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The derivation of the formyl-group oxygen of chlorophyll *b* in higher plants from molecular oxygen

Achievement of high enrichment of the 7-formyl-group oxygen from $^{18}O_2$ in greening maize leaves

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The mechanism of formation of the formyl group of chlorophyll *b* has long been obscure but, in this paper, the origin of the 7-formyl-group oxygen of chlorophyll *b* in higher plants was determined by greening etiolated maize leaves, excised from dark-grown plants, by illumination under white light in the presence of either $H_2{}^{18}O$ or $^{18}O_2$ and examining the newly synthesized chlorophylls by mass spectroscopy. To minimize the possible loss of ^{18}O label from the 7-formyl substituent by reversible formation of chlorophyll *b*-7[1]-*gem*-diol (hydrate) with unlabelled water in the cell, the formyl group was reduced to a hydroxymethyl group during extraction with methanol containing $NaBH_4$: chlorophyll *a* remained unchanged during this rapid reductive extraction process.

Mass spectra of chlorophyll *a* and [7-hydroxymethyl]-chlorophyll *b* extracted from leaves greened in the presence of either $H_2{}^{18}O$ or $^{18}O_2$ revealed that ^{18}O was incorporated only from molecular oxygen but into both chlorophylls: the mass spectra were consistent with molecular oxygen providing an oxygen atom not only for incorporation into the 7-formyl group of chlorophyll *b* but also for the well-documented incorporation into the 13[1]-oxo group of both chlorophylls *a* and *b* [see Walker, C. J., Mansfield, K. E., Smith, K. M. & Castelfranco, P. A. (1989) *Biochem. J. 257*, 599–602]. The incorporation of isotope led to as much as 77% enrichment of the 13[1]-oxo group of chlorophyll *a*: assuming identical incorporation into the 13[1] oxygen of chlorophyll *b*, then enrichment of the 7-formyl oxygen was as much as 93%. Isotope dilution by re-incorporation of photosynthetically produced oxygen from unlabelled water was negligible as shown by a greening experiment in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

The high enrichment using $^{18}O_2$, and the absence of labelling by $H_2{}^{18}O$, unequivocally demonstrates that molecular oxygen is the sole precursor of the 7-formyl oxygen of chlorophyll *b* in higher plants and strongly suggests a single pathway for the formation of the chlorophyll *b* formyl group involving the participation of an oxygenase-type enzyme.

The biosynthesis of chlorophylls (Chls) *a* (**I**) and *b* (**III**), the two major chlorophylls of higher plants, green algae and some prochlorophytes, is now largely understood [1–3], but the mechanism of the formation of the 7-formyl group of Chl *b* has been an outstanding gap in our current knowledge.

Chls *a* and *b* differ only by the substituent at C7: a methyl group in Chl *a* (**I**) and a formyl group in Chl *b* (**III**): the structures of chlorophylls **I–X** are given in Table 1. While the two pigments share most of their biosynthetic pathways, the exact point of their divergence is not clear: Chl *a*, Chl *a* esterified with alcohols other than phytol, as well as chlorophyllide *a* (the related free acid) have all been suggested as possible substrates of formyl group formation (for reviews see [1–3]). A further complication is the finding of parallel pathways of chlorophyll biosynthesis which differ, in particular, by the C8 substituent which can be either an ethyl or vinyl group [4]. The finding of a large number of Chl-*b*-deficient mutants which are allelic suggests that only a few steps are specific to Chl *b* formation [5].

Previous experiments with [U-^{13}C]glutamate (Porra, R. J, Kuchel, P. W. and Bubb, W, unpublished results) have shown

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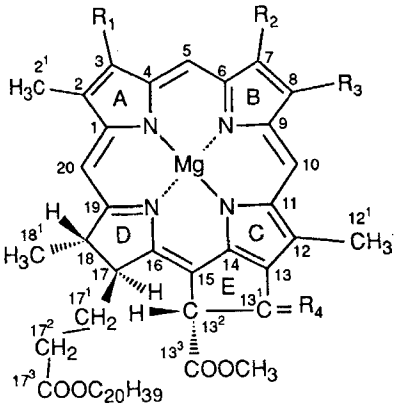
Abbreviations. Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Phe, phaeophytin. The IUPAC-IUB Recommendations 1986 for tetrapyrroles [31] have been used, together with square brackets to indicate substitutions, for chlorophyll and its derivates; for further details see Table 1 which illustrates structures **I–X** referred to in the paper.

Table 1. Structure of chlorophyll derivatives. The IUB-IUPAC approved numbering system for tetrapyrroles [31] has been used with bracket [] nomenclature indicating replacement of substituents in the parent compound. Chlorophylls **IV**, **VIII**, **IX** and **X** can be formally classified as derivatives of either Chl *a* or Chl *b* but are referred to as Chl *b* derivatives since they most frequently arise in this paper as reduction or hydration products of Chl *b*. There is a single bond between C7 and C8 in structures **V**, **VI**, and **VII**.



