

Reduction of the Formyl Group of Zinc Pheophorbide *b* *in vitro* and *in vivo*: a Model for the Chlorophyll *b* to *a* Transformation

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The chemical reduction of the formyl group of pheophorbide *b* with sodium cyanoborohydride in methanol leads to 7¹-methoxy- and 7¹-hydroxy-pheophorbide *a*. The same reaction with zinc pheophorbide *b* yields in addition zinc pheophorbide *a*. This was characterized by mass and ¹H-NMR spectroscopy. Infiltration of zinc pheophorbides *a* and *b* and of zinc 7¹-hydroxy-pheophorbide *a* into etiolated oat leaves yielded phytilyated products. The best yield in the esterification was obtained with 7¹-hydroxy-pheophorbide *a*. Analysis of the products revealed the formation of zinc pheophytin *a* from all infiltrated compounds. The significance for the transformation of chlorophyll *b* into chlorophyll *a* is discussed.

Introduction

Chlorophylls *a* and *b* are the main photosynthetic pigments in higher plants and green algae. Biosynthesis of chlorophyll *a* is well established. The last steps are the light-dependent hydrogenation of protochlorophyllide *a* to chlorophyllide *a*, and the subsequent esterification leading to chlorophyll *a* (review: Rüdiger and Schoch, 1988). Biosynthesis of chlorophyll *b* is less well known. The structural difference is a formyl group at C-7 in chlorophyll *b* instead of a methyl group in chlorophyll *a*. The assumption of an oxygenase reaction was supported by the demonstration of incorporation of gaseous dioxygen from the air into the formyl group of chlorophyll *b* in *Chlorella vulgaris* (Schneegurt and Beale, 1992) and in *Zea mays* (Porra *et al.*, 1994). It is not clear, however, which compound is the receptor for oxygen incorporation. As possible candidates, protochlorophyllide *a*, chlorophyllide *a* and chlorophyll *a* have been discussed. The 7¹-hydroxy compound was suggested as an intermediate (Porra *et al.*, 1994).

There are several reports in the literature indicating the reverse reaction, namely the chlorophyll *b* to *a* transformation. Several authors observed that etiolated plants after a short period of greening in the light, when placed back in the dark, accumulate chlorophyll *a* on the cost of chlorophyll

b (Seybold and Egle, 1938; Rudoi *et al.*, 1977; Tanaka and Tsuji, 1981). Whereas Seybold and Egle (1938) discuss their results only in the terms of higher stability of chlorophyll *a* than of chlorophyll *b*, later authors interpreted their results as a direct chlorophyll *b* to *a* transformation. Since such a reaction implies the reduction of a formyl group to a methyl group without reduction of the carbonyl group at the isocyclic ring, the interpretation of a direct reaction has been met with scepticism.

The assumption of a direct chlorophyll *b* to chlorophyll *a* transformation, deduced from quantitative determination of both chlorophylls, is based on the assumption of complete lack of chlorophyll synthesis in the dark in angiosperms. According to Adamson and coworkers (reviewed by Schulz and Senger, 1993) this assumption is wrong. Chlorophyll synthesis can occur in angiosperms not only in the light, but also in the dark, especially after preirradiation. A careful study with light-grown barley seedlings indicated degradation preferentially of chlorophyll *b* in the dark and synthesis preferentially of chlorophyll *a* in the dark (Walmesley and Adamson, 1989; 1995). The degree of synthesis and degradation varied with seedling age. To distinguish between endogenous pigments and pigments derived from exogenous precursors, Vezitskii and Shcherbakov (1988) investigated the metabolism of infiltrated zinc pheophorbide *b*, the zinc analogon of the magnesium containing chlorophyllide *b*. The authors found esterification and

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a spectral shift from the *b*-type to the *a*-type absorption. They interpreted this as a *b* to *a* conversion but overlooked that it could have been as well a reduction of the formyl group to a hydroxymethyl group leading to the same spectral shift.

In several papers, Ito *et al.* (1993, 1994) described the conversion of chlorophyll *b* into chlorophyll *a* with isolated cucumber etioplasts. The etioplasts were incubated with chlorophyllide *b*, phytol and ATP. The products of esterification were isolated and analyzed. The product with the spectral properties of chlorophyll *a* had the correct retention time in HPLC analysis, the mass spectrum exhibited the mass of pheophytin *a* (apparently magnesium was lost). Thus the chlorophyll *b* to *a* transformation can occur without doubt in isolated etioplasts. We asked therefore whether the reduction of the formyl group to the methyl group can be achieved in chlorophylls already under extremely mild conditions. We describe here a mild chemical reduction of zinc pheophorbide *b* to zinc pheophorbide *a* and *in planta* formation of zinc pheophytin *a* after infiltration of zinc pheophorbide *b* or zinc 7^l-hydroxy-pheophorbide *a* into etiolated oat seedlings.

