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Identification of 8^1-hydroxychlorophyll *a* as a functional reaction center pigment in heliobacteria

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Chromatographic analysis of membranes and antenna-reaction center complexes of *Heliobacterium chlorum* and membranes of *Heliobacillus mobilis* revealed the presence of a small amount of a polar pigment with an absorption spectrum which was essentially identical to that of chlorophyll (Chl) *a*. Its structure was elucidated by means of ^{1}H-NMR Correlated Spectroscopy, ^{13}C-NMR and ^{252}Cf-Plasma Desorption Mass Spectrometry as 8^1-hydroxychlorophyll *a* (8^1-OH-Chl *a*), esterified with farnesol. It is concluded that 8^1-OH-Chl *a* is responsible for the band near 670 nm in the absorption spectrum of heliobacteria. The molar ratio bacteriochlorophyll (BChl) *g*: 8^1-OH-Chl *a* was approx. 17 for rapidly growing cells of *H. chlorum* and *Hb. mobilis*. On the assumption that the antenna consists of 35–40 BChls *g*, this means that two molecules of 8^1-OH-Chl *a* are present per reaction center. At least one of them functions as electron acceptor in the primary charge separation.

Introduction

Bacteriochlorophyll *g*, although the major pigment [1], is clearly not the only chlorophyllous pigment present in heliobacteria. In an earlier publication [2] we demonstrated that both *H. chlorum* and *Hb. mobilis* contain significant amounts of the 13^2-epimer of BChl *g*, BChl *g'*. Evidence for the presence of yet another pigment is given by the absorption spectra which show a band near 670 nm for both organisms [3,4]. This band is present in isolated membranes as well as in solubilized antenna-reaction center complexes [5,6], and a bleaching near 670 nm observed by flash spectroscopy in the sub-nanosecond region indicated that the absorption must be partly due to the primary electron acceptor [7,8]. A similar bleaching has been observed in membranes of green sulfur bacteria [9,10] where the corresponding pigment, BChl 663 has been isolated by means of HPLC and characterized as a lipophilic form of BChl *c* or a BChl *c*-like pigment [11,12].

The nature of the pigment or pigments absorbing at 670 nm in heliobacteria has not yet been determined. Whereas the amplitude of the 670 nm band in membranes of green sulfur bacteria is relatively constant [13], in heliobacteria the height of the band, relative to the Q_y-maximum of BChl *g*, is variable and depends on the culture conditions, conditions less favorable for growth tending to enhance its amplitude [4]. This suggested that part of the absorption might be due to photoisomerization or degradation products of BChl *g*, and that at least in some cultures several compounds might contribute to the absorbance at 670 nm.

This communication concerns the isolation and properties of the 670-nm pigments. We reported already [2] that HPLC-analysis of extracts of *H. chlorum* and *Hb. mobilis* revealed the presence of chlorophyllous pigments of high polarity. One of these is present at a ratio of 1 per 17 BChls *g* in rapidly growing cells of both species. Its chemical structure was determined by

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Abbreviations: A-RC, antenna-reaction center; BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; Chl, chlorophyll; HPLC, high-performance liquid chromatography; P-798, primary electron donor of heliobacteria; PS, photosystem.

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means of optical, mass and NMR spectroscopy and found to be 8^1-hydroxy chlorophyll *a*, with farnesol as esterifying alcohol. Other pigments were found to be present in variable amounts, but only in cells grown under less favorable conditions. It is concluded that 8^1-OH-Chl *a* functions as primary electron acceptor in the reaction center, whereas the other pigments are degradation products which are not essential for photosynthesis.

Materials and Methods

Pigment isolation

H. chlorum and *Hb. mobilis* were grown in medium No. 1552 of the American Type Culture Collection, containing 2.5 mM sodium ascorbate, as described in Ref. 5. The cells were harvested by centrifugation and washed once with 10 mM Tris-HCl buffer (pH 8.0) containing 10 mM sodium ascorbate.

Membrane fragments were prepared as described in Ref. 2. The A-RC complex of *H. chlorum* was prepared as described before [2,5] using sulfobetaine-12 as detergent. Subsequent purification was performed by means of sucrose gradient centrifugation and preparative scale size-exclusion HPLC.

