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CHROMATOGRAPHIC SEPARATION AND ANALYTICAL CHARACTERIZATION OF BACTERIOCHLOROPHYLLS *ap*, *agg* AND *b*

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SUMMARY

A preparative separation method involving precipitation of the polymeric 1,4-dioxane coordination complexes and adsorption chromatography on a dextran gel has been developed for bacteriochlorophylls *ap*, *agg* and *b*. The analytical characterization of the pure compounds was achieved with reversed-phase (RP) thin-layer chromatography utilizing triglyceride-impregnated cellulose layers and RP-C_{18} high-performance liquid chromatography with 5-μm material and acetonitrile–water mixtures as eluent. The purity tests with both methods are reliable and informative. The existence of seven diastereomers of bacteriochlorophylls *ap* and *agg* and more than one diastereomer for bacteriochlorophyll *b* could be demonstrated by combining partition and RP-C_{18} liquid chromatography. These results are backed by the absorption spectra of pure bacteriochlorophyll *agg* diastereomers.

INTRODUCTION

All green plants contain the dihydroporphyrins chlorophyll *a* and *b* (CHL *a* and *b*), which participate in the photosynthetic process. In contrast, in phototrophic microorganisms the primary photosynthetic processes are based on bacteriochloro-

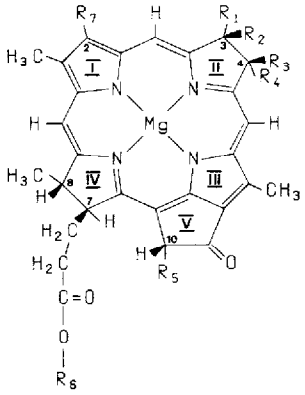

Fig. 1. Structure of bacteriochlorophylls. Substituents R_1–R_7 are identified in Table I.

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phylls (BCHLs), which are tetrahydroporphyrins[1] (Fig. 1). A consequence of the structural differences between these two classes of porphyrins is, *e.g.*, the high reduction capacity of all tetrahydroporphyrins. This is one of the main reasons why the preparation of pure bacteriochlorophylls is difficult and laborious. For studying basic properties such as the molecular interactions of the bacteriochlorophylls as a means of understanding the bacterial photosynthetic processes it is necessary to prepare pure BCHLs.

TABLE I

SUBSTITUENTS R_1–R_7 IN FIG. 1

Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7	*Additional double bond*
CHL *a*	CH_3	–	C_2H_5	–	$O=\underset{\vert}{C}-OCH_3$	Phytyl	$H_2C=\underset{\vert}{CH}$	Between C-3 and C-4
CHL *b*	$C(=O)H$	–	C_2H_5	–	$O=\underset{\vert}{C}-OCH_3$	Phytyl	$H_2C=\underset{\vert}{CH}$	Between C-3 and C-4
BCHL *ap*	CH_3	H	C_2H_5	H	$O=\underset{\vert}{C}-OCH_3$	Phytyl	$H_3C-\underset{\vert}{C}=O$	–
BCHL *agg*	CH_3	H	C_2H_5	H	$O=\underset{\vert}{C}-OCH_3$	Geranyl-geraniol	$H_3C-\underset{\vert}{C}=O$	–
BCHL *b*	CH_3	H	$=CH-CH_3$	–	$O=\underset{\vert}{C}-OCH_3$	Phytyl	$H_3C-\underset{\vert}{C}=O$	–
2-Desvinyl-2-acetyl-CHL *agg*	CH_3	–	C_2H_5	–	$O=\underset{\vert}{C}-OCH_3$	Geranyl-geraniol	$H_3C-\underset{\vert}{C}=O$	Between C-3 and C-4
2-Desvinyl-2-acetyl-proto-CHL *agg*	CH_3	–	C_2H_5	–	$O=\underset{\vert}{C}-OCH_3$	Geranyl-geraniol	$H_3C-\underset{\vert}{C}=O$	Between C-3 and C-4; and between C-7 and C-8; (H-7 and H-8)
2-Desvinyl-2-acetyl-4-(α-hydroxy)ethyl-CHL *ap*)	CH_3	–	$-\underset{\vert}{CH}-CH_3$ $\vert$ OH	–	$O=\underset{\vert}{C}-OCH_3$	Phytyl	$H_3C-\underset{\vert}{C}=O$	Between C-3 and C-4