Materials and Methods

Preparation of pigments

In general all reactions were carried out under inert gas (N₂ or Ar) and dim-green light. UV/Vis spectra were measured on a Lambda 2 (Perkin Elmer). ¹H-NMR spectra were recorded in d₅-pyridine on a 360 MHz instrument (Bruker). Liquid secondary ion mass spectroscopy (LSIMS) was performed on a MAT 900 (Finnigan-MAT, Bremen) in a m-nitrobenzyl alcohol matrix. For preparative column chromatography a reversed phase column was used as described by Helfrich *et al.* (1994).

Preparation of zinc pheophorbide *a*, zinc pheophorbide *b*, their respective phytol esters and 7^l-hydroxy-pheophytin *a* are described by Helfrich *et al.* (1994), Helfrich (1995) and Schoch *et al.* (1995). zinc 7^l-hydroxy-pheophorbide *a*, zinc 7^l-hydroxy-pheophytin *a* and zinc 7^l-methoxy-pheophorbide *a* were obtained from the corresponding *b*-type pigments by the following reduction procedure (shown for zinc pheophorbide *b*):

zinc pheophorbide *b* (2.2 µmol) was dissolved in 40 ml methanol containing ca. 60 mg fresh sodium cyanoborohydride. Methanolic HCl (1 M) was added in small portions under stirring until the UV/Vis spectrum changed from the *b*-type to the *a*-type (λ_{\max} : 640 nm to 654 nm in methanol). Pigments were extracted into ethyl acetate and either analysed by HPLC (see above) or isolated by preparative column chromatography. Zinc 7^l-hydroxy-pheophorbide *a*, zinc 7^l-methoxy-pheophorbide *a*, and zinc pheophorbide *a* were successively eluted from the RP column. The 13²(S)-epimers (pigments *a'*) were eluted immediately after their corresponding 13²(R)-epimers (pigments *a*).

Zinc 7^l-hydroxy-pheophorbide *a*

This pigment can alternatively be prepared in higher yields by reduction of pheophorbide *b* and subsequent zinc insertion (according to Helfrich *et al.*, 1994). UV/Vis: λ_{\max} [nm] (relative absorbance) in diethyl ether: 426 (1.00), 520 (0.03), 566 (0.05), 603 (0.10), 649 (0.60).

LSIMS: *m/z* (% ion intensity) = 670 (100), 671 (79), 672 (79), 673 (59), 674 (54), 675 (28), 676 (8).

Fragmentations (highest peak of cluster) at *m/z* = 654 (23%; M⁺-OH) and 593 (35%; M⁺-OH-CO₂CH₃).

Zinc 7^l-methoxy-pheophorbide *a* was the main product (together with the epimeric *a'* about 50% yield) of reduction of zinc pheophorbide *b*. UV/Vis: λ_{\max} [nm] (relative absorbance) in diethyl ether: 425 (1.00), 520 (0.06), 564 (0.06), 601 (0.11), 648 (0.58).

LSIMS: *m/z* (% ion intensity) = 684 (100), 685 (79), 686 (82), 687 (59), 688 (57), 689 (30), 690 (10).

No fragmentations detectable.

Zinc 7^l-hydroxy-pheophytin *a*

UV/Vis: analogous to the respective pheophorbide derivative.

LSIMS: *m/z* (% ion intensity) = 948.5 (100), 949.5 (83), 950.5 (88), 951.5 (63), 952.5 (58), 953.5 (33), 954.5 (13).

Fragmentations (highest peak of cluster) at *m/z* = 931.5 (11%; M⁺-OH), 670 (17%, M⁺-C₂₀H₃₉+H), 611 (11%; M⁺-C₂₀H₃₉-CO₂CH₃+H).

Plant material

Oat (*Avena sativa* L. cv Irlbach, Baywa, München) seedlings were grown on moist vermiculite for 7 d in the dark at 25–30°C in a closed chamber at high humidity. Primary leaves that protruded already 3–4 cm out of the coleoptiles were harvested under dim-green safe light. The tips (0.5 cm) were removed and the leaves carefully cut to a uniform length of 2.5 cm with a razor blade.