Compound		R_1 (at C3)	R_2 (at C7)	R_3 (at C8)	R_4 (at C13^1)
I	Chl *a*	$-CH=CH_2$	$-CH_3$	$-C_2H_5$	=O
II	[7^1-hydroperoxy]-Chl *a*	$-CH=CH_2$	$-CH_2OOH$	$-C_2H_5$	=O
III	Chl *b*	$-CH=CH_2$	$-CHO$	$-C_2H_5$	=O
IV	[7-hydroxymethyl]-Chl *b*	$-CH=CH_2$	$-CH_2OH$	$-C_2H_5$	=O
V	[7-Methylidene]-[8-ethylidene]-Chl *a*	$-CH=CH_2$	$=CH_2$	$=CH-CH_3$	=O
VI	[7-methylidene]-[8-hydro]-Chl *a*	$-CH=CH_2$	$=CH_2$	$-H,-C_2H_5$	=O
VII	[7,8-dihydro]-[7-hydroxymethyl]-Chl *a*	$-CH=CH_2$	$-H,-CH_2OH$	$-H,-C_2H_5$	=O
VIII	Chl *b*-*gem*-diol (or hydrate)	$-CH=CH_2$	$-CH(OH)_2$	$-C_2H_5$	=O
IX	[3-ethyl]-[7-hydroxymethyl]-Chl *b*	$-C_2H_5$	$-CH_2OH$	$-C_2H_5$	=O
X	[7-hydroxymethyl]-[13^1-hydroxy]-Chl *b*	$-CH=CH_2$	$-CH_2OH$	$-C_2H_5$	$-H,-OH$

that the formyl-group carbon of Chl *b*, like that of the methyl group of Chl *a*, arises from glutamate and not from an exchange route involving, for instance, a C_1 fragment from the C2 of glycine mediated via a formyl-tetrahydrofolate-dependent enzyme of C_1 metabolism: thus, the formyl group must arise by oxidation of the methyl substituent of Chl *a*. Oxygen incorporation into organic molecules is generally considered to arise from molecular oxygen or water by either oxygenase or hydratase mechanisms [6]. In this paper, mass spectroscopy and ^{18}O-labelling were employed to distinguish between either oxygenase (Fig. 1; Schemes A1 and A2) or hydratase mechanisms (Fig. 1; Schemes B1 and B2) for the conversion of the methyl group of Chl *a* to the formyl group of Chl *b*. These techniques have also been employed in the study of chlorophyll biosynthesis [7] and porphyrin ring opening [8]; and ^{18}O-labelled chromophores can be introduced in reconstitution or exchange experiments to probe binding sites in chlorophyll-protein complexes and to determine the effects of various substituents on chlorophyll function [9–14].

The oxygenase-type mechanism could involve a di-oxygenase (i.e. peroxidase) [6] (see Scheme A1) or, alternatively, a mixed-function mono-oxygenase [6] (see Scheme A2). If either oxygenase scheme (A1 or A2) operates, the 7-formyl-group oxygen would be labelled by $^{18}O_2$, like the 13^1-oxo group of protochlorophyllide, which is also known to arise from molecular oxygen [7]. Greening in the presence of $^{18}O_2$, therefore, would lead to an increase of 4Da in the molecular mass of Chl *b*; however, in the absence of such an oxygenase, labelling of the 13^1-oxo group would lead to an increase of only 2Da in both Chls *a* and *b*.

At least two hydratase-type mechanisms (see Schemes B1 and B2) can be envisaged. One (Scheme B1) could involve a dehydrogenase to form 7-methylidene-8-ethylidene-Chl *a* (**V**) as an intermediate while the other (Scheme B2) employs an isomerase to form [7-methylidene]-[8-hydro]-Chl *a* (**VI**). Hydratases directed at the C7 exocyclic double bond lead to the formation of a 7-hydroxymethyl derivative and eventually lead to the formation of Chl *b* (see Schemes B1 and B2). Exocyclic double bonds are known in other chlorophylls, such as bacteriochlorophyll *b* [15] and *g* [16], which possess an ethylidene group at C8: such double bonds have been postulated to play an important role in the biosynthesis of other chlorophylls [16]. If either hydratase scheme (B1 or B2) operates, the 7-formyl oxygen would arise from water and greening in the presence of $H_2{}^{18}O$ would lead to an increase of 2Da in the molecular mass of Chl *b*: the molecular mass of Chl *a* would be unaffected.

In this paper, etiolated maize leaves were greened in the presence of $H_2{}^{18}O$ and $^{18}O_2$ and the chlorophylls extracted for mass spectroscopic investigation. To minimize loss of ^{18}O from the 7-formyl group of Chl *b* by reversible formation of the 7^1-*gem*-diol (**VIII**) with leaf-cell unlabelled water, the formyl substituent was reduced to a hydroxymethyl group on extraction with methanol containing $NaBH_4$: the Chl *a* was extracted unchanged and the Chl *b* as [7-hydroxymethyl]-Chl *b*. Our experiments show that in higher plants the formyl-group oxygen of Chl *b* is derived directly from molecular oxygen; as expected (cf. [7]), it is also confirmed that the 13^1-oxo group of both Chl *a* and *b* arise from molecular oxygen. A brief report of part of this work has been presented

