The Unexpected Reduction of the Vinyl Group of Chlorophyll *b* by Sodium Borohydride in Methanolic Extracts of Maize Leaves and Its Inhibition by 8-Hydroxyquinoline

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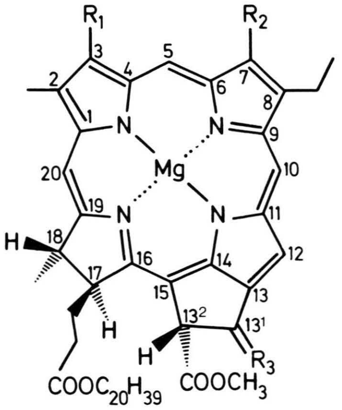
During rapid extraction of chlorophylls from maize leaves under reducing conditions with methanol containing $NaBH_4$, chlorophyll *a* remained unchanged but chlorophyll *b* yielded [7-hydroxymethyl]-chlorophyll *b*. The 3-vinyl group of chlorophyll *b* was also reduced forming significant amounts, up to 60%, of [3-ethyl]-[7-hydroxymethyl]-chlorophyll *b*. This was unexpected since this reduction of the 3-vinyl group does not occur when isolated chlorophyll *b* is treated in an identical manner with methanolic borohydride. The vinyl-group of chlorophyll *a* is not reduced during the same extraction conditions suggesting that the presence of a formyl or hydroxyethyl group at C-7 is necessary. The presence of 8-hydroxyquinoline and $NaBH_4$ in equimolar (16.5 mM) concentrations strongly inhibits the reduction of the 3-vinyl group of chlorophyll *b* in leaf extracts.

Introduction

The reduction of pure Chl *a* (Fig. 1; structure **I**) and Chl *b* (**II**), and of bacteriochlorophylls by $NaBH_4$ has been extensively studied [1–6]. In the plant chlorophylls, the 7-formyl group of Chl *b* is rapidly reduced within seconds to form [7-hydroxymethyl]-Chl *b* (**III**): the reduction of the 13^1-oxo group of both Chls *a* and *b* is much slower taking several hours at room temperature for completion [6]. No concomitant reduction of conjugated double bonds, a well known side-reaction of borohydrides [7], has been reported so far. We have now found that as much as 60% of the Chl *b* was reduced to [3-ethyl]-[7-hydroxymethyl)-Chl *b* (**IV**) during extraction of the newly-formed chlorophylls in greening maize seedlings with methanol containing 16.5 mM $NaBH_4$: this reductant was employed to convert the formyl group to a hydroxymethyl group in biosynthetic ^{18}O-incorporation studies which showed that the precursor of the formyl-group oxygen of Chl *b* is molecular oxygen [8].



		R_1	R_2	R_3
I	Chl *a*	$-CH=CH_2$	$-CH_3$	$=O$
II	Chl *b*	$-CH=CH_2$	$-CHO$	$=O$
III	[7-hydroxymethyl]-Chl *b*	$-CH=CH_2$	$-CH_2OH$	$=O$
IV	[3-ethyl]-[7-hydroxymethyl]-Chl *b*	$-CH_2-CH_3$	$-CH_2OH$	$=O$
V	[7-hydroxymethyl]-[13^1-hydroxy]-Chl *b*	$-CH=CH_2$	$-CH_2OH$	$-H,-OH$

Fig. 1. The structures of chlorophyll derivatives referred to in this paper. The IUB-IUPAC approved numbering system for tetrapyrroles [14] has been used with bracket [ ] nomenclature for substitutions. [7-hydroxymethyl]-Chl *b* (**III**) is identical to [7-hydroxymethyl]-Chl *a* but will be referred to here as a Chl *b* derivative to indicate its origin.

Abbreviations: Chl, chlorophyll; DEAE-cellulose, diethylaminoethyl-cellulose; EDTA, ethylenediamine-tetra-acetic acid; NMR, nuclear magnetic resonance.

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Experimental

Chemicals

$NaBH_4$ and 8-hydroxyquinoline (technical grade) were obtained from Merck-Schuchardt, Darmstadt, Germany. NaB^2H_4 was obtained from Cambridge Isotope Laboratories, Cambridge, MA., U.S.A. Solvents and other chemicals were analytical reagent grade or purified by standard techniques. DEAE-cellulose (DE 52), supplied by Whatman Laboratory Division, Maidstone, England, was prepared as a methanolic suspension [9] which was then equilibrated with $CHCl_3$. Pure Chls *a* and *b* were prepared from green maize leaves as previously described [10].

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 [11]. Etiolated leaves, excised from these seedlings, were then placed in H_2O and greened by illumination ($50-60\ \mu E \cdot m^{-2} \cdot s^{-1}$) with white light (Philips TLD 18 W/84 tubes) for up to 26 h at 27 °C [12].