Infiltration and pigment extraction

The wells of a microtiter plate were filled with the mixture of 190 µl buffer (50 mM Hepes/KOH, pH 7.85) and 10 ml acetone containing 20 nmol of the respective zinc pheophorbide. The following steps were performed in the dark or under dim-green safelight. Ten leaf segments (ca. 130 mg fresh weight) were placed in each well. Infiltration for a short period of time (0.5–3 h) was supported by a ventilator, whereas infiltration for the long period (16 h) used only the natural transpiration of leaves. The leaf samples were then washed with buffer (50 mM Hepes/KOH), pH 7.85) on a Büchner funnel under suction, transferred into a 2 ml Eppendorf tube and frozen with liquid nitrogen. The frozen leaf material was then ground to a fine powder in the Eppendorf tube. The pigments were extracted by further grinding with quartz sand and 450 µl acetone (final concentration ca. 75%). The homogenate was centrifuged at 13,000×g for 2 min and the pellet was again extracted with 550 µl 80% acetone. The acetone extracts were combined. For calculation of total infiltrated pigment, the absorption spectrum was determined between 550 and 750 nm. The esterified pigments were transferred into n-hexane and separated from the non-esterified zinc pheophorbides with the anion exchange resin DE 52 as described by Helfrich *et al.* (1994). The absorption spectrum of the hexane phase was determined between 550 and 750 nm. For the quantification of pigments, the following absorption coefficients according to Jones *et al.* (1977) were used in n-hexane or diethyl ether: zinc phe(ide) *a*: $\lambda_{\max} = 654$ nm ($\epsilon_{\text{mM}} = 90.3$); zinc phe(ide) *b*: $\lambda_{\max} = 638$ nm ($\epsilon_{\text{mM}} = 60.2$); zinc 7¹-hydroxy-phe(ide) *a*: $\lambda_{\max} = 651$ nm ($\epsilon_{\text{mM}} = 90.3$). Slightly different values were used for the pigments in 80% acetone or ethyl acetate: zinc phe(ide) *a*: $\lambda_{\max} = 656$ nm ($\epsilon_{\text{mM}} = 77.3$);

zinc phe(ide) *b*: $\lambda_{\max} = 640$ nm ($\epsilon_{\text{mM}} = 51.3$); zinc 7¹-hydroxy-phe(ide) *a*: $\lambda_{\max} = 653$ nm ($\epsilon_{\text{mM}} = 77.3$). For analysis of mixtures of zinc pheophorbides *a* and *b* the following equations were applied:

$$C_a = (A_{654} \cdot \epsilon_b^{638} - A_{638} \cdot \epsilon_b^{654}) / (\epsilon_a^{654} \cdot \epsilon_b^{638} - \epsilon_a^{638} \cdot \epsilon_b^{654})$$

$$C_b = (A_{638} \cdot \epsilon_a^{654} - A_{654} \cdot \epsilon_a^{638}) / (\epsilon_b^{638} \cdot \epsilon_a^{654} - \epsilon_b^{654} \cdot \epsilon_a^{638})$$

HPLC

For HPLC of the esterified pigments of each infiltration sample, the solvent (hexane containing about 30% acetone) was removed under a stream of dry nitrogen gas. The residue was dissolved in 30 µl acetone. Twenty µl were applied to the injection loop of the HPLC unit (model 480, Gynkoteck, Germering/München). Separation occurred on a column (250×4.6 mm) filled with RP18 material (Rosil C-18.5 µm) with a gradient of acetone/water, that had been acidified to pH 3.5 with acetic acid, starting with 70% acetone and increasing the acetone concentration stepwise to 100% within 20 min (see Fig. 3 for details). Zinc pheophorbide derivatives were separated isocratically at 60% acetone for 23 min, followed by a linear gradient to 100% acetone within 6 min. Detection was achieved with an absorption detector (SP-6V, Gynkoteck) and a fluorescence detector (RF-551, Shimadzu).

Results and Discussion

Chemical reduction of *b*-type pigments

Fischer and Gibian (1942) applied the Wolff-Kishner reduction to chlorophyll *b*. Not only the 7¹-formyl group but also the 13¹-oxo and the 3-vinyl group were reduced under these drastic conditions (see Fig. 1 for numbering of the tetrapyrrole ring). Reduction of the formyl group to a hydroxy group in chlorophyll *b* and its derivatives was first described by Holt (1959) with sodium borohydride as reducing agent. Besides the 7¹-hydroxy derivatives, 13¹-desoxo-7¹,13¹-dihydroxy derivatives were detected as by-products. A more selective reducing agent for the formyl group was introduced into chlorophyll chemistry with sodium cyanoborohydride by Boxer and Bucks (1979). This agent can also be used for reductive amination of carbonyl groups (Borch *et al.*, 1971). When we used this method to reduce zinc pheophorbide

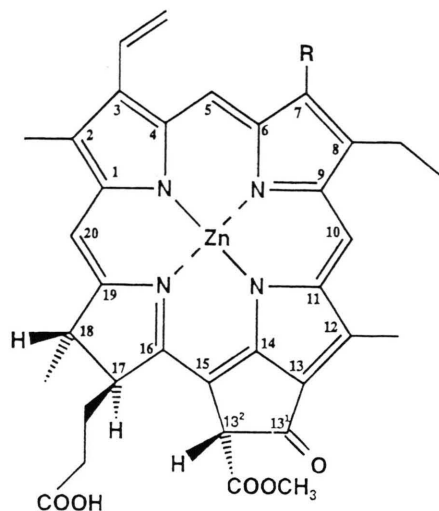


Fig. 1. Structure of the zinc pheophorbides used for infiltration. R = CH₃: zinc pheophorbide *a*; R = CHO: zinc pheophorbide *b*; R = CH₂OH: zinc 7¹-hydroxy-pheophorbide *a*.