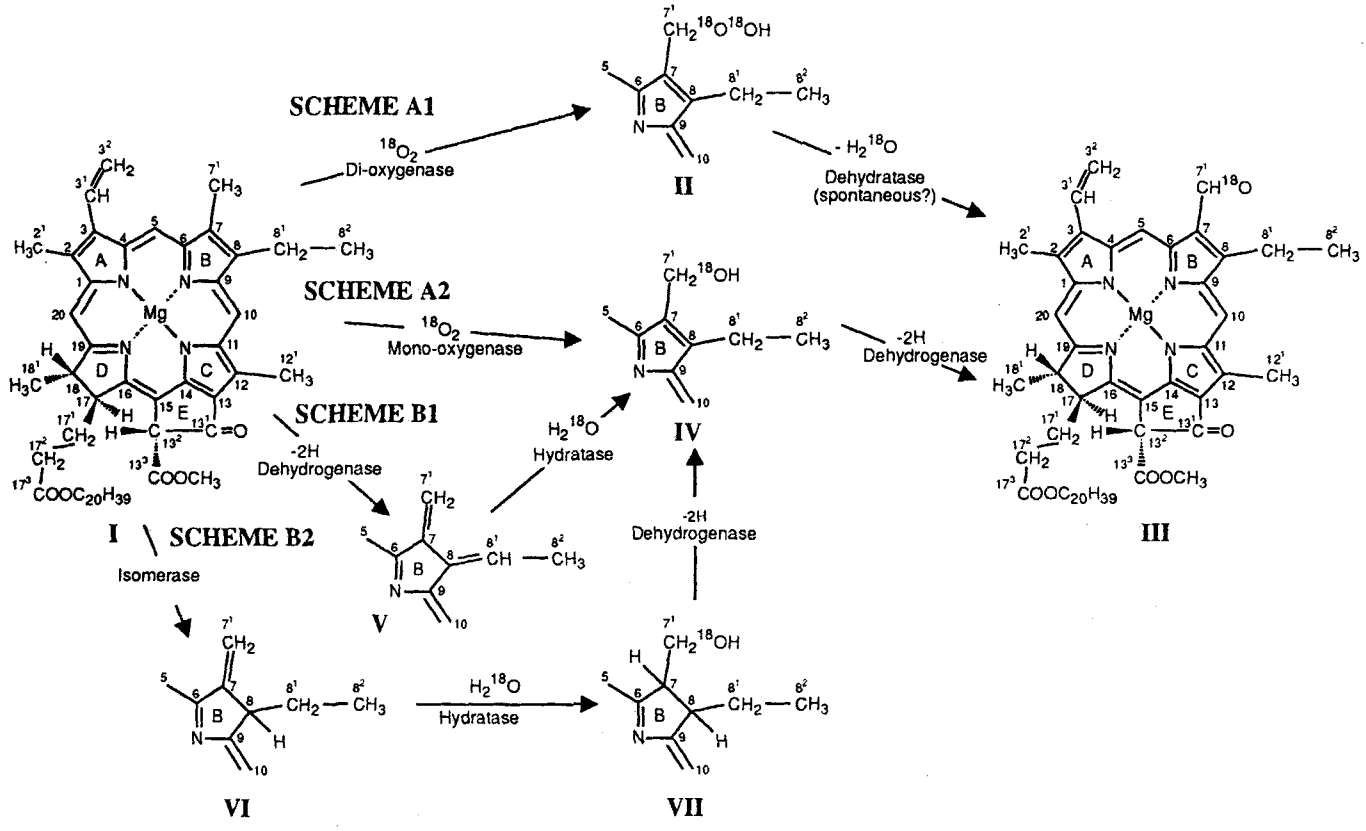


Fig. 1. Different hypothetical pathways for Chl *b* formation from Chl *a*. The reactions only involve ring B: the bold roman numerals identify the structures of the intermediates in Table 1. Formally, it is irrelevant whether the substrate of Chl *b* formation is chlorophyllide *a* or Chl *a* esterified with phytol or with a precursor alcohol of phytol (see [1]); however, enzymes reacting with chlorophylls are known to be partially affected by the structure of the long-chain fatty-alcohol-ester substituent [4], and are expected to clearly distinguish between the free acid and esterified forms of Chl a. No pathways originating from [8-vinyl]-Chl *a* (cf. [4]) have been considered but compound **V** could arise directly from the latter by isomerization.

[17]. Re-incorporation of photosynthetically produced $^{16}O_2$ from $H_2{}^{16}O$ has been shown to be negligible in experiments with the photosystem II inhibitor, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU).

Recently and independently, Schneegurt and Beale [18] reported a 34% incorporation of label from $^{18}O_2$ into the formyl group of Chl *b* in a mutant of the green alga, *Chlorella vulgaris*: they also used DCMU but the only precaution against *gem*-diol formation was rapid extraction at low temperature rather than chemical modification of the chromophore.

EXPERIMENTAL PROCEDURES

Chemicals

$H_2{}^{18}O$ and $^{18}O_2$ with 96.1% and 99.5% certified isotope enrichment, respectively, and NaB^2H_4 were supplied by Novachem Pty Ltd (Melbourne, Australia). $NaBH_4$ and 8-hydroxyquinoline were obtained from Merck-Schuchardt (Darmstadt, Germany). Solvents and other chemicals were analytical grade reagents or purified by standard techniques. DEAE-cellulose (DE 52) was supplied by Whatman Laboratory Division and a suspension in methanol was prepared as described by Sato and Murata [19]; for chromatography of Chls *a* and *b*, the DEAE-cellulose was then equilibrated with $CHCl_3$. Chl *b* was prepared as previously described [20]. DCMU was a gift from Professor P. Dittrich (Botanisches Institut, Universität München).

Organisms, growth and greening conditions

Etiolated maize seedlings (*Zea mays* hybrid var. Dekalb XL689) were grown in the dark at 18°C for 18 days [21]. As specified in the text, etiolated leaves were excised from these seedlings and placed in either H_2O or $H_2{}^{18}O$ (0.3–0.6 ml as required) in small tubes (10×75 mm) and greened under white light (50–60 μmol photon · m^{-2} · s^{-1}, Philips TLD 18 W/84 tubes) for up to 26 h at 27°C [22] in front of a fan to enhance transpiration. For greening experiments in $^{18}O_2$, three etiolated leaves were placed in a Thunberg tube (approximate total volume: 50 ml) containing 2 ml de-oxygenated water, briefly evacuated to 2.7 kPa and filled with approximately 70% $^{18}O_2$ and 30% N_2 by standard vacuum-line techniques: the final pressure in the tubes was 67–100 kPa. The sealed Thunberg tubes were then illuminated as above for up to 26 h.