Pigment extraction was performed as described earlier [2]. In short, the extraction was accomplished by sonication of a suspension in a approx. 100-fold volume of acetone during 2 min at 4°C in the dark. The extract was filtered and subsequently dried on a rotary evaporator. This procedure was completed within 5 min.

The brownish-green solid material obtained by the extraction procedure was dissolved in chloroform. Approx. 3 μl of the solution was injected into a silica HPLC column (Senshupak 1251-N, 250 × 4.6 mm i.d.) cooled to 4°C in an ice-water bath. The pigments were eluted isocratically with degassed *n*-hexane/ 2-propanol/methanol (100:1.85:0.2, v/v) at a flow rate of 0.8 ml min^{-1}. Reversed phase-HPLC was performed as described in Ref. 14 using a Microspher C-18 column (Chrompack, 200 × 4.6 mm i.d.), packed with 3 μm particles and operated at 0.9 ml min^{-1}. Acetone was used to dissolve the material before injection. Pigment elution was monitored by means of an Applied Biosystems Spectroflow 757 ultraviolet-detector.

Sufficiently pure pigments for the measurement of absorption, mass and ^{1}H-NMR spectra were prepared by means of preparative-scale HPLC (Senshupak 5251-N, 250 × 20 mm i.d.) with *n*-hexane/2-propanol/methanol (100:1.5–2.3:0.2, v/v) at a flow rate of 4.9 ml min^{-1}. The solvents used for analytical and preparative HPLC were analytical and reagent grade, respectively, and kept under a nitrogen atmosphere after degassing.

Spectroscopy

Absorption spectra were measured as described earlier [2]. ^{1}H-NMR spectral measurements were performed on a Bruker WM-300 spectrometer. ^{13}C-NMR spectra were recorded on a Bruker MSL-400. All NMR experiments were conducted in [$^2H_6$]acetone (Janssen Chimica, Belgium), usually at low temperature to reduce degradation reactions and to enhance spectral resolution. For room temperature measurements, the solutions were repeatedly frozen and degassed at 10^{-3} Torr.

^{252}Cf-Plasma Desorption Mass Spectrometry was performed by means of a Bio-Ion 20 spectrometer from Applied Biosystems, Uppsala, Sweden. The pigment was dissolved in acetone and transferred onto a nitrocellulose target where the solvent was evaporated.

Results

Chromatographic analysis

Fig. 1 shows a typical elution pattern obtained by normal phase-HPLC of an extract of cytoplasmic membranes of *H. chlorum*, harvested before the end of the exponential growth phase. In addition to the earlier identified pigments neurosporene, BPhe *g*, its epimer BPhe *g'*, BChl *g'* and BChl *g* [2] which all elute within 25 min, there is also a peak of a much more polar pigment visible, with an elution time of 130 min. The absorption spectrum of this component in eluent is shown in Fig. 2. It is very similar to that of Chl *a* in diethyl ether, acetone or benzene [15] and it was virtually identical to that of Chl *a* in eluent (unpublished data). Minor amounts of other polar pigments were observed, with elution times of 30–100 min; their total absorption was less than 1% of that of BChl *g*. The same result was obtained with a rapidly growing culture of *Hb. mobilis*.

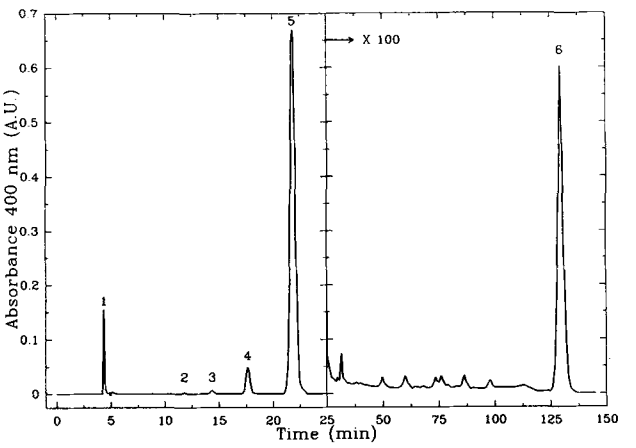

Fig. 1. HPLC tracing of an acetone extract of membranes of *H. chlorum*. Detection wavelength, 400 nm. Peak 6 is due to the Chl *a*-like pigment; peaks 1–5 to neurosporene, BPhe *g'*, BPhe *g*, BChl *g'* and BChl *g*, respectively (see Ref. 2).