b in the presence of a substituted aniline, we isolated a by-product in about 17% yield with the typical UV/Vis-spectrum of zinc pheophorbide *a* ($\lambda_{\text{max}} = 423, 654 \text{ nm}$ in diethyl ether). In comparison, zinc 7¹-hydroxy derivatives show slightly blue-shifted Q_y bands ($\approx 649 \text{ nm}$). Mass analysis (LSIMS) of this fraction showed a molecular peak cluster at $m/z = 654$, which is identical to that of authentic zinc pheophorbide *a*. To confirm the unexpected reduction of the formyl group to a methyl group, we recorded a ¹H-NMR spectrum of the pigment fraction in d₅-pyridine (Table I). All δ -values were identical with those of zinc pheophorbide *a*, which was obtained from chlorophyll *a* by demetallation, ester hydrolysis and subsequent zinc insertion (see Materials and Methods). The resonance of the 7-methyl group (singlet) is located at 3.25 ppm. The three other singlets in this range at 3.25, 3.71 and 3.83 ppm can be assigned to the 2-, 12- and 13²-methyl protons. Other possible reduction products with an *a*-type electronic spectrum, as 7¹-hydroxy or 7¹-amino derivatives should have resonances for the 7-CH₂ group shifted to lower field (e.g. 7¹-hydroxy-pheophytin *a* has a doublet at 6.09 ppm for the 7-CH₂ group). The reduction of a formyl group to a methyl group is usually only achieved by strong reducing agents. Surprisingly, the mild re-

Table I. Comparison of the ¹H NMR spectra of X (a reduction product of Zn-pheophorbide *b*) and authentic Zn-pheophorbide *a*. Chemical shifts δ are given in ppm (against tetramethylsilane as internal standard).

Proton	Multiplicity	X	Zn-pheophorbide <i>a</i>
10-H	s	9.93	9.93
5-H	s	9.73	9.73
20-H	s	8.67	8.67
3 ¹ -H _x	(d)dd	8.28	8.27
13 ² -H	s	6.79	6.79
3 ² -H _B	(d)d	6.31	6.31
3 ² -H _A	(d)d	6.08	6.08
18-H	m	4.56	4.56
17-H	m	4.48	4.48
13 ² -CO ₂ CH ₃	s	3.83	3.82
8-CH ₂	q	3.76	3.76
12-CH ₃	s	3.71	3.71
2-CH ₃	s	3.35	3.36
7-CH ₃	s	3.25	3.25
17-CH ₂ -CH ₂	4×m	3.53–2.96	3.53–2.95
18-CH ₃	d	1.70	1.70
8 ¹ -CH ₃	t	1.66	1.66

ducing agent NaBH₃CN can reduce the 7-formyl group to a methyl group without affecting the 13¹-oxo or other groups of the tetrapyrrole.

To study the mechanism of this reduction, we analysed the reduction products of several *b*-type pigments by HPLC: The typical product pattern obtained by reduction of zinc pheophorbide *b* under conditions described in Materials and Methods is shown in Fig. 2 A. Since the 13²(*R*) educt *b* contained the 13²(*S*) epimer *b'* in considerable amounts, all products consist of a mixture of epimers in the same ratio [13²(*R*)/13²(*S*) = 3:1] The reaction is very rapid; no educt was detectable after the reaction. The main product was the 7¹-methoxy compound (peaks 3 and 4, yield about 55%) followed by the 7¹-hydroxy compound (peaks 1 and 2, yield about 12%). These two products were also obtained by reduction of the metal-free pheophorbide *b* under the same conditions and subsequent zinc insertion (results not shown). The third product (peaks 5 and 6, yield about 30%) was only observed when we started the reduction reaction with the zinc complex. This product was identified as zinc pheophorbide *a* (see above). We tested then the possibility that the 7¹-methoxy and 7¹-hydroxy compounds are intermediates in the chemical reduction of the formyl group to a methyl group. The mixture obtained under stan-