Three methods for isolating these pigments from the corresponding microorganisms have been described: the classical method with sugar columns as the stationary phase[2], separation on silica gel columns[3] and separation on columns of polyethylene particles[4]. A characteristic of all of these methods is the necessity to change the mobile phase for the elution of all BCHLs and accompanying compounds, which is time consuming and impractical. The recently described separation on the dextran Sephasorb HP ultrafine[5,6] combined with a prior precipitation of the BCHL as a coordination polymer with dioxane or triazine eliminates these disadvantages and was used in this work to prepare the BCHLs.

It has been shown that the purity of CHL *a* and *b* preparations can be tested

best by high-performance liquid chromatographic (HPLC) and thin-layer chromatographic (TLC) methods[5,6]. With TLC, in the reversed-phase (RP) technique separation on oil-impregnated cellulose layers[7] is prefered to the use of silica gel layers[3], a stationary phase which irreversibly destroys BCHLs[8]. The separation of bacteriochlorophylls *agg* (BCHL *agg*) and *ap* (BCHL *ap*) into single diastereomers by RP-HPLC has recently been published[9].

Many scientists regard the absorption spectra of di- and tetrahydroporphyrins as a criterion of the purity of these compounds[10,11]. However, one can easily show that impurities and degradation products can constitute up to 10% of the sample without appreciably changing the absorption spectra of the chlorophylls. The absorption spectra of pure compounds and degradation products can be distinguished only if the compounds differ in the macrocycle[9]. Therefore, the absorption spectra of pure Pheophytin *a* (PHEO *a*) and CHL *a* are different, whereas the spectra of BCHL *agg* and BCHL *ap* (both compounds are often referred to as BCHL *a*) are identical. These two compounds differ only in the alcohol at the group C7 (Fig. 1).

EXPERIMENTAL

Thin-layer chromatography

Partition chromatography on triglyceride-impregnated cellulose plates using water–acetonitrile mixtures as eluent was performed as described earlier[7]. hR_F values are given in Table IV.

High-performance liquid chromatography

The equipment and conditions for RP-C_{18} liquid chromatography were as follows: columns, stainless steel, 120.0 × 4.5 mm I.D. and 250.0 × 8.0 mm I.D. (Knauer, Berlin, G.F.R.), packed with 5-μm LiChrosorb RP-C_{18} (Merck, Darmstadt, G.F.R.); mobile phase, acetonitrile–water (98:1 to 80:20, nitrogen-purged; flow-rate, 1–4 ml/min; head pressure, 50–250 bar; temperature, 25°C; pump, Type M 6000; detectors, (a) Model 153 spectrophotometer (Altex, U.S.A.), wavelength 356 or 436 nm, (b) Model LC 55 spectrophotometer (Perkin-Elmer, U.S.A.), wavelength 780 nm.

Absorption spectroscopy of bacteriochlorophylls

The absorption spectra were recorded on a Cary 219 instrument (Varian, U.S.A.).

Cultivation of the microorganisms

All bacteria were obtained from Deutsche Sammlung Mikroorganismen (DSM), Göttingen, G.F.R. The culture media had the following compositions[12]:

Medium No. 27.

Yeast extract	1.0 g
Ethanol	0.5 ml
Succinic acid, disodium salt	1.0 g
Iron(III) citrate solution	5.0 ml
Potassium dihydrogen phosphate	0.5 g
Magnesium sulphate ($7H_2O$)	0.4 g
Sodium chloride	0.4 g

Ammonium chloride	0.4 g
Calcium chloride ($2H_2O$)	0.05 g
Solution SL 6	1.0 ml
Distilled water to	1000 ml
pH	6.8

Medium No. 45.

Analogous to medium No. 27, plus 0.05% of L(+)-ascorbic acid, disodium salt.