Extraction of chlorophylls from greened maize leaves

Leaves were finely chopped with scissors into a mortar and extracted by grinding with a pestle to a translucent pulp in a freshly prepared solution (14 ml/g fresh wt. of leaves) of $NaBH_4$ (16.5 mM) in methanol. Where specified in the text, the extraction was carried out with methanol containing either 16.5 mM NaB^2H_4 or equimolar (16.5 mM) concentrations of $NaBH_4$ and 8-hydroxyquinoline. Grinding was completed in three minutes when an excess of glucose (150 mg) was added to remove unspent $NaBH_4$. The clear methanolic supernatant contained Chl *a* and reduced derivatives of Chl *b*.

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 then washed with a further large volume of saturated brine, dried over solid NaCl and evaporated to dryness at approximately 40 °C under vacuum in a rotary evaporator. The dry chlorophylls were redissolved in a minimum quantity of $CHCl_3$ and applied to a 4 × 65 mm high column of DEAE-cellulose equilibrated with $CHCl_3$ (see above). The carotenoids and chlorophylls were eluted with $CHCl_3$ or $CHCl_3$ containing either 2 or 10% methanol as described in the Results section. The elution of the chlorophylls was monitored by adsorption spectroscopy of each ml of chlorophyll-containing eluant between 680–630 nm and 480–400 nm.

Spectroscopy

All absorption spectroscopy was performed in quartz cuvettes (1 ml capacity and 1 cm light path) using a Shimadzu UV 1202 Spectrophotometer. Mass spectra were obtained by fast atom bombardment ionization (*m*-nitro-benzylic alcohol matrix) with a MAT900 Mass Spectrometer (Finnigan MAT, Bremen, Germany). ^1H NMR spectra were recorded in $^2H_5$-pyridine with a model AM 360 MHz instrument (Bruker, Karlsruhe, Germany).

Results and Discussion

The chlorophylls present in methanolic-$NaBH_4$ extracts of greened dark-grown maize leaves as determined by chromatography and absorption spectroscopy

When the pigments extracted in methanol containing 16.5 mM $NaBH_4$ were applied to a DEAE-cellulose column and developed with $CHCl_3$, a yellow carotenoid fraction ($\lambda_{max} = 481$, 454 and 426 nm) was followed by three blue chlorophyll bands. The first contained Chl *a* (**I**) ($\lambda_{max} =$ 666 nm). The second, split band contained two pigments with similar absorption spectra (Fig. 2). The first one ($\lambda_{max} = 659$ and 428 nm, spectrum 2) contained an unknown pigment, the second half ($\lambda_{max} = 659$ and 434 nm, spectrum 3), eluting only slowly with $CHCl_3$, and better with 2% methanol added, contained the expected C-7^1 reduction product of Chl *b*, *viz.* [7-hydroxymethyl]-Chl *b* (**III**) [*cf.* 6]. The slowest-moving third blue band (λ_{max} = 653 and 415 nm), which eluted with $CHCl_3$ containing 10% methanol, contained [7-hydroxymethyl]-[13^1-hydroxy]-Chl *b* (**V**), which is additionally reduced at the 13^1-oxo group [*cf.* 6].

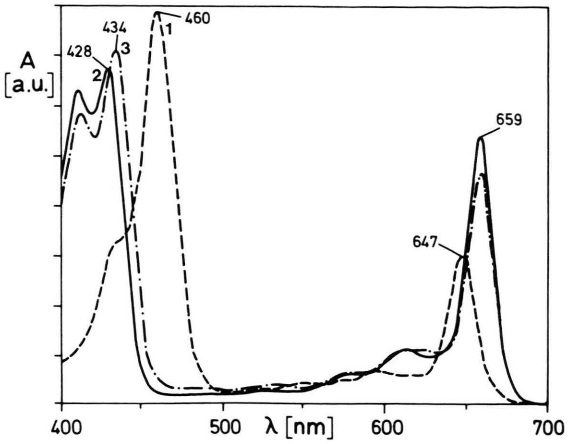

Fig. 2. UV-Vis absorption spectra of Chl *b* and its reduction products in chloroform. Chl *b* (**II**) (– – –, spectrum 1); [3-ethyl]-[7-hydroxymethyl]-Chl *b* (**IV**) (——, spectrum 2); and [7-hydroxymethyl]-Chl *b* (**III**) (–·–·–, spectrum 3). Peak positions of the main bands (nm) are indicated.

Consistent with previous studies [2–4, 6], we showed that pure Chl *b* (**II**), when treated rapidly with $NaBH_4$ in methanol, was all reduced to [7-hydroxymethyl]-Chl *b* (**III**). We speculated that the additional band in extracts of leaves ($\lambda_{max} = 659$ and 428 nm) was [3-ethyl]-[7-hydroxymethyl]-Chl *b* (**IV**) in which both the 3-vinyl and 7-formyl groups were reduced: both the more rapid elution of the 428 nm-absorbing material from the column and the shift of the Soret peak by 6 nm to shorter wavelengths relative to that of [7-hydroxymethyl]-Chl *b* (**III**) are consistent with the reduction of a vinyl group. Assuming equal absorption coefficients of **III** and **IV** at 659 nm, the latter comprised up to 60% of reduction products.