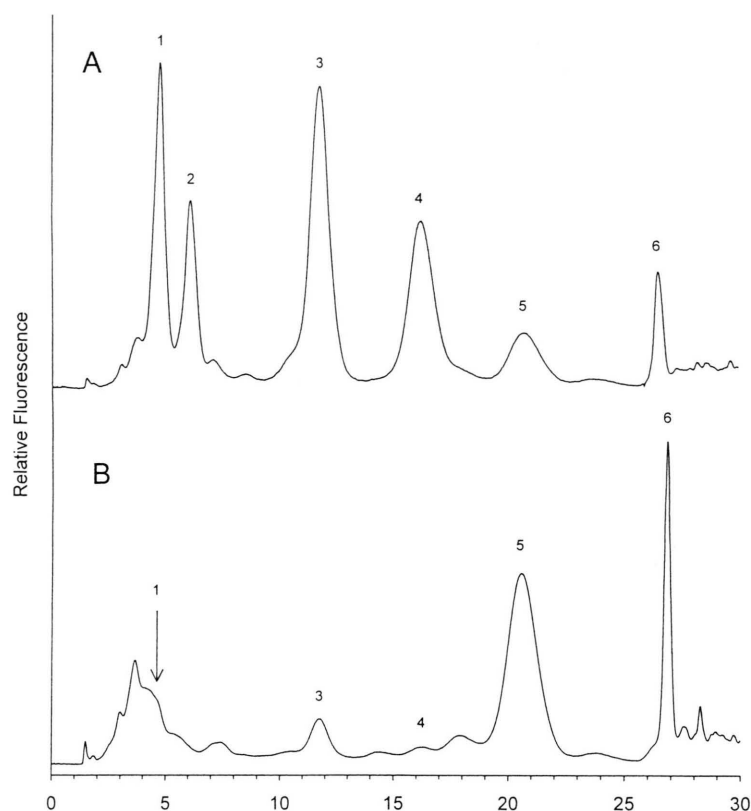


Fig. 2. HPLC analysis of reduction products of the mixture zinc pheophorbide *b/b'* obtained with sodium cyanoborohydride. Separation was achieved isocratically with 60% acetone for 23 min, followed by a linear gradient to 100% acetone within 6 min. Fluorescence emission at 665 nm was used for detection. The products were identified by their absorption and fluorescence maxima and co-chromatography with authentic compounds, 1 = zinc 7¹-hydroxy-pheophorbide *a*; 2 = zinc 7¹-hydroxy-pheophorbide *a'*; 3 = zinc 7¹-methoxy-pheophorbide *a*; 4 = zinc 7¹-methoxy-pheophorbide *a'*; 5 = zinc pheophorbide *a*; 6 = zinc pheophorbide *a'*.

dard conditions of reduction (Fig. 2A) was therefore treated again with sodium cyanoborohydride. The product pattern (Fig. 2B) clearly showed a decrease in the 7¹-methoxy and the 7¹-hydroxy compounds to less than 10% yield (peaks 1–4) and an increase in zinc pheophorbide *a* to about 60% yield (peaks 5 and 6). After a third treatment with sodium cyanoborohydride, peaks 1–4 disappeared completely and the yield of peaks 5 and 6 was nearly 90% (results not shown). *This proves the role of zinc 7¹-hydroxy-pheophorbide a and the 7¹-methoxy analog as intermediates in the chemical b to a transformation.* Several minor peaks in the region of zinc 7¹-hydroxy-pheophorbide *a* (Fig. 2B) could be products of reduction at C-13¹; they

were not further investigated. Purified zinc 7¹-hydroxy-pheophorbide *a* yielded also zinc pheophorbide *a* under identical conditions; no 7¹-methoxy product was obtained although the reaction was carried out in methanol (results not shown).

Infiltration and pigment analysis

After success with the chemical reduction of the formyl group of zinc pheophorbide *b* to a methyl group, we asked whether the same reaction could occur also in plants. The method of choice for such a test was infiltration of pigments into leaf segments under conditions that we had used pre-