Solution SL 6.

Zinc sulphate ($7H_2O$)	0.1 g
Manganese(II) chloride ($4H_2O$)	0.03 g
Boric acid	0.3 g
Cobalt(II) chloride ($6H_2O$)	0.2 g
Copper(II) chloride ($2H_2O$)	0.01 g
Nickel(II) chloride ($6H_2O$)	0.02 g
Sodium molybdate ($2H_2O$)	0.03 g
Distilled water to	1000 ml

Extraction of the bacteriochlorophylls from the cellcultures

The bacteria were cultivated in 2-l glass bottles (Table II). Although all photosynthetic bacteria are anaerobes it is not necessary to exclude oxygen completely. During the growing phase (about 2 weeks) the suspensions were irradiated for 12 h a day with white light (about 600 W). After growth has levelled off, the following work-up is recommended:

TABLE II

MICROORGANISMS USED AND BACTERIOCHLOROPHYLLS ISOLATED

Microorganisms	*Isolated BCHL*	*Culture method*
Rhodospirillum rubum, S1-ATCC	BCHL *agg*	No. 27
Rhodospirillum rubum, R.r.G9	BCHL *agg*	No. 27
Rhodospirillum fulvum, 1360	BCHL *ap*	No. 45.
Rhodospeudomonas viridis, ATCC 19567	BCHL *b*	No. 27

(a) Centrifugation of the suspensions for about 15 min at 9000 *g*. The clear supernatent solution is discarded.

(b) The precipitated cells are suspended in 100 ml of 2-propanol and the suspension is sonicated for about 20 min to break the cell membranes.

(c) The suspension from (b) is centrifuged at 5400 *g* for 30 min. In the supernatant the BCHLs are dissolved. The dried and weighed precipitate gives the total cell mass. An ultracentrifugation step on the BCHL solution considerably reduces the amount of high-molecular-weight proteins. However, this additional clean-up step is not absolutely necessary because the dissolved contaminants are separated through the subsequent chromatographic column separation. To the blue BCHL solution (about 100 ml) are added 25 ml of dioxane followed by 40 ml of water dropwise. The sample is then stored for 1 h in a refrigerator to precipitate the BCHL–dioxane coordination polymer. Under these conditions BCHL *b* is not precipitated as a polymeric 1,4-dioxane complex.

TABLE III

YIELDS OF BACTERIOCHLOROPHYLLS BASED ON DRY MASS OF BACTERIA

Type	*Bacteria dry mass from 5 l suspension*	*Yield of bacteriochlorophyll–dioxane complex*
BCHL *agg*	3.8 g	*ca.* 290 mg = 7.6%
BCHL *agg*	4.2 g	*ca.* 300 mg = 7.1%
BCHL *ap*	1.5 g	*ca.* 150 mg = 10.0%
BCHL *b*	11.0 g	—

(d) The precipitate is centrifuged and dried at room temperature under reduced pressure (15 Torr) in a desiccator. The usually greenish supernatant is rejected. The yield of BCHL–dioxane complexes is between 5 and 10% (Table III) based on the dried cell mass.

(e) A 20-mg amount of BCHL–dioxane complex is dissolved in 1 ml of carbon tetrachloride. Small amounts of contaminants still present, such as carotenoids, pheophytins and xanthophylls are separated by column chromatography on Sephasorb HP ultrafine (Pharmacia, Uppsala, Sweden) using cyclohexane containing 1.0–1.5% of tetrahydrofuran as the mobile phase[5,6]. Because of the ready oxidation of the compounds, flushing of the eluents with nitrogen or argon to purge off the oxygen is essential.

