, iodine and iron(III) chloride (anhydrous) will provide allomerization without molecular oxygen[1,27,28]. The reaction products under these conditions correspond to radical interaction with the solvent or nucleophilic reagent in solution, or both[10,28].

The function of Li^+ as allomerization accelerant in both the presence and absence of extraneous nucleophile may be due to stabilization of the superoxide ion (O_2^{-}) formed in step 1, stabilization of the enol form of the chlorophyll, or both. Such stabilization would prevent the reverse reaction from occurring and render "naked" $Chl^{\ddagger}$, or enolate, accessible to further attack by either molecular oxygen, or nucleophilic agent (or both). Further studies are needed to clarify this complex situation.

Exploratory studies on the atmospheric allomerization of Bchl*a* in the presence and absence of extraneous nucleophile have also been carried out. As expected, in THF solution only one major oxidation product is observed. This product has been identified as 10-OH Bchl*a* by TLC comparison with authentic sample[5] (Table I) and co-chromatography via HPLC (Fig. 7; Table II). However, unlike the allomerization

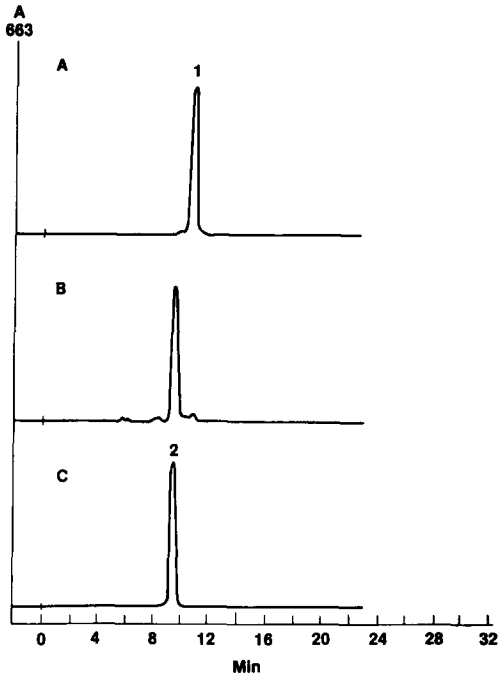


Fig. 7. Co-chromatography. (A) Chromatogram of intact Bchl*a* on Ultrasphere-ODS (25 × 0.46 cm I.D.) columns. The mobile phase consisted of THF–methanol–water–acetonitrile (23.3:28.5:5.0:43.2, by vol.). Flow-rate was 1 ml/min and detection was at 773 nm. Peak: 1 = Bchl*a*. (B) Chromatogram of the exclusive allomerization product of Bchl*a*. Chromatographic parameters as in A. (C) Chromatogram of authentic 10-OH Bchl*a*. Chromatographic parameters as in A. Peak: 2 = 10-OH Bchl*a*.

of Chl*a* in the presence of extraneous nucleophile (methanol), Bchl*a* under identical conditions results only in production of 10-OH Bchl*a*. The importance of this observation is in relation to the accepted structural formulas of Chl*a* and Bchl*a* in the vicinity of ring V, where reaction occurs. Proposed structures are identical in this region (Fig. 1). Failure to detect the 10-MeO lactone of Bchl*a* raises very puzzling questions. The differences may be related to the known smaller tendency of Bchl*a* to undergo enolization, or perhaps a smaller tendency of Bchl*a* to become oxidized by molecular oxygen to the radical cation ($Bchla^{+}$), or both. These data, however, do suggest that a re-examination of the structural formula of Bchl*a* may be in order.

Finally, we have verified that 10-OH and 10-MeO lactone derivatives of chlorophyll are formed independently of one another during allomerization in the presence of extraneous nucleophile (methanol). Studies on authentic 10-OH Chl*a* under such conditions do *not* indicate any significant amount of further reaction.

CONCLUSION

The results of TLC, HPLC, NMR and ^{252}Cf-PDMS investigation on the products of atmospheric allomerization of the chlorophylls have been presented. Results indicate that the allomerization reaction proceeds spontaneously in methanol solution but metal ion catalysts (Li^{+}) can also initiate reaction in other solvents. The

10-hydroxy derivative of the chlorophylls are major allomerization products and are formed in both the presence and absence of extraneous nucleophiles. The 10-MeO lactones are formed only when chlorophylls are allowed to allomerize in polar solvents.

Contrary to the case of most chlorophylls, allomerization of Bchl*a* in the presence of extraneous nucleophile results *only* in production of the 10-hydroxy derivative (10-OH Bchl*a*). No 10-methoxy lactone of Bchl*a* has been observed. As both Chl*a* and Bchl*a* are thought to possess identical structure in the vicinity of the reaction site (ring V), a question arises as to why Chl*a* spontaneously forms both the 10-MeO lactone and 10-hydroxy derivatives whereas Bchl*a* forms only the latter.

Instrumentally, analytical reversed-phase HPLC on Ultrasphere-ODS columns has been shown to be a rapid and effective method for determining the presence of allomerization products in chlorophyll preparations; it is even able to separate stereoisomeric forms efficiently. Analytical reversed-phase HPLC has also been shown to be a useful tool in following the course of allomerization reactions over time. Semi-preparative reversed-phase HPLC on Zorbax-ODS columns was quite easily able to separate quantities of allomerization products large enough to conduct NMR and other necessary studies. ^{252}Cf-PDMS was shown to be an effective tool for determining the molecular mass of allomerized species and, when coupled with NMR data, enabled us conclusively to identify and structurally define the products of chlorophyll allomerization.

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