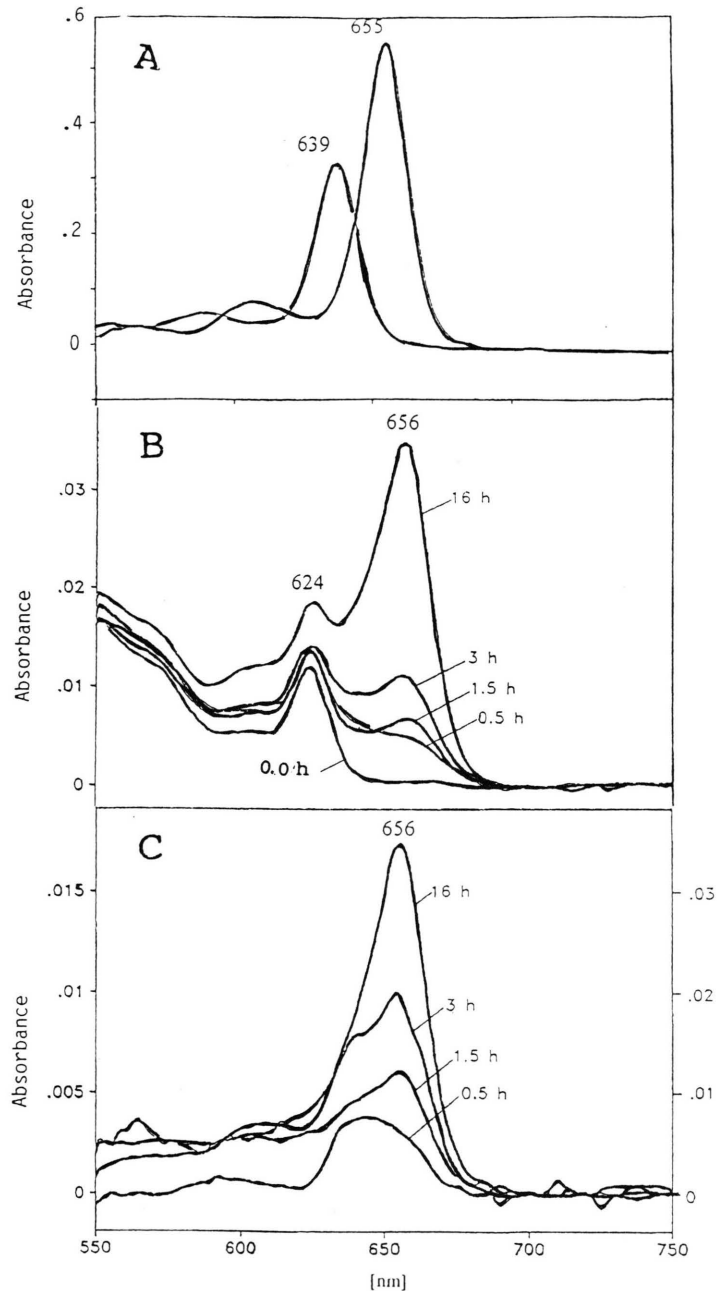


Fig. 3. Absorption spectra of educts before and products after infiltration. A: zinc pheophorbide *a* ($\lambda_{\text{max}} = 635 \text{ nm}$) and zinc pheophorbide *b* ($\lambda_{\text{max}} = 639 \text{ nm}$) in ethyl acetate. B: esterified pigments in hexane/acetone (7:3) obtained after infiltration of zinc pheophorbide *b* for 0.5–16.0 h into etiolated primary leaves of *Avena sativa*. The absorption maximum of endogenous protochlorophyll at 624 nm is present already before infiltration. C: The same spectra as in B after subtraction of the control spectrum (0.0 h) resulting in elimination of the absorption of endogenous protochlorophyll. The scale at the right is for 16 h infiltration.

viously for studying the esterification of chlorophyllides and zinc pheophorbides (Schoch *et al.*, 1990). To avoid any confusion with endogenous pigments, we used zinc pheophorbides throughout in the present study. The pigment solution was taken up by the leaf segments via the natural transpiration stream, in some experiments supported by artificial ventilation. We avoided vacuum infiltration because we had already shown that essential biochemical activities (e.g. transcription rate) dropped dramatically after vacuum infiltration, probably a consequence of anaerobiosis within the tissue (Schoch *et al.*, 1990). The zinc pheophorbides could not readily be dissolved in the buffer. They were dissolved in acetone. Subsequent dilution with buffer resulted in a clear solution, i.e. it did not lead to any precipitation of the pigments. The final acetone concentration (5%) did not impair biochemical reactions (e.g. reactions that are essential for greening of etiolated plants). The number and size of leaf segments and the amount of buffer had been optimized with the dye Fuchsin (not shown). The leaf segments were carefully washed with buffer to remove all zinc pheophorbides from the outside of the leaves. The remaining pigment was considered as „infiltrated pigment“ and was taken as basis for the calculation of the percentage of pigment that was metabolized.

Metabolism of the infiltrated zinc pheophorbide *b* is shown in Fig. 3. The amount of esterified pigment, i.e. pigment in the hexane phase, increased steadily with time of infiltration (determined up to 16 h, Fig. 3B). The controls were treated in the same way as the other samples except that infiltration was performed with buffer containing only 5% acetone but no pigment. The absorption peak at 624 nm present in the controls and in all other samples belongs to esterified protochlorophyll that is present in etiolated leaves in small amounts (Schoch *et al.*, 1977). The lack of any peak in the red region of the absorption spectrum of the controls shows also the quality of the green safe light. Undesired light would phototransform endogenous protochlorophyllide to chlorophyllide which would immediately be esterified to chlorophyll. Since no chlorophyll can be detected, the esterified pigment must be formed from infiltrated non-esterified zinc pheophorbide *b*.

The absorption spectrum of the esterified product is different from that of the infiltrated zinc

pheophorbide *b*, it resembles the absorption spectrum of zinc pheophorbide *a* (Fig. 3A and B). This is even more evident after subtraction of the contribution of endogenous protochlorophyll (Fig. 3C). The difference spectrum shows clearly the shift of the maximum from 639 nm to 656 nm with increasing time of infiltration. This signifies the disappearance of the formyl group at C-7.