In the DCMU experiment, the cut ends of the excised leaves were placed in 5 mM DCMU in a Thunberg tube and illuminated as above: to ensure the leaves were thoroughly permeated with DCMU prior to illumination, the cut ends were placed in the DCMU solution for 4 h at 18°C in the dark in front of a fan to enhance transpiration.

Table 2. The theoretical distribution of the clusters derived from the natural abundances of the C, H, N, O and Mg isotopes. Phe = phaeophytin.

Compound	Formula	$(M)^+$	$(M+1)^+$	$(M+2)^+$	$(M+3)^+$	$(M+4)^+$
Chl *a*	$C_{55}H_{72}N_4O_5Mg$	100	77.2	43.8	16.7	4.4
Phe *a*	$C_{55}H_{74}N_4O_5$	100	64.3	21.4	4.8	0.8
[7-hydroxymethyl]-Chl *b*	$C_{55}H_{72}N_4O_6Mg$	100	72.2	44.0	16.9	4.5
[7-hydroxymethyl]-Phe *b*	$C_{55}H_{74}N_4O_6$	100	64.4	21.6	4.9	0.8

Extraction of chlorophylls from greened maize leaves

Leaves were finely chopped with scissors into a chilled mortar and extracted in dim light by grinding with a pestle to a fine translucent pulp in a freshly prepared extractant (14 ml/g fresh mass leaves) of methanol containing $NaBH_4$ and 8-hydroxyquinoline in equimolar (16.5 mM) concentrations [23]. Extraction was completed in 3 min when glucose was added to react with unused $NaBH_4$.

Column chromatography of extracted chlorophyll derivatives

The chlorophylls in the methanolic supernatant were transferred to diethylether by adding large volumes of saturated brine. The ether solution was washed with large volumes of brine, dried over solid NaCl and evaporated to dryness at approximately 30–40 °C in a rotary evaporator. The chlorophyll residue was redissolved in a minimum quantity of $CHCl_3$ and applied to a column (4×65 mm) of DEAE-cellulose equilibrated with $CHCl_3$. The elution of the chlorophylls was monitored by absorption spectroscopy of each 1-ml fraction of chlorophyll-containing eluant between 680–630 nm and 480–400 nm.

Spectroscopy

Absorption spectroscopy was performed in quartz cuvettes (1 ml; 1-cm light path) using a Shimadzu UV1202 spectrophotometer. ^{1}H-NMR spectra were recorded in ($^2H_5$)pyridine with a Bruker model AM 360-MHz instrument. Fast-atom-bombardment (FAB) mass spectra of Chl *a* and [7-hydroxymethyl]-Chl *b* were recorded using liquid surface ionization technique in a *m*-nitrobenzyl alcohol matrix in a Finnigan model MAT9000 with a caesium gun (20 kV, ≈1 μA) and 1200-Da resolution. The pigments were dissolved in methylene chloride before deposition on the ion source. After a survey spectrum recorded in the exponential scan mode, 20–25 spectra of the molecular ion region in the linear scan mode were averaged. For the analysis, the natural-abundance-based intensity distributions of the ion clusters shown in Table 2 were used. Further, adjustment was made for the varying amounts of M^+, $(M+H)^+$ and $(M-H)^+$ ions arising in the spectrometer [24]. This was done by an iterative procedure assuming that the relative intensities of these three ions within a cluster was constant. Since partial demetallation occurs during FAB-MS of chlorophylls, both the molecular ion region for the chlorophylls (i.e. Mg complexes) and phaeophytins (Mg-free pigments) were analysed.

RESULTS

Formation of [3-ethyl]-[7-hydroxymethyl]-Chl *b* in leaf extracts

Because aldehydes are prone to reversible *gem*-diol formation, we initially sought to minimize loss of ^{18}O label from the formyl group of Chl *b* by rapidly extracting the leaf pigments with methanol containing 16.5 mM $NaBH_4$. Chromatography on a DEAE-cellulose column with $CHCl_3$ solvents containing 0–10% methanol revealed the presence of not only the expected Chl *a* (**I**; λ_{max} 666 and 332 nm), [7-hydroxymethyl]-Chl *b* (**IV**: λ_{max} 659 and 434 nm) and [7-hydroxymethyl]-[13^1-hydroxy]-Chl *b* (**X**: λ_{max} 635.5 and 415 nm) but also [3-ethyl]-[7-hydroxymethyl]-Chl *b* (**IX**: λ_{max} 659 and 428 nm) which is a diethyl derivative (see Table 1): no [3-ethyl]-Chl *a* could be found in these extracts. Although reduction of conjugated double bonds by borohydride is known [25], previous experiments with isolated Chl *b* and $NaBH_4$ revealed no 3-vinyl group reduction [23]. Up to 60% of the original Chl *b* was converted to this diethyl derivative [23] and interfered with detection of ^{18}O labelling by mass spectroscopy which cannot readily distinguish between the addition of 2Da by incorporation of two protons or by replacement of ^{16}O by ^{18}O. With extreme care, separation of [7-hydroxymethyl]-Chl *b* and [3-ethyl]-[7-hydroxymethyl]-Chl *b*, as their corresponding phaeophytins, was achieved by HPLC at low temperature on a silica gel column using toluol/methanol/isopropanol gradients but reproducible results were difficult to obtain.

After extensive investigations of the 3-vinyl-group reduction, it was found that addition of 8-hydroxyquinoline to the extractant, in equimolar concentration with the $NaBH_4$, almost completely inhibited the reduction of the 3-vinyl group but not of the 7-formyl group [23] and this inhibitory technique (see Experimental Procedures) was used in all experiments (see below). Although the use of the chelator suggests that a metal ion is involved in vinyl-group reduction, the requirement for equimolar 8-hydroxyquinoline and $NaBH_4$ indicates that an equimolar complex with modified reducing properties may be formed: not only is the 3-vinyl group not reduced under these conditions but also the 13^1-oxo group is no longer reduced [23].

