

Chlorophyll *b* to Chlorophyll *a* Conversion Precedes Chlorophyll Degradation in *Hordeum vulgare* L.*

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This study reveals by *in vivo* deuterium labeling that in higher plants chlorophyll (Chl) *b* is converted to Chl *a* before degradation. For this purpose, de-greening of excised green primary leaves of barley (*Hordeum vulgare*) was induced by permanent darkness in the presence of heavy water (80 atom % ^2H). The resulting Chl *a* catabolite in the plant extract was subjected to chemical degradation by chromic acid. 3-(2-Hydroxyethyl)-4-methylmaleimide, the key fragment that originates from the Chl catabolite, was isolated. High resolution ^1H -, ^2H -NMR and mass spectroscopy unequivocally demonstrates that a fraction of this maleimide fragment consists of a mono-deuterated methyl group. These results suggest that Chl *b* is converted into Chl *a* before degradation. Quantification proves that the initial ratio of Chl *a*:Chl *b* in the green plant is preserved to about 60–70% in the catabolite composition isolated from yellowing leaves. The incorporation of only one deuterium atom indicates the involvement of two distinguishable redox enzymes during the conversion.

Chl 1b occurs as an accessory pigment of the light harvesting systems in higher plants, green algae, Euglenaceae and Prochlorophyta and comprises up to 30% of the total Chls (1). As a matter of fact, although both Chl *a* and *b* catabolites were found in the green alga *Chlorella protothecoides*, only catabolites originating from Chl *a* were isolated from higher plants (2, 3). The following observations have led to the hypothesis that in higher plants Chl *b* is converted into Chl *a* before degradation: (i) A system comprising isolated etioplasts of cucumber *Cucumis sativus* showed that chlorophyllide *b* is converted to Chl *a* (4). Subsequent studies of a system consisting of barley (*Hordeum vulgare*) etioplasts showed that chlorophyllide *b* is converted via 7^1 -hydroxy Chl *a* to Chl *a*; both steps required the presence of ATP in the incubation mixture (5). (ii) *In vitro* chlorophyll degradation experiments with membrane fractions of senescent chloroplasts of rape cotyledons (*Brassica napus*) have shown that Pheo *b* is refused as substrate of the ring cleaving pheophorbide oxygenase under the condition that Pheo *a* is accepted (2). (iii) Zn(II) Pheo *b* was converted to Zn(II)

7^1 -hydroxy Chl *a* in intact barley etioplasts, the reduction required NADPH or NADH. NADH was found to be less effective, ATP was not essential (6). (iv) Fully senescent cotyledons of rape (*B. napus*) contain amounts of Chl *a* catabolites **7a-c** accounting for 90% of total Chls originally present in the mature leaf tissue (2, 7).

Chl *a* to Chl *b* conversion appears now to be part of a general Chl *a(b)* interconversion cycle, which is considered to play an important role in the formation and reorganization of the photosynthetic apparatuses (5) and which enables plants to adapt to high and low light conditions by adjusting the ratio Chl *a*:Chl *b* from 3.8–4.8 to 2.7–3.0, respectively (8).

In vivo labeling experiments with heavy water to elucidate biogenesis mechanisms are barely mentioned in the literature. The method has been applied to study peripheral changes of the porphyrin system during the biosynthesis of bacteriochlorophyll *a* in the photosynthetic bacterium *Rhodospirillum rubrum* (9, 10) and to follow the insertion of a deuteron during the light induced cyclization of dark synthesized acyclic carotenoid precursors in the green alga *Senedesmus obliquus* (11). Recently we have demonstrated by *in vivo* deuterium labeling experiments with *C. protothecoides* that in the last step of the macrocyclic ring cleavage a hydrogen atom is highly stereoselectively inserted in the catabolite (12).

In this work we evince by spectroscopic methods that during the de-greening of barley leaves (*H. vulgare*) in heavy water a fraction of the Chl *a* catabolite is deuterium labeled; specifically one deuterium atom is incorporated in the methyl group of the apparent chlorophyll *a* catabolite. The results suggest that Chl *b* is converted to Chl *a* by two different cofactors.

EXPERIMENTAL PROCEDURES

Chemicals and Materials—All chemicals were reagent grade; all solvents were distilled before use. Heavy water containing 80 atom % deuterium was supplied from Armar AG, CH-5312 Döttingen (Switzerland). Thin layer chromatography aluminum foils pre-coated with silica gel 60PF₂₅₄₊₃₆₆ (0.2 mm) and silica gel 60PF (0.040–0.063 mm) for column chromatography were purchased from Merck, Darmstadt, Germany and 35cc Sep-Pack® Vac C-18, 10 g from Waters (Milford, MA).