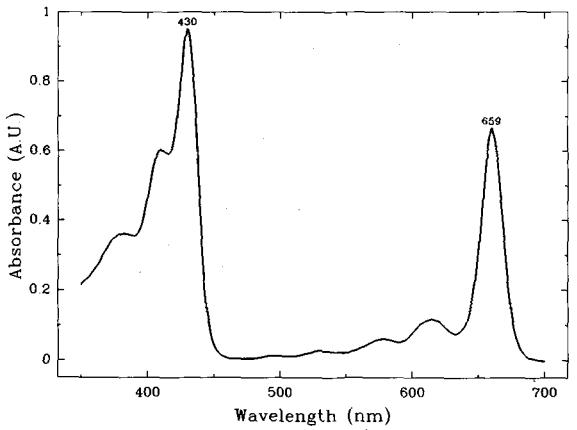

Fig. 2. Absorption spectrum in eluent of the Chl *a*-like pigment.

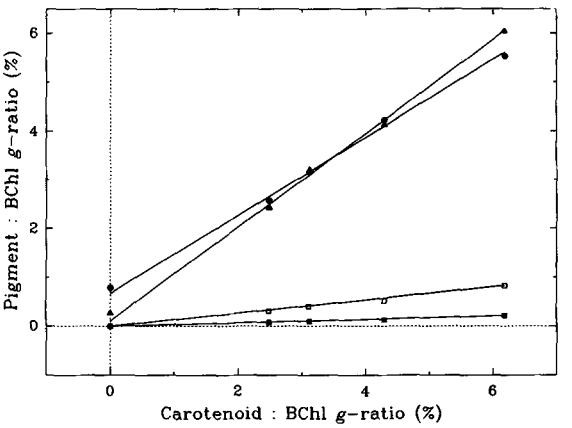

Fig. 3. Pigment content relative to BChl *g* measured by HPLC analysis after the admixing of increasing amounts of an acetone solution of 98.6% pure BChl *g* to a suspension of *H. chlorum* membranes before extraction. The pigment ratios on both scales are plotted as the ratios of the areas under the corresponding peaks in the chromatograms, measured at 400 nm. Triangles: BChl *g'*; open squares: BPhe *g*; solid squares: BPhe *g'*; circles: Chl *a*-like pigment. The abscissa gives the carotenoid: BChl *g* ratio of the solution after extraction; 6.2% corresponds to the ratio in membranes, 0% to the composition of the BChl *g* solution added.

By means of reversed-phase-HPLC it was checked that pigments of still higher polarity were not present in membranes of *H. chlorum*; the first pigment to be eluted was found to be identical to the Chl *a*-like pigment, isolated by preparative scale normal phase-HPLC. This shows that the Chl *a*-like pigment must be solely responsible for the band near 670 nm in the in vivo absorption spectrum of rapidly growing cultures of *H. chlorum* and *Hb. mobilis*.

Nevertheless, it was necessary to eliminate the possibility that some of the Chl *a*-like pigment was a conversion product of BChl *g* formed during the extraction procedure or column chromatography. We used the method described before [2] by adding various amounts of an acetone solution of 98.6% pure BChl *g*, containing BChl *g'* and the Chl *a*-like pigment as main impurities, to a suspension of membranes of *H. chlorum* and submitting this mixture to the normal extraction procedure and HPLC analysis. Using the neurosporene peak as an internal standard, straight lines were obtained for each pigment as shown in Fig. 3, with intercepts corresponding to the composition of the BChl *g* added. We therefore conclude that the elution pattern gives a proper representation of the in vivo pigment composition.

Structure determination

Several tens of milligrams of the Chl *a*-like pigment were obtained by preparative scale HPLC for the determination of its molecular structure by means of mass and NMR spectroscopy.

The high-mass part of the ^{252}Cf-Plasma Desorption Mass Spectrometry spectrum of the pigment is shown in Fig. 4. The two dominant peaks are approx. 203 mass units apart, indicating that the esterifying alcohol is farnesol, as already established for BChl *g* [16]. The total molecular weight of the pigment is 835 mass units, whereas Chl *a* with farnesol would be about 17 mass units lighter. Considering the higher polarity of the pigment as compared to Chl *a* (see Refs. 2 and 17), and taking into account the accuracy of the measurement, the most likely possibilities to explain the mass difference are therefore an extra NH_2, O or OH group. Since the ultraviolet/visible absorption spectrum is essentially identical to that of Chl *a*, this group cannot be located on the resonant part of the macrocycle.