Besides the formation of a methyl group (transformation of *b*-type to *a*-type pigment) this could alternatively mean reduction of the formyl group to a hydroxymethyl group (transformation of *b*-type to 7¹-hydroxy *a*-type pigment). The Qy band of zinc 7¹-hydroxy-pheophytin *a* is blue-shifted compared with the Qy band of zinc pheophytin *a* only by 3 nm in hexane/acetone. One cannot distinguish between these two pigments by determination of only the absorption spectrum of the hexane phase. We therefore investigated the pigments in the hexane phase also by HPLC (Fig. 4). Infiltration of zinc pheophorbide *b* for 3 h resulted in zinc pheophytin *b* and zinc pheophytin *a*; no 7¹-hydroxy compound could be detected (Fig. 4A). Longer infiltration time (16–19 h) led to nearly complete disappearance of the *b*-compound. By-products of zinc pheophytin *a* (peak 3) are the 13²-hydroxy derivative (peak 2) formed by allomerization of zinc pheophytin *a* and the epimer zinc pheophytin *a'* (peak 4); both are secondary products of the primary product of esterification (peak 3). Infiltrated zinc 7¹-hydroxy-pheophorbide *a* is completely transformed into the *a* compound within 16–19 h so that the pattern of esterified pigments (Fig. 4C) is identical with the result after infiltration of zinc pheophorbide *b* (Fig. 4B). *This result shows that the 7¹-hydroxy compound is an intermediate not only in the chemical (see above), but also in the biological b to a transformation.* We cannot decide at present whether the reduction steps occur before or after esterification. The enzyme chlorophyll synthase accepts zinc pheophorbide *b*, zinc pheophorbide *a* and the 7¹-hydroxy derivative equally well for the esterification reaction (Helfrich, 1995).

Kinetics of esterification after infiltration of zinc pheophorbide *a*, zinc pheophorbide *b* and zinc 7¹-hydroxy-pheophorbide *a* are shown in Fig. 5. Quantitative absorption spectra were determined for „total infiltrated pigment“ and for the fraction of esterified pigments. In the case of the *a*-type

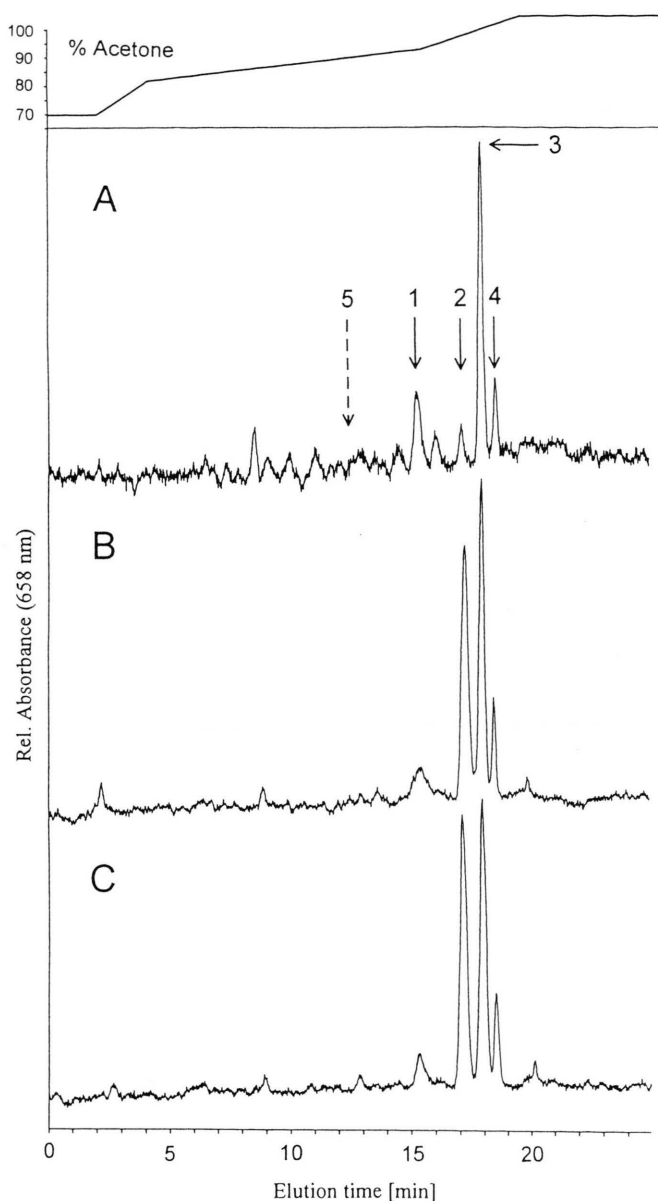


Fig. 4. HPLC of esterified pigments after infiltration of zinc pheophorbide *b* for 3 h (A), for 16–19 h (B) and after infiltration of zinc 7¹-hydroxy-pheophorbide *a* for 16–19 h (C). The pigments were identified via retention time and by co-chromatography with authentic pigments as follows: 1 = zinc pheophytin *b*, 2 = zinc 13²-hydroxy-pheophytin *a*, 3 = zinc pheophytin *a*, 4 = zinc pheophytin *a*'. The peak between peaks 1 and 2 belongs probably to zinc pheophytin *b*'. The position of zinc 7¹-hydroxy-pheophytin *a* is indicated with the dashed arrow 5; this pigment was not detected in the mixture of esterified pigments after infiltration. The acetone concentration of the step gradient is indicated on top.