Plant Material—Barley seeds (*H. vulgare* L. cv. Gerbel) were a gift from Florimond Desprez, Cappelle-en-Pévèle 59242 Templeuve, France. The seeds were germinated in high density (5 seeds/cm²) in moist garden soil, purchased from a local market, and grown for about 7–10 days in natural light until the primary leaves reached about 10–15 cm in height.

NMR— ^1H -, ^{13}C -, and ^2H -NMR measurements were performed on a Bruker Avance DRX-500 spectrometer operating at the frequencies of 500.13, 125.75, or 76.77 MHz, respectively. Samples were dissolved in CDCl₃. Chemical shifts were recorded in ppm downfield from tetramethylsilane except for deuterium NMR in which CDCl₃, δ = 7.27 ppm was used as internal standard. ^1H -Spectra for the labeled material were recorded at a resolution of $7.63 \cdot 10^{-5}$ ppm/data point. Gaussian multiplication of the free induction decay (13, 14) was performed on the NMR-Unix station with UXNMR version 2.1. (Parameters used: Gaussian broadening = 0.5 Hz, line broadening = –0.55 Hz).

Mass Spectroscopy (MS)—Mass spectra were obtained with a Bruker FTMS 4.7T BioAPEXII, using chemical ionization (CI) or electrospray ionization techniques in the positive mode. Electrospray ionization

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¹ The abbreviations used are: Chl, chlorophyll; Chlid, chlorophyllide; Pheo, pheophorbide; AcOEt, ethyl acetate; AcMe, acetone; AcOH, acetic acid; CH₂Cl₂, dichloromethane; EtOH, ethanol; MeOH, methanol; d_w , h_w , number of attached deuterium or hydrogen atoms in a molecule; ppm, parts per million; MS, mass spectroscopy; amu, atomic mass units.

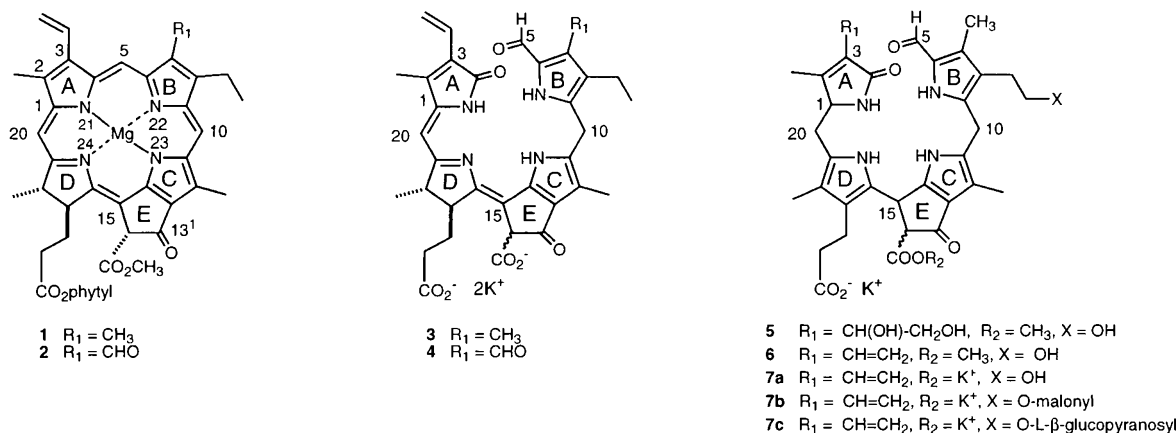


FIG. 1. **Formulae of Chl catabolites isolated from green plants.** *Chlorella protothecoides* **3**, **4** (20, 21), *Hordeum vulgare* **5** (16), *Liquidambar spec.* (22) and *Cercidiphyllum japonicum* **6** (3), and *Brassica napus* **7a-c** (7, 23). The structures **1** for Chl *a* and **2** for Chl *b* show the numbering system commonly in use for chlorophyll and its derivatives according to IUPAC-IUB 1979 (24, 25). This convenient numbering system is likewise applied to the ring cleavage products of the chlorophyll macrocycle, which are denominated as *seco* derivatives in accordance to IUPAC-IUB rule R-1.2.6.2. (26, 27).

spectra were expanded in the range of the molecular ion up to a resolution of 150,000. The most recent IUPAC data for atomic mass and natural abundance of the elements were used to calculate exact molecular masses (15).

De-greening of Primary Leaves of Barley—The procedure was similar as described previously (16) but with the following modifications: To arrest chlorophyll biosynthesis the green intact shoots were left at 25 °C for 12 h in permanent darkness. Afterward, batches of green primary leaves (100 g wet weight; 12 g dry weight) were cut 10–15 cm from the apex and immersed with their ends in heavy water (100 ml, 80 atom % ^2H). The opening of the 1,000 ml beakers was covered with punctured aluminum foil to allow gas exchange. The leaves were subsequently incubated at 25 °C during 7–8 days in permanent darkness. When the green color of the Chls had vanished, the yellowish still turgid leaves were collected and stored frozen until use. Unlabeled natural material was obtained accordingly using tap water instead of heavy water.