^{1}H-NMR experiments were conducted as described before [2] in [$^2H_6$]acetone at low temperature. By manipulating the temperature in the range of 192 to 240 K, the H_2O line could be shifted until there was no overlap with lines from the compound investigated. The best result was obtained at 233 K (Fig. 5A). Detailed Correlated Spectroscopy (COSY) experiments yielded an almost complete interpretation of the spectrum (Table I), although the nature of the broad peak around 5.9 ppm and the cause for the broadening of

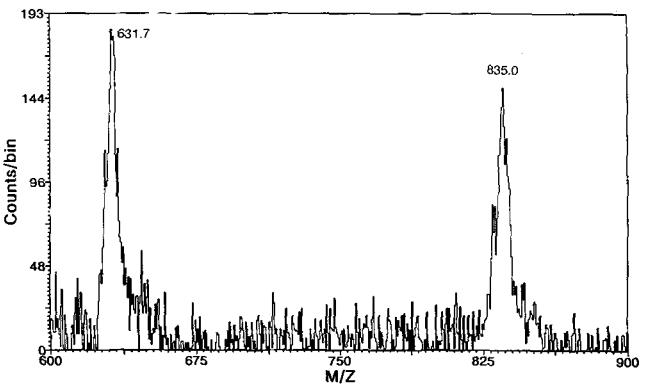

Fig. 4. High-mass part of a ^{252}Cf-PDMS spectrum of the Chl *a*-like pigment recorded on a nitrocellulose target.