RESULTS AND DISCUSSION

Liquid chromatography of bacteriochlorophylls

Partition thin-layer chromatography of bacteriochlorophylls. The BCHLs, like

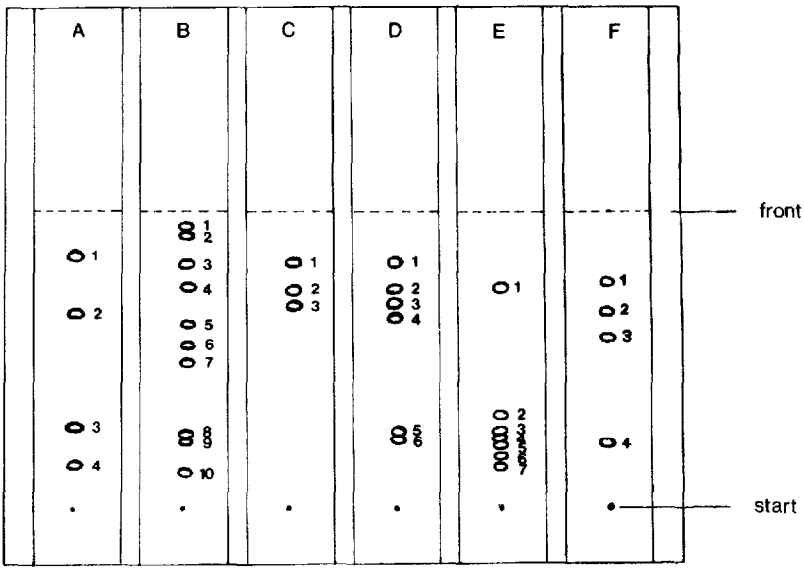


Fig. 2. RP-TLC chromatograms of: (A) BCHL *b* Isopropanol extract; (B) BCHL *agg* isopropanol extract; (C) BCHL *agg* after dioxane precipitation and column chromatography; (D) as C, but heated at 90°C for 5 min; (E) red pigments of *Rhodospirillum rubrum*, S1-ATCC (Nos. 2–7); (F) commercially available pure BCHL *ap* from Sigma Chemie, Munich, G.F.R. For identification of spots, see Table IV and text.

the chlorophylls, can be separated by a type of partition TLC that utilizes oil-impregnated cellulose as the stationary phase. Acetonitrile–tetrahydrofuran–carbontetrachloride–water (70:15:10:5) is used as the eluent. The details of this separation method have been described earlier[7]. The thin-layer chromatograms of all three BCHLs are shown in Fig. 2. Table IV gives the hR_F values and colours of the spots.

TABLE IV

hR_F VALUES AND COLOURS OF BCHL *g* AND OXIDATION PRODUCTS

Compound	*Chromatogram*	*Spot No.*	*Colour in visible light*	hR_F *value*
BCHL *agg*	Fig. 2C	1	Blue	83
		2	Blue	75
		3	Blue	70
BCHL *ap*	Fig. 2F	1	Blue	68
		2	Blue	64
		3	Blue	54
BCHL *b*	Fig. 2A	2	Green	60
BPHEO *agg*	Fig. 2D	5	Violet	29
BPHEO *ap*	Fig. 2F	4	Violet	27
2-Desvinyl-2-acetylprotochlorophyll *ap*	Fig. 2B	6	Green	53
2-Desvinyl-2-acetyl-CHL *ap*	Fig. 2B	7	Green	47
2-Desvinyl-2-acetylprotochlorophyll *agg*	Fig. 2D	4	Green	69

The chromatograms of the crude extracts of BCHL *b* and BCHL *ap* are shown in Fig. 2A and B, respectively. BCHL *b* itself gives only a single spot (No. 2, Fig. 2A). As BCHL *b* is very unstable, it is difficult to test the purity of this compound by TLC. In chromatogram 2B two other spots in addition to BCHL *ap* (spot No. 5) can be seen, which we assigned to 2-desvinyl-2-acetylprotochlorophyll *ap* (spot No. 6) and 2-desvinyl-2-acetylchlorophyll *ap* (spot No. 7). Whether these oxidation products originate during handling of the compounds (*e.g.*, through contact with oxygen) or are elements of the photosynthetic apparatus of these microorganisms is inclear. All other compounds in chromatograms 2A and 2B are unknown.