Identification of the major reduction products of Chl *b* in the absence of 8-hydroxyquinoline

The ^{1}H-NMR spectra of [7-hydroxymethyl]-Chl *b* (**IV**) and [3-ethyl]-[7-hydroxymethyl]-Chl *b* (**IX**) agreed with the structures and a full set of signals for both is shown in Table 3. The most important differences between the spectra of these two compounds involve the signals of the 3-vinyl group. Whereas in Chl *b* in ($^2H_6$)acetone they occur at 7.85 ppm (H_X), 6.15 ppm (H_A) and 5.98 ppm (H_B) [26], they are shifted in [7-hydroxymethyl]-Chl b to 8.06 ppm (H_X) 6.38 ppm (H_A) and 6.06 ppm (H_B) but are absent from the spectrum of the diethyl compound (see Table 3) where the ring current is also slightly reduced. The OH signal, which is somewhat variable and known to be very solvent-dependent, occurs at approximately 7.45 ppm in both products (see Ta-

Table 3. ^{1}H-NMR chemical shifts of 7-[hydroxymethyl]-Chl *b* and [3-ethyl]-[7-hydroxymethyl]-Chl *b* in ($^2H_5$)pyridine observed in this work. The 13^2-epimer ratio is approximately 4.2:1.

Proton	Chemical shift in	
	[7-hydroxymethyl]-Chl *b*	[3-ethyl]-[7-hydroxymethyl]-Chl *b*
	ppm	
10-H	10.36 (s)	10.35 (s)
5-H	9.96 (s)	9.87 (s)
20-H	8.78 (s)	8.75 (s)
2-CH_3	3.30 (s)	3.21 (s)
3^1-H	8.09 (dd)	3.42 (m)[e]
3^2-H_A	6.40 (d)	–
3^2-H_B	6.07 (d)	1.59 (t)[c]
7-CH_2[b]	6.10 (d,br)	6.20 (s,br)
7^1-OH[a]	7.45 (s,br)	n.d.
8-CH_2	3.88 (q)	3.9[d]
8-CH_3	1.74 (t)	1.65 (t)
12-CH_3	3.65 (s)	3.65 (s)
13^2-H	6.79 (s)	6.76 (s)
13^2-$COOCH_3$	3.91 (s)	3.90 (s)
17-H	4.48 (d,br)	4.50[d]
17-CH_2-CH_2	2.6–3 (m)	2.5-3 (m)
18-H	4.73 (q)	4.70[d]
18-CH_3	1.78 (d)	1.78 (d)
P_2	5.38 (t)	n.d.
P_1	4.68, 4.52 (m)	n.d.

[a] Exchangeable with ^2H_2O.
[b] Intensity 50% reduced when prepared with NaB^2H_4.
[c] 3^1-CH_3 group.
[d] Weak multiplet.
[e] 3-CH_2 group.

ble 3) and was identified by $^1H/^2H$ exchange with ^2H_2O. The mass spectrum always showed mixtures of M^+ and $(M+H)^+$ ions. In [7-hydroxymethyl]-Chl *b* the base ions were at *m/z* = 906 and 907 with the isotope peaks as expected (see Experimental Procedures). Upon reductive extraction with NaB^2H_4 in methanol, there was an increase in mass by 1 Da and a decrease in the intensity of the 7-CH_2 signal at 6.10 ppm by 50% in the ^{1}H-NMR spectrum. The masses of the diethyl derivative were 2 Da higher. In addition, the mass spectra of the corresponding phaeophytins showed ion groups 22 Da lower than the corresponding chlorophylls: the demetallation occurred during ionization in the mass spectroscope and is more pronounced in Chl *a* than in these [7-hydroxymethyl]-Chl *b* derivatives. The mass spectrum of [3-ethyl]-[7-hydroxymethyl]-Chl b again showed M^+ and $(M+H)^+$ base ions but located at *m/z* 908 and 909 in an approximately 4:1 ratio. Reductive extraction with NaB^2H_4 in methanol increased the mass by 2 Da indicating the incorporation of one ^{2}H atom into both the 7-hydroxymethyl group and 3-ethyl group.

Greening etiolated maize leaves in the presence of $H_2{}^{18}O$ by illumination with white light

Before testing the hypothesis that the formyl group of Chl *b* is formed via hydration of a methylidene group and subsequent dehydrogenation (Schemes B1 and B2; Fig. 1), it was necessary to conduct several controls.

The first control was designed to determine if a slow exchange of oxygen isotope occurs during the long greening period due to reversible *gem*-diol formation. Several dark-grown maize seedlings were greened in white light. All further manipulations were carried out under white light (see Experimental Procedures). To reduce the $H_2{}^{16}O$ content, the green seedlings were partially dehydrated in a desiccator over P_2O_5 before excising the leaves and inserting the cut ends in $H_2{}^{18}O$ in tubes for periods up to 26 h in front of a fan to enhance transpiration (see Experimental Procedures). Both the diminished $H_2{}^{18}O$ levels in the tubes and the mass of the leaves after this treatment indicated significant uptake of the labelled water (approx. 0.5 ml). In a typical experiment, a leaf weighing approximately 280 mg after partial desiccation weighed 360 mg after uptake of $H_2{}^{18}O$: without allowing for loss by transpiration, this suggests at least 80 mg $H_2{}^{18}O$ has been taken up by the leaf, corresponding to about 25% of the total water content. However, after extractions with methanol containing equimolar 8-hydroxyquinoline and $NaBH_4$, mass spectroscopy of the [7-hydroxymethyl]-Chl *b* revealed no incorporation of ^{18}O isotope above the noise level.

