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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BACTERIOCHLOROPHYLL *b* AND ITS DERIVATIVES AS AN AID FOR STRUCTURE ANALYSIS

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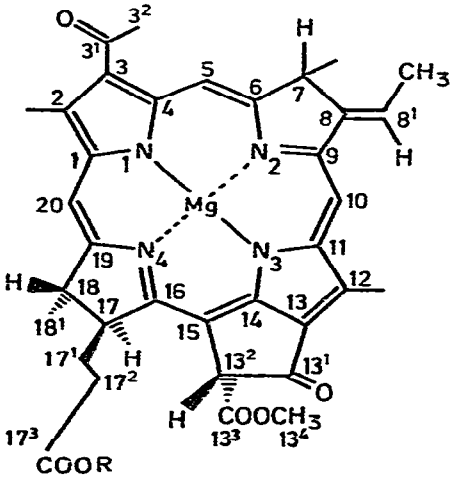
SUMMARY

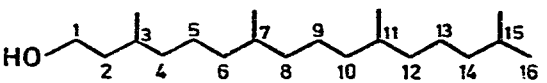
Bacteriochlorophyll *b* is chromatographed without degradation on reversed-phase silica with aqueous methanol containing sodium ascorbate. Like other chlorophylls, pigments differing only in their esterifying alcohols are separated on this column. For bacteriochlorophyll *b* and a variety its derivatives, a constant ratio of the retention times, $q_R = 1.31 \pm 0.1$, has been found for pairs of pigments esterified with either Δ^2-phytanol or $\Delta^{2,10}$-phytadienol. Similar retention-time ratios have been obtained for other sets of chlorophyll pigments differing only in their alcohols.

INTRODUCTION

Bacteriochlorophyll (bchl) *b* occurs in only a few photosynthetic bacteria[1–4], where it functions both in the light-harvesting antennae and in the photosynthetic



Bacteriochlorophyll *b*



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reaction centres. Owing to the presence of an exocyclic $\Delta^{8,8'}$-ethylidene group[5] it is particularly unstable, and its chemistry as yet insufficiently explored. Most of the structural data have hitherto been obtained with bchl *b* isolated from *Rhodopseudomonas viridis*[5–8]. The report of the occurence of bchl *b* (possibly together with bchl *a*) in several halophilic *Ectothiorhodospira* species[9,10] prompted us to investigate high-performance liquid chromatography (HPLC) as an analytical tool for bchl *b* and its derivatives. Bchl *b* is unstable in the commonly used HPLC systems[11,12], but can be stabilized on reversed-phase materials by a variation of the solvent system. At the same time, structural information can be gained concerning the nature of the esterfying alcohol.

EXPERIMENTAL

*Ectothiorhodospira halochloris** was light-grown at 37°C in the medium given by Imhoff and Trüper[9]. *Rhodopseudomonas viridis* and *Rhodopseudomonas spheroides* R 26 were grown in the medium of Gloe[13]. The cells were harvested after 7 days, and the bacteriochlorophylls isolated by standard procedures[14] under dim light. Acetone must be excluded from the extraction owing to the instability of bchl *b* in this solvent[5]. Pheophytins were obtained from the chlorophylls by treatment with 1% methanolic hydrochloric acid under nitrogen for 1 min[15]. For transesterification to the methyl pheophorbides, the pheophytins were refluxed for 15 min in 2% methanolic sulphuric acid under nitrogen. HPLC was performed with the apparatus described earlier[11] for the separation of pheophytins; it consisted of two Waters 6000 A pumps, a Waters injector, 30 cm × 4 mm RP-8 columns (Knauer, Oberursel, G.F.R.) and an LDC II variable-wavelength detector (Latek, Heidelberg, G.F.R.). The absorption detector has been equipped with a stepping motor and an intelligent recorder (Bryans, BS 8000) to allow *in situ* recording of absorption spectra[16]. Retention times are corrected for dead volume. Capillary gas–liquid chromatography was carried out with 20 m FFAP (SP 1000; WGA, Hamburg, G.F.R.) columns in a Model 2300 (Carlos Erba, Milan, Italy) chromatograph, under the previously reported conditions[21]. All solvents were distilled in glass and degassed prior to use. Sodium ascorbate (Merck, Darmstadt, G.F.R.) was reagent grade. Solvents containing sodium ascorbate were prepared freshly every day.