Determination of the Atomic $^1\text{H}/^2\text{H}$ Composition of Water—The watering layer was sporadically sampled, and the deuterium content of the water was determined by standard ^1H -NMR (360 MHz) procedure in which the remaining proton signal in the samples was measured. A sealed capillary filled with acetone was used as external proton standard whose signal area was determined from the area of pure water (H_2O) set to 100 atom % ^1H ; a relaxation delay of 60 s was applied during measurements to the sample.

Isolation of 3-(2-Hydroxyethyl)-4-Methylmaleimide (9**) from Yellow Leaves of *H. vulgare***—De-greened yellow leaves of *H. vulgare* (150 g wet weight) were homogenized in a blender with a mixture of 0.1 M potassium phosphate buffer, pH 6.8:AcMe:MeOH = 1:1:1 (300 ml). The resultant slurry was filtered over two layers of cotton gauze, the residue was washed with the disintegration buffer (2×150 ml). The collected filtrates were centrifuged at $5,000 \times g_{av}$ for 10 min. Pellets were discarded and the supernatants were extracted with CH_2Cl_2 (2×400 ml). After phase separation the aqueous layer was shortly evaporated *in vacuo* to remove residual solvent. The remaining solution (200 ml) was filtered through a reversed phase column (35cc Sep-Pack® Vac C-18 cartridges). Afterward the cartridge was washed with 0.1 M potassium phosphate buffer, pH 6.8 (50 ml) and eluted with a 0.1 M potassium phosphate buffer, pH 6.8:AcMe = 1:1 (150 ml) solution. Retention and elution of the catabolite in the cartridge was controlled by a microscale chromic acid degradation assay using aliquots of the eluate (*vide infra*). Positive reacting fractions were pooled (120 ml), the volatile organic solvent was withdrawn *in vacuo* leaving a dark brown aqueous phase (60 ml). A solution consisting of 2 N H_2SO_4 and 1% CrO_3 (60 ml) was added with stirring at room temperature for 5 min. The resulting solution was continuously extracted overnight with diethyl ether. The sodium sulfate dried ether phase was evaporated to incipient dryness, and the residue was applied to four TLC plates. The plates were developed in $\text{CH}_2\text{Cl}_2/\text{AcOEt}/\text{EtOH}/\text{AcOH}$ (50:10:5:0.5). The section of the TLC foil containing maleimide fragment **9** was cut out and eluted with methanol (50 ml). Evaporation of the filtrate *in vacuo* left a residue, which was two times purified by microcolumn chromatography ($\varnothing = 4$ mm, length = 10 cm) using silica gel as stationary and CH_2Cl_2 /acetone

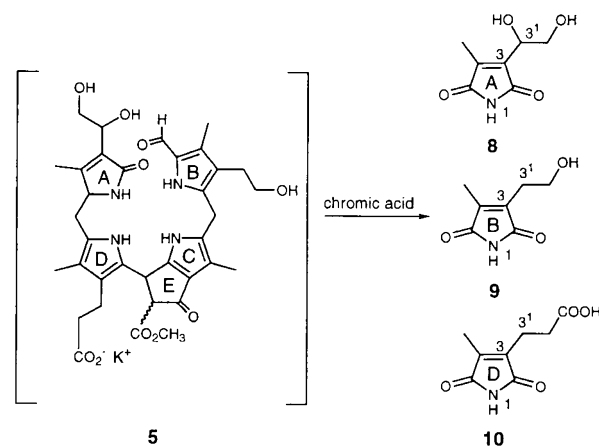


FIG. 2. **Isolated maleimides **8**, **9**, and **10** obtained after CrO_3 oxidation of an extract from de-greened leaves of *H. vulgare*.** Of the three fragments analyzed by ^1H -NMR only a fraction of the methyl group of **9** was deuterium labeled. The constitution of the Chl *a* catabolite from *H. vulgare* **5** (16) is shown in brackets (not isolated).

(8:2) as mobile phase. *Vacuo* evaporation of the effluent afforded 1.7 mg of pure maleimide **9**. Accordingly, maleimides **8** and **10** were isolated from the CrO_3 oxidation mixture, data not shown.