The second control sought to determine whether a rapid exchange of isotopic label could occur by reversible *gem*-diol formation during extraction. Excised green leaves were ground in $H_2{}^{18}O$ and extracted either immediately or after 15 min with the reductive extractant. In both controls, mass spectroscopy of the [7-hydroxymethyl]-Chl *b* showed no incorporation of ^{18}O isotope.

In the actual experiment to determine if the 7-formyl oxygen is derived from water, several dark-grown maize seedlings were partially dehydrated as above, but in the dark. The leaves were then excised, the cut ends immersed in $H_2{}^{18}O$ and illuminated with white light (see Experimental Procedures) for up to 26 h at 27 °C in front of a fan to enhance transpiration. Diminished labelled-water levels and leaf mass again indicated significant uptake of $H_2{}^{18}O$ (see above). The greened leaves were extracted with methanol containing 8-hydroxyquinoline-modified $NaBH_4$: mass spectroscopy of the [7-hydroxymethyl]-Chl *b* showed that no incorporation of ^{18}O from $H_2{}^{18}O$ into the Chl *b* molecule had occurred. These experiments were performed in the absence of DCMU which would inhibit photosynthetic oxygen formation from $H_2{}^{18}O$ (see below).

Greening etiolated maize leaves in the presence of $^{18}O_2$

Excised leaves from dark-grown maize seedlings were greened by illumination in white light in Thunberg tubes in the presence of approximately 70% $^{18}O_2$ and 30% N_2 for 16 h and 26 h at 27 °C (see Experimental Procedures); N_2 was included since previous experiments with atmospheres containing 100% $^{16}O_2$ showed that greening was consistently 50% inhibited while 70% oxygen produced very little inhibition. The final total pressure in the tubes was variable (67–101 kPa) but there was no obvious difference in greening amongst these leaves.

FAB-MS was carried out on Chl *a* extracted from excised leaves greened in an $^{18}O_2$-containing atmosphere. Typical mass spectra are shown in Fig. 2. The low-resolution spectrum (A) shows that Chl *a* is demetallated under FAB conditions to phaeophytin (Phe) *a*. The high-resolution spectra B and C show the relative intensities of the major ions of Chl *a* and Phe *a*, respectively, from leaves greened in the presence of $^{18}O_2$: the superimposed stick spectra show

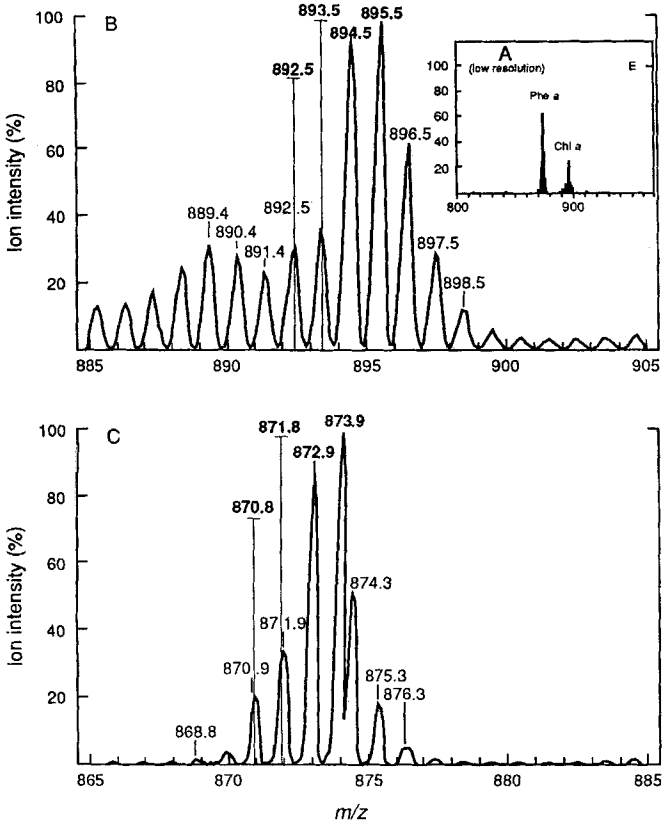


Fig. 2. FAB-mass spectrum of Chl *a* (I) was obtained after reductive extraction of leaves greened for 26 h in the presence of an atmosphere containing 70% $^{18}O_2$ and 30% N_2. Spectrum A is a low-resolution spectrum and shows very considerable formation of Phe *a* during MS. B and C are high-resolution spectra of the molecular ion regions of Chl *a* and Phe *a*, respectively: the actual *m/z* values for the major ions are shown in bold figures. The superimposed 'stick spectra' in B and C show the relative intensities of the M^+ and $(M+1)^+$ ions of Chl *a* and Phe *a*, respectively, after extraction from leaves greened for 26 h in air of normal isotope composition.