RESULTS AND DISCUSSION

HPLC of bchl a, and of bchl b from R. viridis

The solvent mixture containing methanol and water, which has been used to separate pheophytins of the *a*-type[11], can be used for chlorophylls as well[12]. With bchl *a*, optimum separation has been obtained with solvent systems containing 5–11% water (Fig. 1). Bchl *b* isolated from *R. viridis* gave a complex elution pattern with six major peaks. None of them had the typical absorptions of either bchl *b* or bac-

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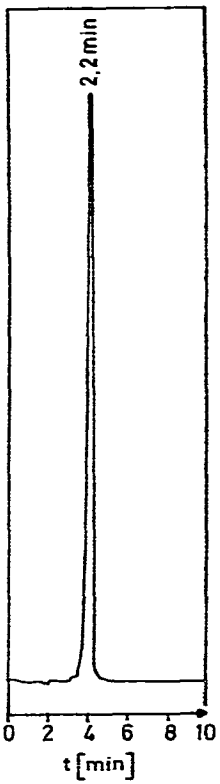

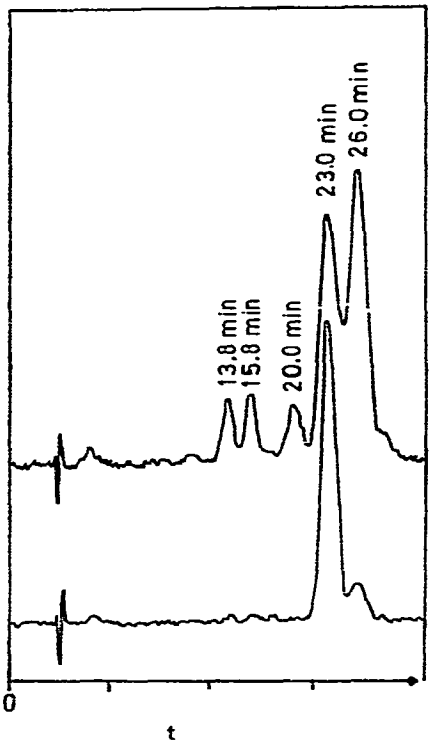

Fig. 1. HPLC of bchl *a* from *R. spheroides* R 26. Conditions: column, 300 × 4.6 mm I.D. RP-8 silica (Knauer); eluent, methanol–water (95:5); flow-rate. 1.5 ml/min; ambient temperature; absorption detector set at 600 nm.

Fig. 2. HPLC of bchl *b* from *R. viridis*. Conditions: columns as in Fig. 1; eluent, methanol–1% aqueous sodium ascorbate (89:11); flow-rate and temperature as in Fig. 1; absorption detector set at 600 nm (lower trace) and 680 nm (upper trace).

teriopheophytin (bphe) *b*, for which absorption maxima at 606* and 530 nm, respectively, have been determined *in situ* in the solvent system containing 11% water. Instead, the peaks all had maximum absorptions around 680 nm, which are characteristic for the isomerization/oxidation products[5,6] of bchl *b* or bphe *b* containing an endocyclic $\Delta^{7,8}$ instead of the exocyclic $\Delta^{8,8^1}$ double bond. These reactions of bchl *b* during the chromatographic separation can be suppressed, if water containing 1% sodium ascorbate is used instead of the pure water in the eluent (Fig. 2). The sodium

* The Q_x bands (corresponding to the low-energy transition oriented across the two reduced pyrrole rings) of bchl and bphe are the best suited for selective detection of the two pigments. The Q_y and B (corresponding to the low-energy transition oriented across the two unsaturated rings, and the higher-energy transitions) bands have much stronger absorptions but overlap in the two pigments; the latter band also overlaps with those of other chlorophylls. It should be noted that the Q_x band of bchl is strongly solvent-dependent and shifted in the eluent by almost 30 nm compared with its position in ether solution. (See also L. Coyne cited in ref. 17.)

ascorbate has a double effect: it acts as a mild buffer to prevent acid-catalysed reactions induced by any remaining free silica surface, and, more important, it acts as an antioxidant. Quantitative analysis of the eluted pigments indicated, however, that at least 50% of the pigments were degradation products. Because bchl *b* is known to degrade during extraction, the question arose as whether the by-products originated from the isolation procedure or from the chromatography. To test the stability of bchl *b* on the column, the peak containing bchl *b* (retention time, t_R = 23 min) was recycled five times. The integrated area under the peak decreased by 4.6% for each run. Under identical conditions, the peak area of pheophytin *a* and pyropheophytin *a*, two stable derivatives of chlorophyll *a*, decreased by 7.5 and 4.0% per run, respectively. This decrease is due to losses during recycling, and to integration errors with the broadening peaks. The data clearly show the stability of bchl *b* in the chromatographic system used, even at extended development times.