Synthetic 3-(2-Hydroxyethyl)-4-Methylmaleimide (9**)**—Synthetic material was isolated as CrO_3 oxidation product of 3-(2-hydroxyethyl)-4-methylpyrrole. The pyrrole was synthesized in two steps² starting from benzyl-3-methyl-4-methoxycarbonylmethyl-5-methoxycarbonyl-2-pyrrolicarboxylate (**18**). The melting point of **9** is 104–109 °C. ^1H -NMR: 2.01 (*t*, $J = 0.9$ Hz, 3H, $\text{H}_3\text{C}(4^1)$), 2.67 (*td*, $J_1 = 6.1$ Hz, $J_2 = 0.9$ Hz, 2H, $\text{H}_2\text{C}(3^1)$), 3.82 (*t*, $J = 6.1$ Hz, 2H, $\text{H}_2\text{C}(3^2)$), 7.17 (*br*, 1H, HN(1)). ^{13}C -NMR: 8.82 ($\text{C}4^1$), 27.53 ($\text{C}3^1$), 60.54 ($\text{C}3^2$), 139.20 + 140.49 ($\text{C}4 + \text{C}3$), 171.25 + 171.96 ($2 \times \text{C}=\text{O}$). MS-Cl: 156 ($[\text{M} + \text{H}]^+$, 100%), 138 ($[\text{M} + \text{H} - \text{H}_2\text{O}]^+$, 58%), 125 ($[\text{M} + \text{H} - \text{CH}_2\text{OH}]^+$, 6%). IR(KBr): 3,398 (very strong), 1,712 (very strong), 1,360 (strong), 1,086 (strong), 1,041 (strong), 738 (strong).

Maleimide Assay—200- μl samples were agitated with 200 μl of ether Et_2O and 200 μl of chromic acid solution (*vide supra*). The ether solution was spotted on thin layer chromatography and developed in CH_2Cl_2 :AcMe (8:2); $R_f(\text{9}) = 0.27$. The maleimide fragments were visualized with Cl_2 /benzidine (19).

RESULTS

General Description—Fig. 1 shows the constitutional formulae of the catabolites isolated so far and reveals that they are

² P. Folly and N. Engel, unpublished results.

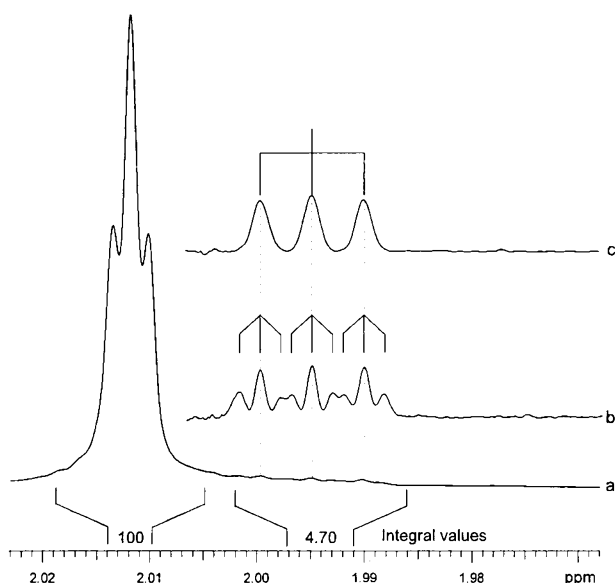


FIG. 3. 500 MHz proton spectrum of the maleimide fragment **9** isolated from *H. vulgare* after de-greening in heavy water (80 atom % ^2H). Only the region of the methyl group is shown. *a*, this trace shows the long range J_{CH} triplet coupling of the tri-protio-methyl group with the methylene group situated at $\text{C}(3^1)$. *b*, Gaussian multiplication of the free induction decay before Fourier transformation and amplification of the signals in close proximity to the tri-protio-methyl group unveils the fraction of molecules having a geminal $^1\text{H}/^2\text{H}$ coupling. *c*, result of a double resonance experiment in which the methylene group $\text{C}(3^1)$ at $\delta = 2.67$ ppm was irradiated. The absence of a quintet in the up-field region excludes the presence of a di-deutero-methyl group.

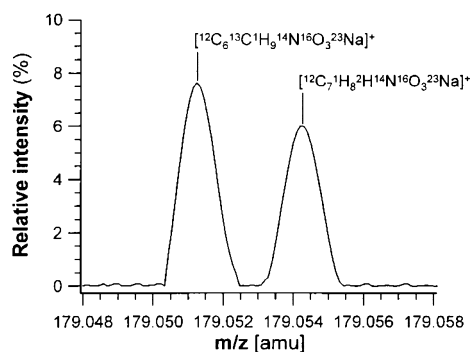


FIG. 4. Electrospray ionization-MS spectrum of the labeled maleimide fragment **9**. The spectrum was recorded on an ion cyclotron resonance instrument. The integral of the molecular peak ion at m/z 178.047, not shown, was set 100 (see Table I). Separation of the signals down to the base line was achieved at a resolution of 150,000. Carbon-13 fraction contains, in addition, the isotopomer with oxygen-17 together the signal integrates to an area of 7.7 rel %.