Product identification

The ^1H NMR spectrum of [7-hydroxymethyl]-Chl *b* (**III**) agreed with the structure. The 3-vinyl signals occur at 8.06 (H_x), 6.38 (H_A) and 6.06 ppm (H_B). The OH signal, which is somewhat variable and known to be very solvent dependent, occurs at approximately 7.4 ppm and was identified by $^1H/^2H$ exchange with ^2H_2O. The mass spectrum showed both M^+ (base at 906 *m/z*) and $(M+H)^+$ ions (base at 907 *m/z*) and the corresponding isotope peaks in an approximately 4:1 ratio. Upon reductive extraction with NaB^2H_4 in methanol, there was an increase in mass by 1 *m/z*, and a decrease in the intensity of the 7-CH_2 signal at 6.08 ppm by 50% in the ^1H NMR spectrum.

In the ^1H NMR spectrum of [3-ethyl]-[7-hydroxymethyl]-Chl *b* (**IV**), the ring current was slightly reduced, and the vinyl signals were no longer present. The mass spectrum showed again M^+ (base at 908 *m/z*) and $(M+H)^+$ ions (base at 909 *m/z*) in an approximately 4:1 ratio. Reductive extraction with NaB^2H_4 in methanol increased the mass by 2 *m/z* indicating the incorporation of one ^2H atom into the hydroxymethyl group and also the 3-ethyl group.

Investigation of the reduction of the vinyl group of Chl b in methanolic-borohydride extracts of maize leaves

Consideration of which components of the leaf extract initiated reduction of the vinyl group of Chl *b* led to investigation of the effect of 10, 20 and 40% water on the reaction of pure Chl *b* with methanolic $NaBH_4$. It was always completely reduced to the [7-hydroxymethyl]-Chl *b* (**III**) with Soret absorption at 434 nm: no [3-ethyl]-[7-hydroxymethyl]-Chl *b* (**IV**), the "3,8-diethyl" derivative with Soret absorption at 428 nm was detected.

To investigate the possibility that metal ions in the cell sap of the maize leaves are involved in the vinyl group reduction, the effect of three metal chelating agents on ethyl group formation during chlorophyll extraction with methanolic $NaBH_4$ were investigated: 8-hydroxyquinoline, 8-hydroxyquinoline-5-sulphonic acid (sodium salt) or EDTA (tetra-sodium salt) were added in equimolar concentrations with the $NaBH_4$. The two 8-hydroxyquinoline derivatives, but not the EDTA, strongly inhibited the formation of the 428 nm-absorbing "3,8-diethyl" derivative (**IV**). However, when the concentration of the $NaBH_4$ exceeded that of the chelating agent, the "3,8-diethyl" compound was again formed. Further, metal ions such as Fe^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+} and Ca^{2+} did not induce the reduction of the vinyl group of pure Chl *b*. We concluded, therefore, that the inhibition is not due to chelation of an activating metal ion; rather, the 8-hydroxyquinoline derivatives, in equimolar proportions with $NaBH_4$, were complexing the reductant and modifying its reducing properties. Indeed the chelator prevented also reduction of the

13^1-oxo group. In the presence of equimolar chelator, the $NaBH_4$ formed a third slow-moving chlorophyll band but it no longer had principal peaks of the doubly-reduced Chl *b* (**V**) (see above) but absorbed at 652.5, 612.5 and 431.5 nm with a shoulder at 411 nm and moved considerably faster during chromatography.

Concluding remarks

The presence of a formyl- or hydroxymethyl-group at C-7 appears to be needed for the reduction of the nearby 3-vinyl group as no evidence could be found for the reduction of the 3-vinyl group of Chl *a* during extraction. Since the 7-formyl and the 3-vinyl substituents of Chl *b* are close to each other and conjugated, a concerted mechanism is feasible. However, the identity of the agents in these extracts which catalyze the reduction are unknown.

Because the above ^1H NMR investigations have shown that ^2H from NaB^2H_4 was incorporated not only into the 7-hydroxymethyl group but also into the 3-ethyl group, it is clear that the majority (if not all) of the "3,8-diethyl" derivative arose by borohydride reduction during extraction and was not originally present in the leaves. This fact, coupled with the failure to find any "3,8-diethyl" derivative of Chl *a* in extracts, supports the view of Rebeiz *et al.* [13] that the natural heterogeneity of Chls *a* and *b* in nature, which includes the so-called "3-mono-vinyl-" and "3,8-di-vinyl-" derivatives, does not extend to "3,8-diethyl" forms.

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