Only one of the additional peaks absorbing around 680 nm has been identified hitherto: a major by-product from bchl *b* is 3-acetyl-3-devinyl-chlorophyll *a*[6]. The same pigment can be prepared by oxidation of bchl *a* with quinones[18]. The pigment obtained by this method from bchl *a* from *R. spheroides* co-migrated with the slowest moving degradation product of bchl *b* from *R. viridis* (t_R = 26.0 min). It should be noted that both starting pigments contain phytol as the esterifying alcohol. Co-migration has also been observed for the pheophytins and the methylpheophorbides, prepared from the two pigments on reversed-phase HPLC as well as on silica HPLC (carbon tetrachloride–acetone, 95:5 and 90:10, respectively). This, and the identical UV–visible spectra of the respective products, suggests that the peak at t'_R = 26 min contains 3-acetyl-3-devinyl-chlorophyll *a*. A second by-product of bchl *b* identified earlier[5] is 3-acetyl-3-devinyl-8-(α-hydroxyethyl)-8-deethylchlorophyll *a*, to which the slowest migrating peak has tentatively been assigned (t'_R = 13.8 min).

Comparison of bchl b from R. viridis and E. halochloris

The bchl *b* from *E. halochloris* gave a similar HPLC pattern, but the retention times for all pigments were considerably reduced, *e.g.* from 23 to 17.6 min for bchl *b*, which corresponds to a ratio q = 1.31 for the t_R values of the two pigments. The identical factor of q = 1.31 ± 0.1 has been found for the retention times of the pheophytins and of most of the by-products (Table I). This constant ratio indicates a common substituent difference in a part of the molecule important for interaction with the reserved-phase adsorbent. The esterifying alcohol at the propionic acid side-chain is such a substituent. HPLC has indeed been used successfully to separate mixtures of pheophytins of the *a*-type esterified with different alcohols[11,28], with their retention times decreasing with an increasing number of double bonds of the esterifying alcohol (Table II).

A third example of pigments differing only in their alcohols is bchl *a* isolated from *Rhodospirillum rubrum* (containing $\Delta^{2,6,10,14}$-phytatetraenol, *i.e.* geranylgeraniol) and from most other purple non-sulphur bacteria (containing Δ^2-phytaenol, *i.e.* phytol)[19]. In every case, the integral pigments move on reversed phases in the way that would be expected for the free alcohols, *e.g.* from comparison with (unsaturated) C-18 acids[20]. The suggestion of bchl *b* from the two different organisms and their reaction products, to differ only in their esterifying alcohols, has been proved by transesterification to the methyl esters. The t'_R values of the product mixtures ob-

TABLE I

HPLC OF bchl *b* AND ITS DERIVATIVES FROM *R. VIRIDAS* AND *E. HALOCHLORIS*

Corrected retention times and their ratios for the respective pigments from both organisms; conditions as in Fig. 2, if not otherwise indicated.

	t'_R (min)		$q = \frac{t'_R \text{ (R. viridis)}}{t'_R \text{ (E. halochloris)}}$
	R viridis	*E. halochloris*	
Bchl *a**	23.0	17.6	1.31
Bphe *b***	5.2	4.0	1.30
3-Acetyl-3-devinyl-8-(α-hydroxyethyl)-8-de-ethylchlorophyll *a****	13.8	10.6	1.30
Unknown oxidation product	15.8	12.0	1.32
Unknown oxidation product §	20.0	15.2	1.31

* λ_{det} = 620 nm; 11% ascorbate in water, 89% methanol.
** λ_{det} = 530 nm; 5% ascorbate in water, 95% methanol.
*** Tentative structure.
§ λ_{det} = 675 nm; 11% ascorbate in water, 89% methanol.

tained from the pheophytins of *E. halochloris* and *R. viridis*, respectively, are identical.

Gas chromatography of unsaturated phytanols

The alcohol of bchl *b* from *R. viridis* is Δ^2-phytaenol, and that of bchl *b* from *E.*

TABLE II

HPLC OF CHLOROPHYLLS AND PHEOPHYTINS ESTERIFIED WITH DIFFERENT (POLY)-UNSATURATED PHYTHANOLS

Conditions as in Fig. 1, if not otherwise indicated.