all bile-pigment-like linear tetrapyrroles derived from an oxygenolytic cleavage at the $\text{C}(4)=\text{C}(5)$ bondage of the former Chls. De-greening experiments were performed with green primary leaves of *H. vulgare* in heavy water (80 atom % ^2H) or in tap water, respectively. The crude enriched plant extract was directly subjected to the chromic acid degradation procedure basically developed for porphyrins (19, 28). The particular maleimide fragment 3-(2-hydroxyethyl)-4-methyl-maleimide (**9**) (Fig. 2), which contains the characteristic β -hydroxyethyl as marker group of the former Chl *a* catabolite of *H. vulgare*, was isolated and spectroscopically analyzed. ^1H - and ^{13}C -NMR and mass spectra of the synthetic and the unlabeled natural material were alike and in accordance with structure **9**. All seven carbon-bound protons were assigned by chemical shift reasoning, coupling constants, and integral values (see "Experimental Procedures"). The N-bound hydrogen atom occurred as single broad signal and the O-bound proton was part of the water peak of the solvent.

Analysis by Nuclear Magnetic Resonance Spectroscopy—The maleimide fragment **9** isolated from the labeling experiment of *H. vulgare* shows a small additional signal group in close proximity to the methyl group $\text{C}(4^1)$ at $\delta = 2.012$ ppm.³ After Gaussian multiplication of the free induction decay and amplification this signal group unambiguously displays the resonance pattern of a mono-deuterated methyl group (Fig. 3). The resonance is centered at $\delta = 1.995$ ppm and appears as a characteristic triplet \times triplet. In a double resonance experiment in which the methylene group $\text{C}(3^1)$ at $\delta = 2.67$ ppm was irradiated, the triplet of the tri-protio-methyl group collapsed as expected into a singlet and the signal group into a simple triplet with a coupling constant of $J_{\text{H}-^2\text{H}} = 2.4$ Hz in a ratio of 1:1:1 (Fig. 3). Both, spin multiplicity and signal ratio immediately evince due to the nuclear spin quantum number $I = 1$ of deuterium that the number of deuterium atoms in the methyl group equals 1. The mono-deuterated methyl group is up-field shifted by $\Delta\delta = 0.017$ ppm relative to the tri-protio-methyl group. This effect is within the range commonly observed for primary isotopic effects (29, 30). Integration of the signal of the tri-protio-methyl group versus the mono-deutero-methyl group revealed a proton ratio of about 100:4.7. From this figure a ratio of $9:9\text{-}d_1$ of 100:7.1 was calculated. The ^2H -NMR spectrum confirmed the presence of one deuterium atom by a triplet ($J_{\text{H}-^2\text{H}} = 2.4$ Hz) centered at $\delta = 2.1$ ppm; no other signals were present except the solvent peak (spectrum not shown).

Analysis by Mass Spectroscopy—Samples were measured in an ion cyclotron resonance spectrometer equipped with an electrospray ionization inlet. Fig. 4 shows the isotopic fine struc-

³ The number of significant figures (4 sf) are not intended to indicate the accuracy of an absolute δ value but serves to calculate chemical shifts differences.

TABLE I

Calculated and measured mass spectroscopic data of the unlabeled and labeled maleimide **9** in the range of the nominal masses 178 and 179

Item	Nominal mass	Possible combinations of isotopic ions	Unlabeled (calculated) ^a		Labeled (measured)	
			Mass exact	Relative natural abundance	Mass exact	Relative abundance
	m/z (amu)		m/z (amu)		m/z (amu)	
1	178	$[^{12}\text{C}_7\text{H}_9^{14}\text{N}^{16}\text{O}_3^{23}\text{Na}]^+$	178.04755	100	178.047 ^b	100
2	179	$[^{12}\text{C}_7\text{H}_9^{15}\text{N}^{16}\text{O}_3^{23}\text{Na}]^+$	179.04449	0.4	— ^c	—
3	179	$[^{12}\text{C}_6^{13}\text{C}^1\text{H}_9^{14}\text{N}^{16}\text{O}_3^{23}\text{Na}]^+$	179.05081	7.6	179.051 ^d	7.7
4	179	$[^{12}\text{C}_7\text{H}_9^{14}\text{N}^{16}\text{O}_3^{17}\text{O}^{23}\text{Na}]^+$	179.05167	0.1	179.054	6.2
5	179	$[^{12}\text{C}_7\text{H}_8^2\text{H}^{14}\text{N}^{16}\text{O}_3^{23}\text{Na}]^+$	179.05365	0.1		

^a Isotopic mass and natural abundance of the constituting elements were taken from the literature (15). For the sake of correctness $0.55 \cdot 10^{-3}$ amu (rest mass of the electron) were subtracted from the uncharged molecular mass ions.

^b The scale was adjusted to the theoretical m/z value of the exact molecular mass ion.

^c Signal hidden in the noise.

^d The signals of the isotopomers containing carbon-13 or oxygen-17 are unresolved.