Fig. 2C and F depict the chromatograms of pure BCHL *agg* (2C) prepared according to the above procedure and pure commercial BCHL *ap* (2F). Three spots are seen (Nos. 1–3) which contain all seven diastereomers of these compounds[9]. Spots Nos. 1–3 (Fig. 2C and F) are easily degraded in about 30 min by light and oxygen. The appearance of three spots for BCHL *agg* and BCHL *ap* were the decisive indication that these two chlorophylls do consist of more than two C-10 epimers. Spot No. 4 in Fig. 2F has been identified as BPHEO *agg*. We did not succeed in achieving a better separation of these BCHL *a* diastereomers by varying the composition of the mobile phase. Fig. 2D is a chromatogram of pure BCHL *agg* which was heated at about 90°C for a short time. A comparison of Fig. 2D and C demonstrates that the other compounds are formed during this heating. In this chromatogram spot No. 4 is 2-desvinyl-2-acetylprotochlorophyll *agg* and spot No. 5 BPHEO *agg*. Spot No. 6 is unidentified. Fig. 2E is the chromatogram of the so far unidentified red dyes from *Rhodospirillum rubrum*, S1-ATCC. Spot No. 1 in Fig. 2E is a small

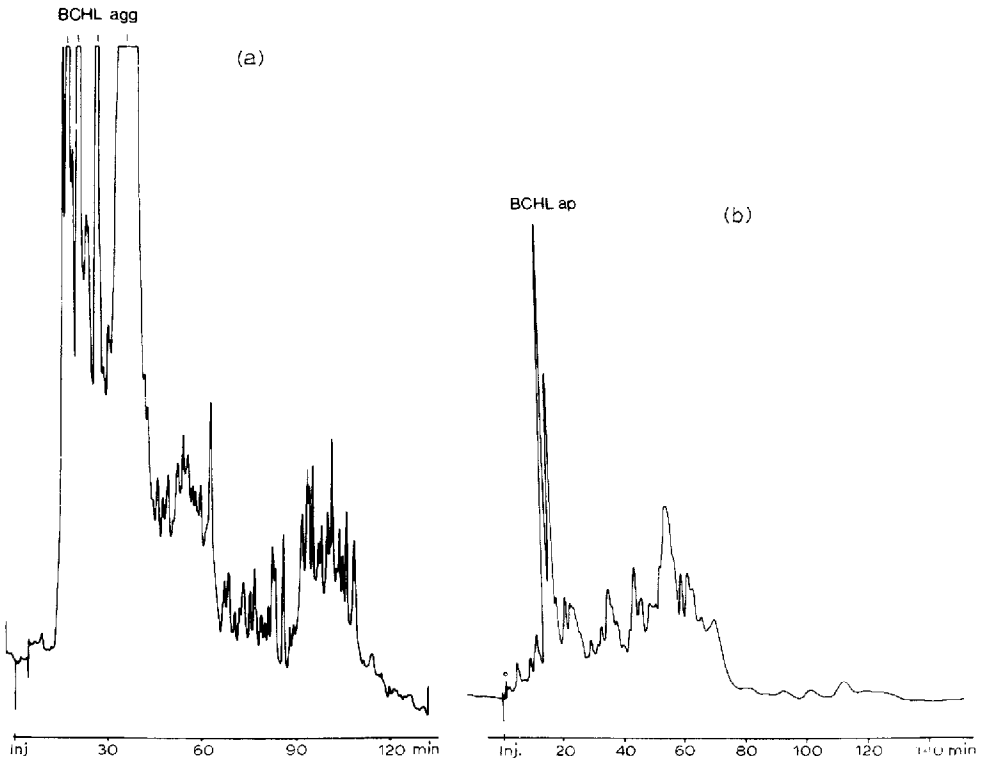


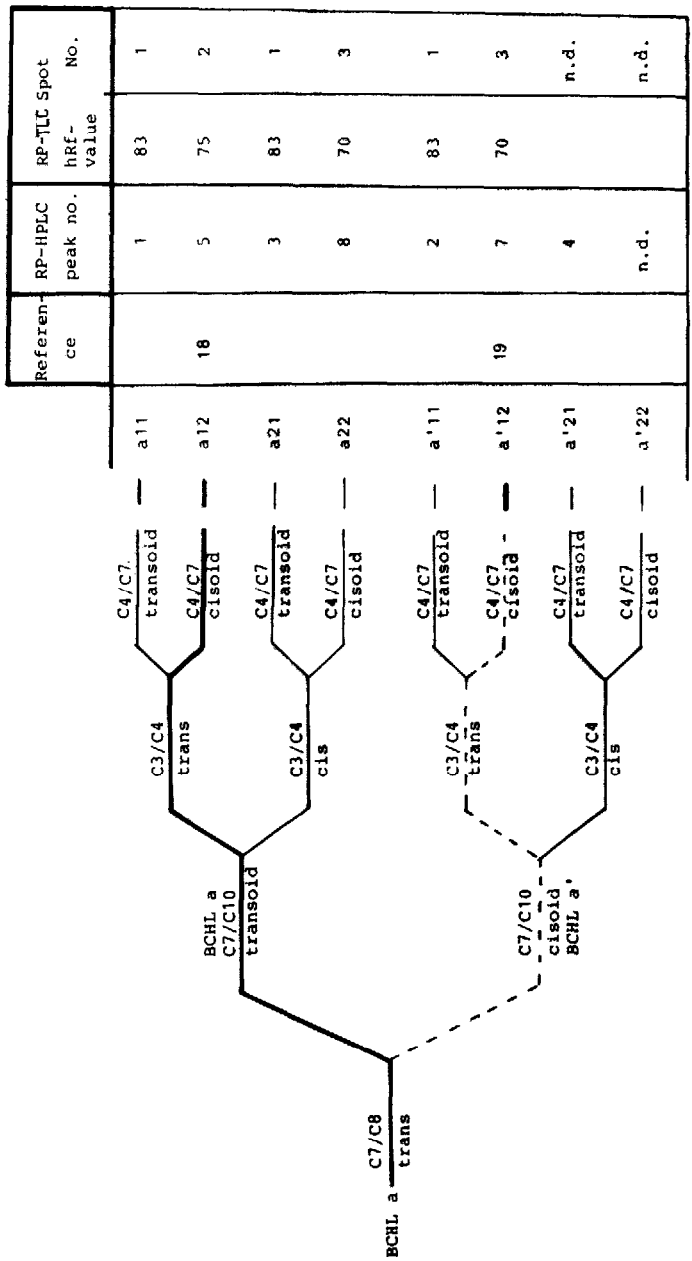
Fig. 3. RP-C_{18} HPLC traces of (a) BCHL *agg*– and (b) BCHL *ap*–dioxane complex precipitate. Mobile phase: acetonitrile–water (88:12).

amount of BCHL *agg*. A better separation of compounds Nos. 1–3 in Fig. 2C and F was possible only with RP-HPLC.

Reversed-phase high-performance liquid chromatography of BCHL agg, BCHL ap and BCHL b. Fig. 3 shows the necessity for a liquid chromatographic separation of the precipitated dioxane–BCHL *a* complex. The unknown compounds in this HPLC trace probably correspond to low-molecular-weight proteins, dissolved in the polar solvents and coprecipitated with the coordination complex. BPHEO *ap* and BPHEO *agg* are not eluted under the conditions given under Experimental. The elution of these compounds takes place only with a reduced water content of 4–8% in the mobile phase (Fig. 4)[5].

Under optimized separation conditions the HPLC trace of the pure compounds BCHL *agg* and BCHL *ap* each consists of seven peaks with different intensities (Fig. 5). The different substituents at C-3, C-4, C-7 and C-8 theoretically allow the existence of eight BCHL *a* diastereomers with the structure shown in Scheme 1. All assignments of theoretically proposed structures to identified BCHL *a* diastereomers in the HPLC trace (Fig. 5) and to the spots in Fig. 2C and F, respectively, are listed in Schemes 1 and 2 (ref. 12).