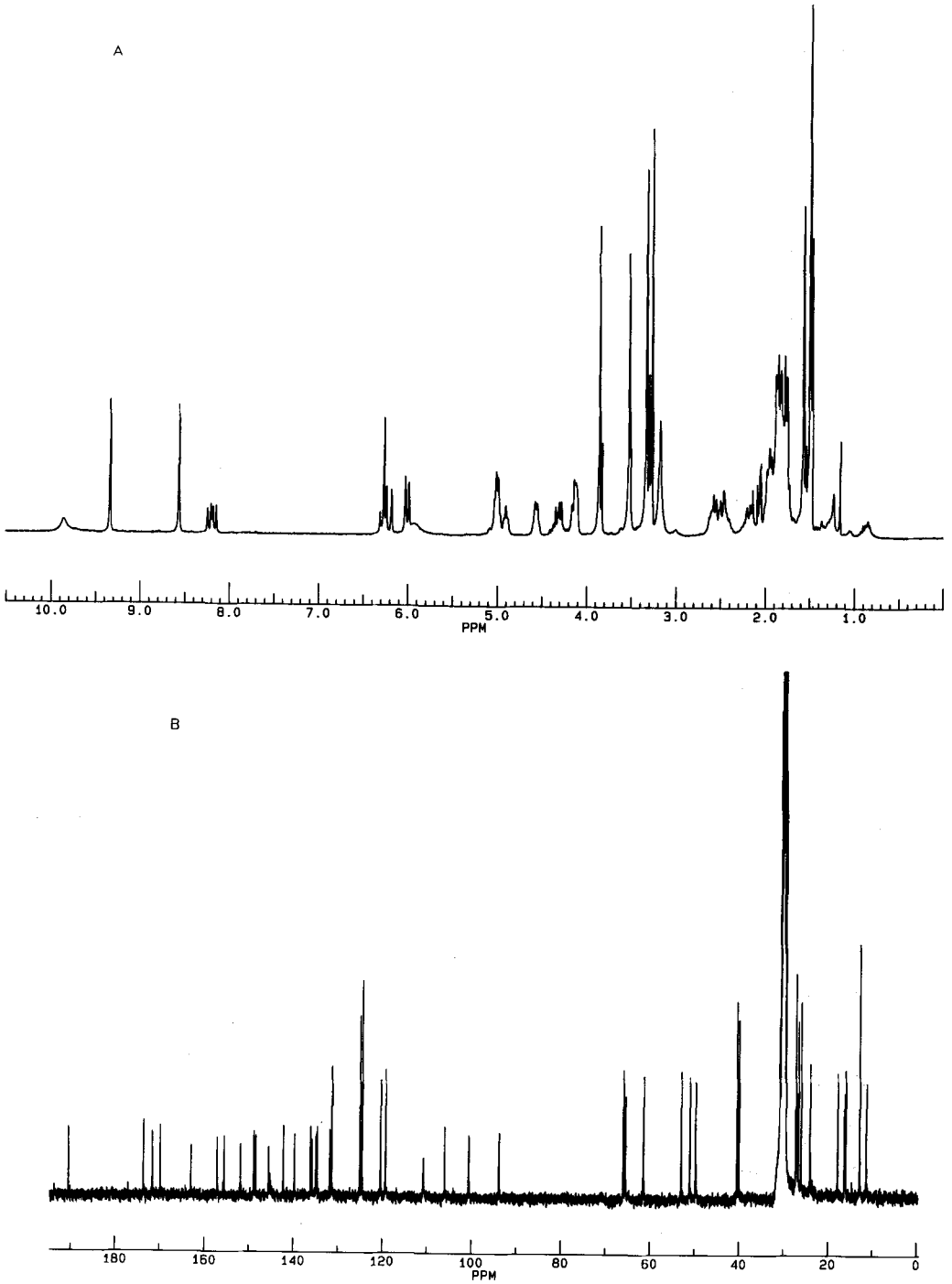

Fig. 5. ^{1}H-NMR spectrum (A), and ^{13}C-NMR spectrum (B) of 8^{1}-OH-Chl *a* in [$^{2}H_6$]acetone at 233 K. The lines at 2.05 ppm and at 3.25 and 3.28 ppm in ^{1}H-NMR are due to acetone and water, respectively. The assignment of the other lines is given in Table I. Lines at 32 ppm and 214 ppm (not shown) in ^{13}C-NMR are due to acetone.

the 10-H and 7-CH_3 lines remained obscure. For that reason, natural abundance ^{13}C-NMR was performed under otherwise identical conditions (Fig. 5B), followed by an Attached Proton Test (APT) which yields positive peaks for carbon atoms with zero or two protons and negative peaks for one or three protons (unpublished data). The interpretation of Lötönen and Hynninen [18] for Chl *a* was used to explain this spectrum and is not listed here for that reason. There is, however, one line at 64.88 ppm (just below the 13^{2} line at 65.37 ppm, see Fig. 5B), which did not occur in the Chl *a* spectrum. Since the APT-spectrum has a negative sign at 64.88 ppm, the most likely possibility is a carbon atom with one proton, one oxygen and two other groups. This strongly suggests the presence of a hydroxyethyl group, as in BChls *c*, *d* and *e*. This

TABLE I

Assignment of the ^{1}H-NMR lines of 8^1-OH-Chl a

The spectra were measured in [$^2H_6$]acetone at 233 K using acetone as internal standard ($\delta = 2.05$). The numbering of the protons is given in Fig. 6.

Protons	δ values in ppm and multiplicity [a]
10-H	9.84 (s)
5-H	9.32 (s)
20-H	8.54 (s)
3^1-H	8.17 (dd)
13^2-H	6.25 (s)
3^2-H_B	6.18 (dd)
3^2-H_A	6.00 (dd)
8^1-H	5.90 (o)
^{6,10}F-H	4.99 (m)
^{2}F-H	4.88 (t)
18-H	4.54 (m)
1F-H_A	4.30 (m)
^{1}F-H_B, 17-H	4.12 (o)
13^2-CO_2CH_3	3.84 (s)
12-CH_3	3.51 (s)
2-CH_3	3.32 (s)
7-CH_3	3.17 (s)
17^1, 17^2-H	2.4–2.6 (m)
8^1-CH_3	1.9–2.0 (o)
^{4,5,8,9}F-H	1.8–2.0 (o)
18-CH_3	1.85 (d)
^{3,7,11}F-CH_3	1.4–1.6 (s)

[a] s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet; o, overlapping with other signals.

implies, however, that we should have been able to monitor a coupling between a quartet at approx. 6 ppm and a doublet at approx. 2.15 ppm [19] in ^{1}H-NMR COSY. At room temperature COSY did show this coupling, while the 10-H and 7-CH_3 lines sharpened strongly (unpublished data). Obviously, at lower temperatures the OH-exchange interferes with the typical resonances of the hydroxyethyl group, obscuring the coupling and broadening the signals of neighboring protons. This gives us the possibility to locate the hydroxyethyl group on ring II at position 8, thus establishing the structure of the Chl *a*-like pigment as 8^1-hydroxy Chl *a* (Fig. 6).