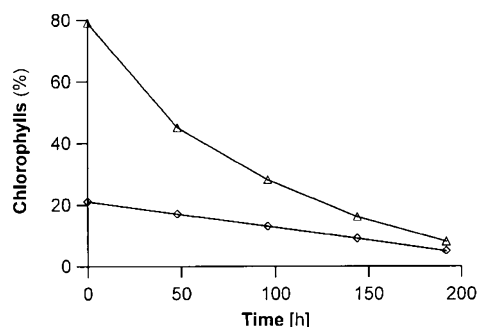


FIG. 5. Decrease of Chl *a* (Δ) and Chl *b* (◇) during the de-greening of barley leaves (*H. vulgare*) in presence of (heavy) water. Both pigments were separated by standard high pressure liquid chromatography techniques in which methanol extracts of the leaves were applied to a RP₁₈ column. The ratio Chl *a* to Chl *b* was determined with the means of a built-in UV-visible diode-array-detector working at the wavelength of 430 and 450 nm, respectively.

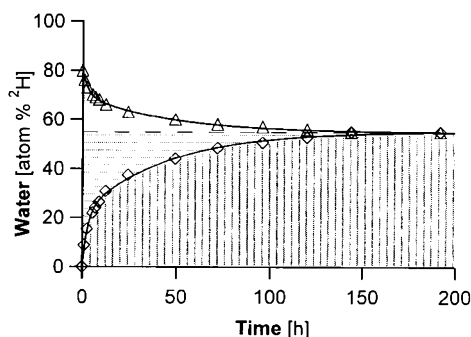


FIG. 6. Exchange rate between the leaves and the heavy water reservoir. Upper curve, time dependent dilution of the heavy water reservoir due to leaking of ordinary water from the leaves. An equilibrium concentration of 55 atom % ²H was reached after about 192 h. Lower curve, the deuterium content in the leaves was calculated from the deuterium content of the watering layer and the equilibrium concentration. The vertically hatched area when subtracted from the total area limited by the dotted equilibrium line furnishes the horizontal hatched area, which represents the total amount of water (H₂O) in the leaves during the de-greening period.

ture at a resolving power of 150,000. The observed molecular peak ion [¹²C₇¹H₉¹⁴N¹⁶O₃²³Na]⁺ was calibrated to *m/z* 178.048 and an integral value of 100. Table I opposes the calculated and measured values. The signals are base line separated exhibiting the expected mass difference of 3×10^{-3} amu. The ion at 179.051 amu [¹²C₆¹³C¹³H₉¹⁴N¹⁶O₃²³Na]⁺ contains carbon-13 and shows the predicted abundance of 7.7 rel %, which is because of the ¹³C natural abundance of 7×1.10 rel %. The second satellite signal at 179.054 amu [¹²C₇¹H₈²H¹⁴N¹⁶O₃²³Na]⁺ contains ²H and presents the number of molecules in which one protium is replaced by one deuterium. This fraction occurs with an abundance of 6.2 rel % from which 0.1 rel % was subtracted because of the natural abundance of deuterium of 9×0.015 . The molar ratio of 9:9-*d*₁ is therefore 100:6.1.

Quantitative Estimation of the Labeling Process—The ratio of Chl *a*:Chl *b* in the leaves during de-greening was followed by high pressure liquid chromatography of a methanol extract (Fig. 5). At the beginning of the incubation experiment this ratio was determined to be 79:21 (100:26). After 8 days in permanent darkness the incubation was terminated. About 10% Chl *a* and 24% Chl *b* of the initial content still remained unchanged in the leaves, which means that about 90% Chl *a* and 76% Chl *b* had vanished. Under the assumption that both Chls are totally converted into catabolites, a ratio Chl *a*:Chl *b* of 81:19 (100:23) should be expected. Random labeling of Chl *b*

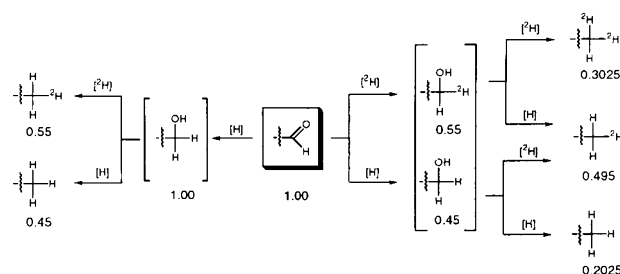


FIG. 7. Calculated deuterium label distribution pattern for a two-step reduction of a formyl (Chl *b*) into a methyl group (Chl *a*) in the presence of heavy water. During the incubation period the concentration in the watering layer diminished from 80 atom % ²H to an equilibrium mixture of 55 atom % ²H (see Fig. 6). Right, statistical [¹H/²H] distribution over both steps. Left, selective reduction of the formyl group by [¹H] to the corresponding alcohol followed by statistical [¹H/²H] labeling in the methyl group formation. The numbers indicate the fraction of moles of individual species carrying none, one, and two deuterium label(s).