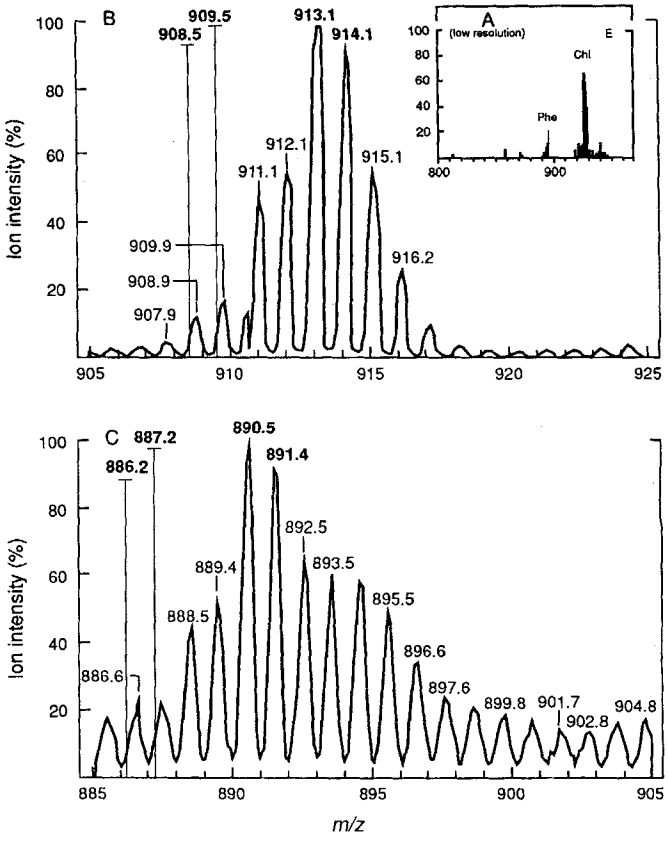


Fig. 3. FAB-mass spectra of [7-hydroxymethyl]-Chl *b* (IV). The low-resolution spectrum (A) and the high-resolution spectrum (B) of the Chl *b* derivative were obtained after reductive extraction of leaves greened for 26 h in the presence of an atmosphere containing 70% $^{18}O_2$ and 30% N_2. The high-resolution spectrum of the Phe *b* derivative (spectrum C) was obtained after 16 h of greening as the spectrum obtained after 26 h was too noisy to be useful (see Table 4): spectrum A, showing the relative intensities of the Chl *b* and Phe *b* derivatives, indicates only minor formation of the Phe *b* derivative during mass spectroscopy. In spectra B and C the actual *m/z* values for the major ions are shown in bold figures. The superimposed 'stick spectra' in B and C show the relative intensities of the M^+ and $(M+1)^+$ ions of the chlorophyll and phaeophytin, respectively, after extraction from leaves greened for the appropriate period in air of normal isotope composition.

the relative intensities of the major ions of Chl *a* and Phe *a* obtained from leaves greened in air. Comparison of each stick spectrum with its partner spectrum clearly shows an increase of 2Da (see Fig. 2) when the pigments were obtained from leaves incubated in the presence of $^{18}O_2$: this indicates the incorporation of one ^{18}O atom/tetrapyrrole molecule. The analyses of the mass spectra are described in the Experimental Procedures. The isotope enrichments in Chl *a* were 75% and 72% after 16 h and 26 h, respectively (Table 4). The Phe *a* molecular ion was analysed in parallel: the slightly higher incorporation values (77% and 76%) allow an estimate of the precision of the mass spectra analyses. This ^{18}O atom is incorporated into the 13^1-oxo group of Chl *a* by an oxygenase activity demonstrated previously by Walker et al. [7].

Typical high-resolution mass spectra of [7-hydroxymethyl]-Chl *b* (spectrum B) and its phaeophytin (spectrum C) are shown in Fig. 3: the low-resolution spectrum (A) shows that less demetallation occurred during mass spectroscopy of the Chl *b* derivative than with Chl *a*. The stick spectra in Fig. 3B and C again show the relative intensities of the major ions of the chlorophyll and its phaeophytin formed during greening in air. Comparison of the stick and partner spectra showed an increase of 4Da (see Fig. 3) in the pigments formed in the presence of $^{18}O_2$ indicating the incorporation of two ^{18}O atoms/tetrapyrrole molecule: the first is again located in the 13^1-oxo group and the second, therefore, must be the 7-formyl oxygen. It is reasonable to assume that the first labelling site is at C-13^1 because the point of divergence between the Chl *a* and Chl *b* pathways occurs after isocyclic ring closure when the C-13^1 ketonic oxygen is introduced to the protochlorophyllide molecule [7]. Thus, when calculating ^{18}O-labelling of the 7-formyl group of Chl *b*, it is also reasonable to assume that labelling at the 13^1-oxo group of Chl *b* is the same as in Chl *a*. With this assumption, the labelling of the 7-formyl oxygen was as high as 93% and 92% after 16-h and 26-h incubations, respectively (see Table 4). The enrichment occurring in the phaeophytin derivative was calculated to be 98% (Table 4). The enrichment of the formyl oxygen, ranging over 92–98%, is close to the isotopic purity of the precursor $^{18}O_2$ (99.9%) and this has important biological implications (see Discussion).

Table 4. ^{18}O-Labelling of chlorophylls and phaephytins during greening of maize leaves. Details of greening excised etiolated maize leaves under an atmosphere of 70% $^{18}O_2$ and 30% N_2 are described in Experimental Procedures. Values are given as mol oxygen-isotope-derived cluster/100 mol total ions. The ratio of Chl *a*/Phe *a* = 1:3, that of Chl *b* derivative/Phe *b* derivative = 2.7:1. MS analysis showed that [7-hydroxymethyl]-Chl *b* contained only insignificant amounts of [3-ethyl]-[7-hydroxymethyl]-Chl *b*. The spectrum of [7-hydroxymethyl]-Phe *b* was noisy and, after a 26-h greening period, was too noisy to be useful.