Pigment contents

In order to determine the amounts of 8^1-OH-Chl *a* relative to that of BChl *g* from the area under the elution peaks, it was necessary first to determine the molar extinction coefficient of BChl *g* in eluent. This was done by dissolving a known amount of BChl *g* in a known volume and measuring the absorbance at the Q_y-maximum. A millimolar extinction coefficient $\epsilon = 89 \pm 5$ mM^{-1}cm^{-1} at 765.6 nm in eluent was obtained, corresponding to $\epsilon = 67$ mM^{-1}cm^{-1} at 400 nm, the detection wavelength. The extinction coefficients for the Q_y-maxima in diethyl ether, benzene and acetone were found to be 96 ± 5 (767.2 nm), 85 ± 4 (776.0 nm) and 76 ± 4 mM^{-1}cm^{-1} (761.6 nm).

Table II lists the BChl *g*: 8^1-OH-Chl *a* ratios for various preparations. It was assumed that the extinction coefficient of 8^1-OH-Chl *a* is the same as that of Chl *a* in eluent, determined to be 85 ± 3 mM^{-1}cm^{-1} in the Q_y-maximum, corresponding to 53 mM^{-1}cm^{-1} at 400 nm. The lowest amounts of 8^1-OH-Chl *a* were found in membranes obtained from rapidly growing cultures, which have a pigment ratio of about 17. Somewhat higher values were found for the A-RC complex of *H. chlorum*. Still higher values were obtained with *H. chlorum* harvested after the end of the exponential growth phase. It should be noted that membranes obtained from these 'old' cells also contained significant amounts of other polar pigments. These pigments eluted around 100 min in a broad band which contained two partially separated pigments. The absorption spectrum of the combined fractions showed maxima at 446 and 660 nm, and was clearly different from that of 8^1-OH-Chl *a*. Their total amount corresponded to a BChl *g*: pigment ratio of 35, assuming the same extinction coefficient at 400 nm as of Chl *a*. The nature of these pigments was not further investigated; we assume that they are degradation products of BChl *g* produced in old or decaying cells.

The relative amounts of 8^1-OH-Chl *a* and of the other polar pigments were reflected by the height of the band near 670 nm in the in vivo absorption spectra. Membranes from rapidly growing cells of *H. chlorum* and *Hb. mobilis* had an absorption spectrum similar to that shown in Fig. 1 of Ref. 5 with A_{670}/A_{788} ratios of 0.134–0.137. For the 'old' culture of Table II this ratio was significantly higher (0.22).

Table II also gives data for the other pigments present in heliobacteria. For BChl *g'* the numbers are in good agreement with those reported earlier [2]. The amounts of BPhes in rapidly growing cells were even

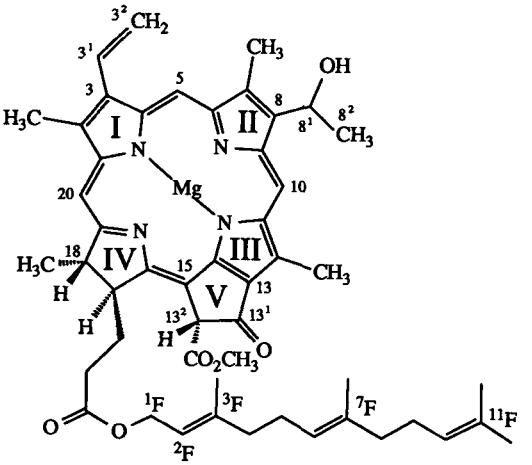

Fig. 6. Structure and partial carbon numbering of 8^1-hydroxy chlorophyll *a*, using the IUPAC system.

TABLE II

Pigment composition of H. chlorum and Hb. moliblis

The amount of pigment in each preparation is given relative to the amount of BChl *g*. A molar absorptivity of 150 mM^{-1} cm^{-1} at 436 nm was used for neurosporene [21] and of 85 mM^{-1} cm^{-1} at 659 nm for 8^1-OH-Chl *a*, identical to that of Chl *a*. Unless otherwise indicated, the numbers are the average of three separate measurements. The extinction coefficients of BPhe *g* and of PBhe *g'* were taken to be equal to that of BChl *g* at 400 nm, BChl *g'* was assumed to have the same extinction coefficient at the Q_y-maximum as BChl *g*.