in a medium of water containing 80 atom % ²H should yield 80% mono-deuterium labeled Chl *a* and 20% of a “protio-labeled” Chl *a* catabolite in a ratio 9:9-*d*₁ of 100:17.6. As a matter of fact, the deuterium content of the heavy water in the beaker slowly diminished during the incubation period from 80 atom % ²H to an equilibrium concentration of 55 atom % ²H due to the dilution with protic water contained in the leaves (Fig. 6). Under the assumption that the number of deuterium atoms and the initial volume of 100 ml of water remains constant during the experiment the deuterium content of the leaves at each sampling point was calculated from the deuterium concentration of the watering layer and the equilibrium concentration by Eq. 1.

$$x_l = \frac{(x_{t=0} - x_{t=s})}{(x_{t=0} - x_{equ})} \cdot x_{equ} \quad (\text{Eq. 1})$$

where x_l = atom % ²H in the leaves at the sampling time; $x_{t=0}$ = atom % ²H in the water layer at zero time (80%); $x_{t=s}$ = atom % ²H in the water layer at sampling time; x_{equ} = atom % ²H in the water layer at equilibrium (55%).

The area under the calculated curve, which presents the time course of the relative amounts of 55 atom % ²H in the leaves, was subtracted from the total area given by the square of the equilibrium concentration (55 atom % ²H) and the incubation time (192 h). The difference assigns the total of the relative portion of pure water (H₂O) in mixture with 55 atom % ²H in the leaves due to the slow exchange rate. This portion (13.6%) together with the protic fraction of the equilibrium mixture (45%) converts Chl *b* into the nonlabeled apparent Chl *a* in an amount of 1.4 and 7.7 rel %, respectively; the sum was therefore subtracted from the portion of Chl *b* and surcharged to Chl *a*. A theoretical ratio of 9:9-*d*₁ of 100:9.5 was calculated from those figures. This result is in close agreement with the spectroscopic measurements of a 9:9-*d*₁ relation of 100:6.1 for MS and 100:7.1 for ¹H-NMR and accounts for 64 and 74%, respectively, of the theoretical value.

The overall yield was calculated as follows. According to the literature, 115 g *H. vulgare* leaves afforded 13 mg of the intact catabolite **5** which represents a recovery rate of 10% of the original Chls (16). Quantitative conversion with CrO₃ oxidation to the maleimide fragment **9** should therefore yield 3.7 mg per 150 g leaves. In this labeling experiment 1.7 mg of purified **9** was isolated from 150 g leaves, which represents a yield of 46%. This loss is in agreement with the low yields of maleimides generally obtained from CrO₃ oxidation of porphyrins (31).

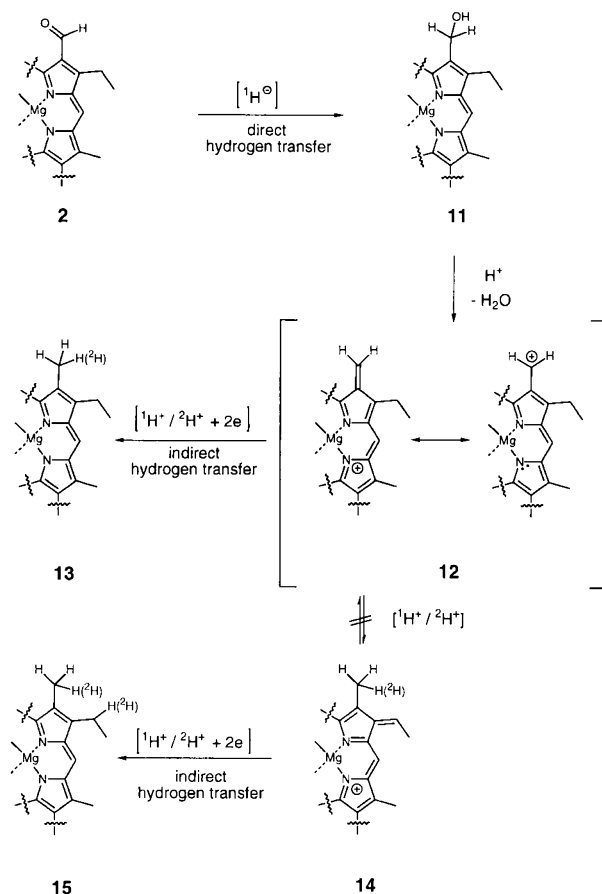


FIG. 8. **Proposed two step reduction of Chl *b* to Chl *a* involving a direct and an indirect hydrogen transfer.** The reduction of the formyl group of Chl *b* to 7¹-hydroxy Chl *a* (11) is mediated by NAD(P)H. The cofactor is regenerated by hydride transfer from a carbon-bond hydrogen source, which was acquired during the phototrophic growth phase of the plant in ordinary water. Elimination of water from the intermediate 11 affords the resonance-stabilized carbocation 12, which is subsequently reduced by two electrons to the corresponding carbanion. Proton/deuteron quenching of the latter with the isotopic composition of the water yields 13, which then contains the observed mono-deuterated methyl group. Isomerization of the carbocation 12 to 14 and following reduction would introduce label in the ethyl group of 15 that has not been observed. For clarity, only the eastern half of the Chl *b* molecule is shown.