pigments, the absorption coefficient for zinc pheophorbide *a* was used for calculation of the pigment amount. In the case of zinc pheophorbide *b*, the equation for determination of *a*- and *b*-type

pigments in *a,b*-mixtures was applied (see Materials and Methods). The values are the sum of all esterified pigments as fraction of „total infiltrated pigment“. Only a small fraction (<0.02) of zinc

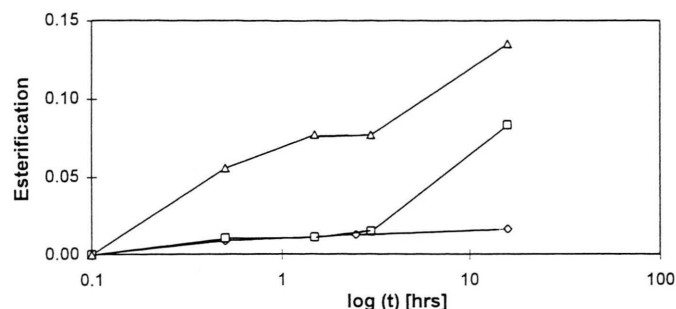


Fig. 5. Kinetics of esterification after infiltration of (◇) zinc pheophorbide *a*, (□) zinc pheophorbide *b*, (△) zinc 7¹-hydroxy-pheophorbide *a*. All values are based on total infiltrated pigment = 1. Quantification was achieved with the absorption spectra (see Fig. 2) and the equation for *a*-type and *b*-type pigments.

pheophorbide *a* was esterified within 0.5 h of infiltration, this amount did not increase significantly with time. In the case of zinc pheophorbide *b*, about the same fraction (<0.02) of infiltrated pigment was esterified within a short time but the esterified pigment increased to about 0.06 within 16 h infiltration time. This increase was mainly caused by the increase in zinc pheophytin *a* that was formed from the *b*-compound with time. Surprisingly, zinc 7¹-hydroxy-pheophorbide *a* was esterified much faster and also to a higher degree (nearly 0.15 within 16 h) than the other pigments (Fig. 5). We assume that the hydroxy compound can penetrate membranes better so that it reaches the site of esterification much faster and in higher amounts than the other compounds. However, the permeability of membranes for the different zinc pheophorbides has still to be investigated in detail.

The biological significance of chlorophyll *b* to *a* transformation must remain speculative at the moment. According to observations made up to now, it could be a facultative biosynthetic step to increase the flexibility of plants for acclimation to changing environmental conditions. Alternatively, it could be considered as obligatory step in chlorophyll breakdown. Hörtensteiner *et al.* (1995) found that the dioxygenase of *Brassica napus* involved in chlorophyll breakdown accepts pheophorbide *a* but not *b* *in vitro*. One possibility to explain the disappearance of chlorophyll *b* in senescing plants is the obligatory transformation of chlorophyll *b* to *a* prior to breakdown reactions. The experimental conditions used in our study require another explanation since we did not observe chlorophyll

breakdown. Etiolated plants when placed into light form chlorophyll *a*-containing protein complexes (core complexes) prior to chlorophyll *b*-containing protein complexes. The demand for chlorophyll *a* as constituent of the core complexes could be the driving force for the *b* to *a* transformation under our experimental conditions.

The possibility to transform not only chlorophyll *a* into chlorophyll *b* but also chlorophyll *b* into chlorophyll *a* should enable also green plants to acclimate in a relative fast response to changing environmental conditions. It is well known that the chlorophyll *a/b* ratio is lower in shade than in sun leaves. A rapid transition from shade to sun happens quite often in nature (e.g. under trees or branches that break down). The increase in the chlorophyll *a/b* ratio that must follow such an event can be achieved by breakdown of chlorophyll *b*, new synthesis of chlorophyll *a* or, more rapidly, by transformation of chlorophyll *b* into chlorophyll *a*. It should be kept in mind, however, that the stabilisation of the new state depends on formation of chlorophyll protein complexes, i.e. on the parallel formation the corresponding apoproteins. It has been demonstrated, however, that apoproteins *per se* are not stable but have to be stabilized by incorporation of the correct set of pigments, especially chlorophylls. This has been shown for plastid-encoded apoproteins (Eichacker *et al.*, 1992, Kim *et al.*, 1994) as well as for nuclear-encoded apoproteins (review: Paulsen, 1995). The chlorophyll *b* to *a* transformation could be the most rapid reaction for stabilization of apoproteins under appropriate conditions.

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