Preparation	Molar ratio of BChl *g* to:	Neurosporene (Peak 1) [a]	BPhe *g'* (Peak 2) [a]	BPhe *g* (Peak 3) [a]	BChl *g'* (Peak 4) [a]	8^1-OH-Chl *a* (Peak 6) [a]
H. chlorum membranes [b,d]		14.9±0.6	462±48	122 ± 7	17.6±0.7	14.3±0.7
membranes [b]		12.5±0.5			17.4±0.4	17.6±0.4
membranes [c]		6.2±0.5	109± 7	30.1± 0.6	16.4±0.4	10.5±0.6
A-RC complex		8.5±0.8 [e]	825±44 [e]	123 ±19 [e]	14.1±0.5 [e]	13.6±0.9
Hb. mobilis membranes		20.9±0.9	621±18	143 ± 3	17.1±0.2	17.1±1.0

[a] Numbering as given in Fig. 1.
[b] Different batches of rapidly growing cultures.
[c] Harvested approx. one day after the end of the exponential growth phase.
[d] Six measurements.
[e] Data from Ref. 2, after correction for the different extinction coefficient used for BChl *g*.

lower than observed previously, especially in *Hb. mobilis*, again demonstrating that they cannot be functional photosynthetic pigments. The neurosporene content was variable.

The above data again stress the importance of well-defined, optimal growth conditions for a representative pigment analysis.

Discussion

In this communication we present a method for the pigment analysis of heliobacteria which yields full separation of all photosynthetic pigments. The amount of artifact created during extraction and subsequent chromatography was shown to be too small to interfere with a quantitative determination of all pigments present in heliobacterial preparations with respect to the dominant pigment, BChl *g*.

The goal of the present study was to identify the pigment that absorbs at 670 nm in vivo which is believed to be the intermediate acceptor as indicated by sub-nanosecond and nanosecond absorbance difference measurements [7,8,20]. The results presented here demonstrate that, in rapidly growing cells of *H. chlorum* and *Hb. mobilis*, 8^1-hydroxy Chl *a* is the only pigment which is responsible for the band at 670 nm in the in vivo absorption spectrum. No other pigments besides BChl *g*, BChl *g'* and neurosporene were detected in significant amounts. This also means that 8^1-OH-Chl *a* must be the primary electron acceptor in heliobacteria.

The pigment analyses reported here, as well as those reported earlier [2] also show the importance of optimal growth conditions. In more or less aging cultures not only higher amounts of 8^1-OH-Chl *a* were found, but also other polar pigments which contribute to the 670 nm absorption band in vivo. The level of BPhes *g*, present in insignificant amounts in healthy cultures, showed a considerable increase upon aging of the cells (Table II) and under less favorable growth conditions [2]. An increase of the level of BChl *g'* was also observed [2].

As far as we know, our observations provide the first demonstration of the presence of a Chl *a*-derivative in anoxygenic photosynthetic bacteria. Since 8^1-OH-Chl *a* can be obtained from BChl *g* by a simple isomerization and oxidation at ring II, it seems likely that the latter pigment is the precursor for synthesis of 8^1-OH-Chl *a* in vivo. It thus appears that the 'general' scheme for Chl and BChl synthesis proposed by Michalski et al. [16] may be valid for heliobacteria. This does not mean, of course, that it also applies to other organisms. The increased amount of the pigment in old cultures may be due to uncontrolled, non-specific conversion of BChl *g* by release of enzymes from lysed cells. It may be noted that a photoisomerization of BPhe *g* to pheophytin *a* in vitro has been reported [16], while Brockmann and Lipinski [1] reported 8^1-OH-Chl *a* to be one of the oxidation products of BChl *g*, formed in a pyridine extract.

As discussed earlier [2] there is, unfortunately, a considerable uncertainty as to the size of the photosynthetic unit in heliobacteria. For a BChl *g*: reaction center ratio of 35–40 [5], our data for those samples which may be assumed to contain the lowest amount of 'non-specific' 8^1-OH-Chl *a* indicate the presence of two 8^1-OH-Chls *a* per reaction center. It is not clear if both pigment molecules are associated with the reaction center, but at least one of them must be assumed to function as primary electron acceptor.

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