DISCUSSION

The ratio unlabeled to labeled **9** reflects both the isotopic composition of the deuterium concentration in the medium and the exchange and hydrogen transfer reaction, which occur during biodegradation. Formally two hydride ions are required to reduce an aldehyde group to a methyl group. Depending on the mechanism, up to two deuterium atoms per methyl group can be incorporated during the catabolic process of Chl *b* in the presence of heavy water (Fig. 7). We found that only one deuterium atom was incorporated.

This fact demonstrates that of the hydrogen isotopes forming the methyl group of the labeled Chl *a* catabolite one originates from the formyl group of the former Chl *b*, one arrives by hydride transfer from an as yet unidentified carbon-bond hydrogen source, and another enters the methyl group as proton/deuteron from the surrounding water (Fig. 8). Enzymatic reduction of an aldehyde group to the corresponding alcohol is generally accomplished by nicotinamide-dependent hydride carriers NAD(P)H (32, 33). It is a hallmark of these coenzymes not to exchange with the deuterium of the surrounding medium (34). We assume that a hydride ion is mediated by a NAD(P)⁺/

NAD(P)H between a carbon-bond hydrogen such as for example (poly)saccharides, which were acquired during the phototrophic growth phase of the plant in ordinary water and the formyl group. This assumption is supported by *ex vivo* experiments in which NAD(P)H was essential to reduce Zn(II) pheophorbide *b* to the corresponding 7¹-hydroxy-Chl *a* (6).

Reductive elimination of a hydroxyl group is generally a more difficult task due to the strong carbon-oxygen bond. This is the reason why enzymatic examples are rarely found in the literature (5, 35). Nevertheless, the unique electronic arrangement of the cyclic 18- π electron porphyrin system facilitates the formation of a resonance-stabilized carbocation **12** (Fig. 8). This elimination process demands an activator capable to transform the hydroxyl group into a better leaving group. In this context it is noteworthy that ATP, which can act as activator was required for the transformation of chlorophyllide *b* to Chl *a* in barley etioplasts (5) but not for the first reduction step to 7¹-hydroxy Pheo *a* (7). Most recently, it has been demonstrated that the final reduction step is achieved when reduced spinach ferredoxin is added to lysed etioplasts (36). Ferredoxins participate in electron transfer reactions, electrons are typically provided by an electron transfer chain involving NADH and/or flavoproteins (33). Therefore, we regard cation **12** as terminal electron acceptor, which becomes reduced by two electrons to the corresponding carbanion. Final quenching of the latter with a proton/deuteron from the aqueous medium would account for the observed statistically mono-deuterium labeling of the methyl group. It has been suggested that **12** and/or **14** (Fig. 8) are possible intermediates (37). However, ¹H- and ²H-NMR spectra show no deuterium label in the ethyl group of the maleimide fragment **9**; isomerization occurs, if at all, only very slowly.

The catabolic sequence Chl *b* → Chl *a* → Chl *a* catabolite is more likely to proceed than a subsequent conversion of an assumed Chl *b* catabolite for the following reasons: (i) Pheo *a* oxygenase appears to be highly specific, the enzyme uses Pheo *a* as substrate but refuses Pheo *b* (*vide supra*). (ii) Independent proofs have shown that the Chl *a*(*b*) converting enzymes are present in higher plants (*vide supra*). (iii) The proposed cation **12** is stabilized by resonance through the extended electronic 18 π -system of the Chl macrocycle, whereas the formation of a corresponding cation from 7¹-hydroxy-Chl *b* catabolite would be less favored by resonance.

Chromic acid oxidation of the bile-pigment-like chlorophyll catabolite **5** of *H. vulgare* present in the enriched plant extract afforded in addition to **9** the corresponding maleimides **8** and **10** (Fig. 2). ¹H- and ²H-NMR spectroscopic investigation showed that of the three maleimides isolated from the plant extract only maleimide **9** was partially mono-deuterium labeled at C(4¹), all others (data not shown) were not. During *de novo* biosynthesis of the Chls all methyl groups should become evenly mono-deuterated in heavy water because of the consecutive decarboxylation of uroporphyrinogen III to coproporphyrinogen III by uroporphyrinogen III decarboxylase. This result confirms apart from Chl *a*(*b*) interconversion the general assumption that angiosperms form no Chls in the dark (17).

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