Pigment	Greening period	Molecular ion intensity at (*m/z*)						^{18}O-labelling at	
		^{16}O		^{18}O					
				1 atom/ tetrapyrrole molecule		2 atoms/		C13¹	C7¹
	h	(*m/z*)	%	(*m/z*)	%	(*m/z*)	%	%	
Chl *a*	16	(892.5)	24	(894.5)	76	–	–	76	–
	26		28		72		–	72	–
Phe *a*	16	(870.5)	23	(872.5)	77	–	–	77	–
	26		24		76		–	76	–
[7-hydroxymethyl]-Chl *b*	16	(908.5)	6	(910.5)	29	(912.5)	65	76	93
	26		8		33		59	74	92
[7-hydroxymethyl]-Phe *b*	16	(886.5)	7	(888.5)	27	(890.5)	66	76	98
	26		–		–		–	–	–

In the above experiment, the photosynthesis inhibitor, DCMU, was not used to prevent dilution of $^{18}O_2$ by inhibiting $^{16}O_2$ production from $H_2{}^{16}O$ in the cell sap. A comparison of the isotope enrichment after 16 h and 26 h showed a decrease of 1–4% over this of 10-h period indicating minor dilution by photosynthetically produced $^{16}O_2$. Assuming rapid equilibration of the photosynthetically produced $^{16}O_2$ with the $^{18}O_2$, this 1–4% decrease in isotope enrichment corresponds to production of about 0.5–2.0 ml $^{16}O_2$/50-ml Thunberg tube over the 10-h period. It has been shown that C_4 plants, when photosynthesizing maximally, evolve 0.07 μmol O_2/μmol photon absorbed [27]. Thus, three leaves with a total area of 25 cm^2 in a Thunberg tube would produce 8.5 ml $^{16}O_2$ in 10 h at 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$. However, after 26 h in the light, the leaves are not fully greened and there is considerable self shading in the Thunberg tube. Also, there is a significant lag in the development of O_2-evolving activity in greening leaves [28]. Thus formation of 0.5–2.0 ml $^{16}O_2$ over 10 h in this experiment is not unreasonable.

When the experiment was repeated in the presence of 5 mM DCMU (see Experimental Procedures), there was no significant increase in the amount of ^{18}O incorporation from $^{18}O_2$ in either the C-7¹ or C-13¹ positions (data not shown). Inspection of the mass spectra of the Chl *a* and *b* derivatives from leaves greened in the absence of $^{18}O_2$ shows clearly that little or no addition of ^2H occurs in the mass spectrometer.

DISCUSSION

Possible involvement of a mono-oxygenase in Chl *b* formation

Up to 93% ^{18}O enrichment of the 7-formyl oxygen of Chl *b* was achieved using $^{18}O_2$ (99.5%) as substrate by careful optimization of the greening conditions and the extraction procedure. High enrichment of the 7-formyl group oxygen of Chl *b* in the presence of $^{18}O_2$ and the absence of detectable label from $H_2{}^{18}O$ excludes all hydratase mechanisms and unequivocally demonstrates that molecular oxygen is the sole precursor of the formyl oxygen and indicates an oxygenase reaction is involved. Probably, the oxidation of the precursor 7-methyl group is catalysed by a relatively common mixed-function oxygenase pathway (see Scheme A2). The genetic analysis of pigment-deficient mutants of *Chlamydomonas reinhardii* by Chunaev et al. [5] suggests that the final step of Chl *b* biosynthesis is controlled by a single gene possibly indicating a single enzyme. Since a mono-oxygenase would be expected to produce a 7-hydroxymethyl derivative (Scheme A2), either a single enzyme with unusual properties leading directly to a carbonyl group is required or an additional dehydrogenase (Scheme A2): if there are non-specific dehydrogenases in the chloroplast which can catalyse the last putative reaction of Chl b formation then this two-enzyme process may appear to be controlled by one gene. Similarily, the dioxygenase pathway, exemplified by α-oxidation of fatty acids, involves a peroxide intermediate (Scheme A1) which may be degraded to simulate single-gene control.

Important biological implications of the high enrichment of the 7-formyl oxygen of Chl *b* by $^{18}O_2$

High enrichment of the 7-formyl group of Chl *b* shows that reversible *gem*-diol formation during the long 26-h greening period with unlabelled cellular water is slow. This suggests that oxidation of the methyl group occurs in a very hydrophobic location within the thylakoid membranes, possibly when the substrate, Chl *a* (or a close precursor), is already in the antenna pigment-protein complex or in a membrane-bound transport protein where water may be absent or have different properties so that reversible *gem*-diol formation is minimal.

The high enrichment indicates that the reduction of the formyl group of Chl *b* by 8-hydroxyquinoline-modified $NaBH_4$ in methanol is necessary to prevent isotope loss by reversible *gem*-diol formation during extraction: recent work with a *Chlorella vulgaris* mutant [18] showed comparable ^{18}O enrichment of the 13^1-oxo group of Chl *a* but enrichment of the 7-formyl-group oxygen was only about one third of that demonstrated here. The high enrichment of the 7-formyl group relative to the 13^1-oxo group in this work indicates that isotope loss by exchange may occur during extraction at $C13^1$; however, keto groups are less amenable to exchange. It is more probable, therefore, that the lower enrichment at $C13^1$ reflects the occurrence of an isotope exchange during the oxygen-incorporating cyclization process of isocyclic ring E formation [7] which is catalysed in a hydrophilic space in the chloroplast by soluble-stromal enzymes in association with membrane-bound enzymes [29, 30].

All oxygenase reactions are strongly exothermic and hence irreversible [6]. This may be important when considering light acclimation in higher plants when Chl *a/b* ratios change without significant change in total chlorophyll. When acclimating to higher light intensity the increase in Chl *a/b* ratio must be due either to selective Chl *b* degradation or due to the conversion of the 7-formyl to a 7-methyl group by a different enzyme or enzyme system because there can be no conversion of Chl *b* to Chl *a* by reversal of the oxygenase-catalysed reaction.

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