Pigment	*Esterifying alcohol*	t_R (min)	$q = \frac{t_R (\Delta^2)}{t_R (\times)}$
Pheophytin *a**	Δ^2	8.0(13.6)**	1.0(1.0)
	$\Delta^{2,14}$	6.7(11.5)**	1.19(1.18)
	$\Delta^{2,10,14}$	5.6(9.4)**	1.43(1.45)
	$\Delta^{2,6,10,14}$	4.6(7.4)**	1.7(1.84)
Bphe *a*	Δ^{2}***	6.0	1.0
	$\Delta^{2,6,10,14}$ §	3.9	1.54
Bchl *a*	Δ^{2}***	3.0	1.0
	$\Delta^{2,6,10,14}$ §	1.5	2.0
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* From refs. 11 and 28.
** Methanol–acetone (90:10) on RP-18 (unbracketed), and methanol–water (95:5) on RP-8 (values in brackets).
*** From *R. spheroides* R 26.
§ From *Rhodospirillum rubrum*.

halochloris has recently been identified as $\Delta^{2,10}$-phytadienol[21]. A second phytadienol ($\Delta^{2,14}$) has been found in chlorophyll *a*, of greening higher plants[11,29], and a third one ($\Delta^{2,6}$) as esterifying alcohol of bchl *c* in *Chlorobium limicola*[22]. Apart from geometric isomers, these are the three possible isomers with one (apparently conservative) double bond fixed at C-2.

The gas chromatography (GC) of these three isomers and of phytapolyenols with different degrees of unsaturation (Table III) suggests that it will also be useful in structure elucidation. In the cases where series of chlorophylls are known to differ only in their esterifying alcohols, the GC elution order from a polar column is opposite to that of the integral pigments on a reversed-phase column. This points again to the prime importance of the esterifying alcohol in reversed-phase chromatography of chlorophylls.

TABLE III

GC OF (POLY)UNSATURATED PHYTHANOLS AND THEIR RETENTION TIME RATIO WITH RESPECT TO Δ^2-PHYTANOL (PHYTOL)

Conditions as in Fig. 3.

Alcohol	t_R *(min)*	$q = \frac{t_R(\Delta^2)}{t_R(\times)}$
Δ^2*	11.2	1
$\Delta^{2,6}$*	13.4	1.20
$\Delta^{2,10}$**	14.1	1.26
$\Delta^{2,14}$***	15.5	1.38
$\Delta^{2,10,14}$*	18.6	1.66
$\Delta^{2,6,10,14}$*	23.2	2.07

* From chl *a* from greening oats[11].
** From bchl *c* of *Chlorobium limicola*[22].
*** From bchl *b* of *E. halochloris*[21].

In particular, the still limited data clearly show two trends: the GC retention times increase with the number of double bonds, and for the three isomers with two double bonds it increases with their increasing separation on the hydrocarbon skeleton (Fig. 3).

HPLC was probably the most important tool in the recent identification of a variety of "new" chlorophylls[11–13,21–29]. Their structure analysis is in many cases still incomplete, owing to their occurence in only trace amounts. Under favorable conditions, HPLC can yield valuable structural information on these pigments.

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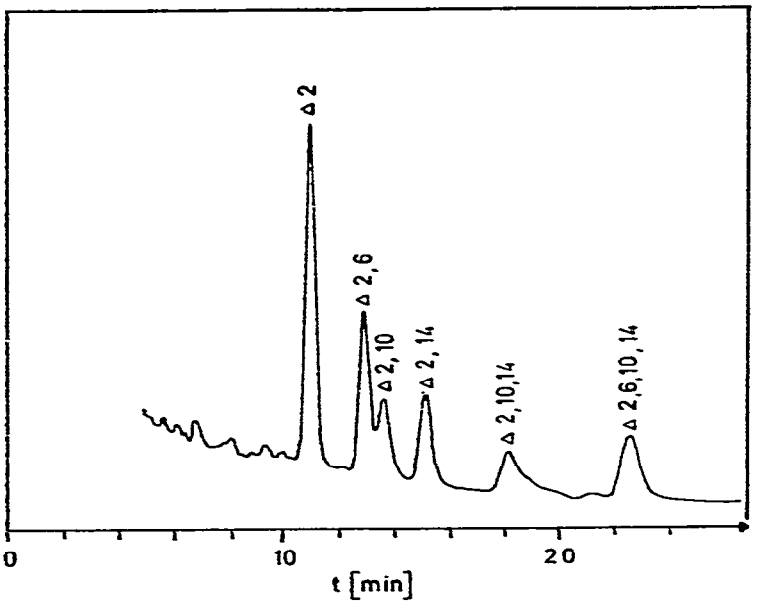

Fig. 3. GC of (poly)unsaturated phytanols. The numbers on the peaks indicate the points of unsaturation. Conditions: column, 20 m FFAP (SP 1000, WGA); carrier gas, helium; flow-rate, 4 ml/min; injection, split-less 130°C; column temperature, isothermal 186°C.

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