In contrast to BCHL *a* (BCHL *agg* or BCHL *ap*), BCHL *b* theoretically exists as four diastereomers, whose structure are shown in Scheme 3. The HPLC trace of crude BCHL *b* material is shown in Fig. 6. Because of the great instability of this



Scheme 1. Structures of BCHL *a* diastereomers.

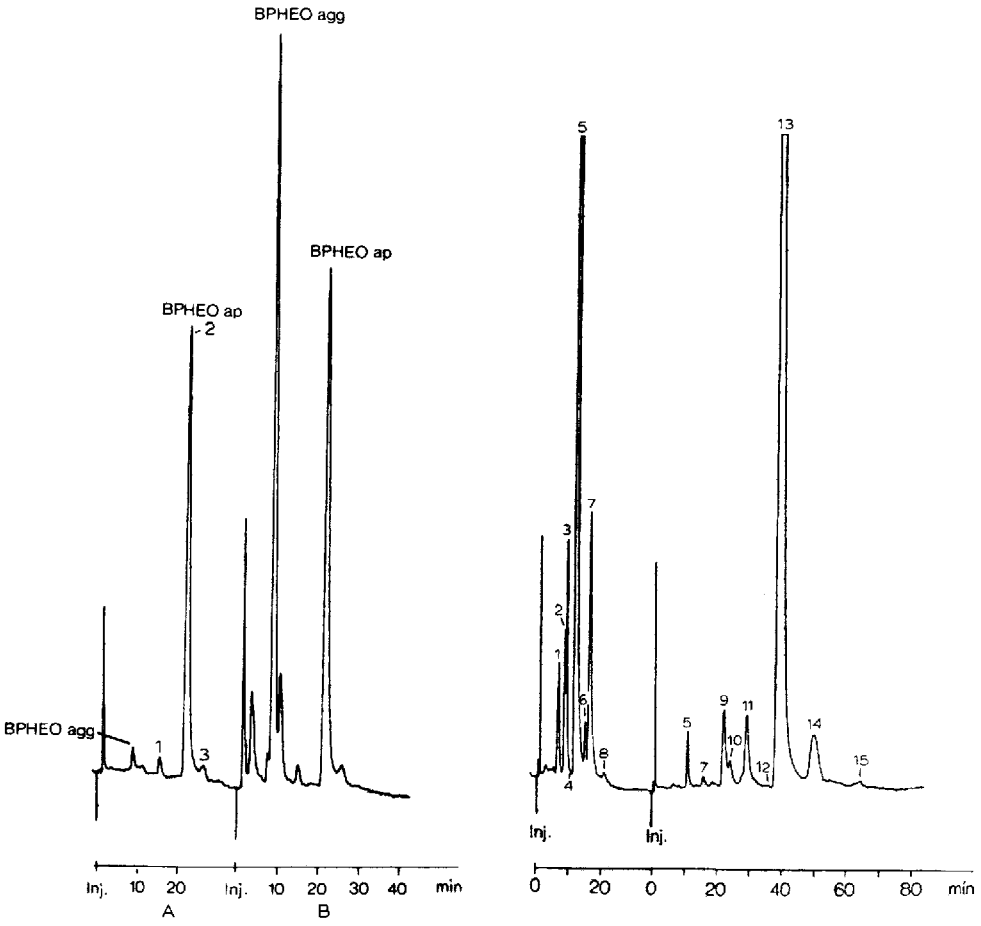
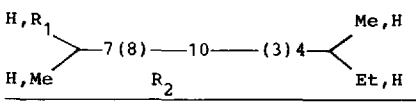
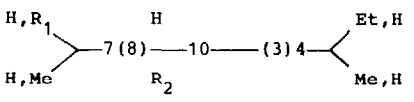
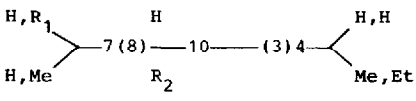
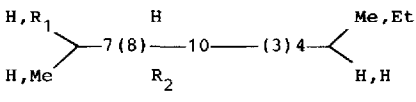
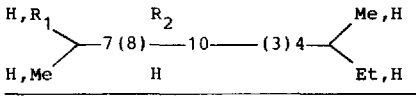
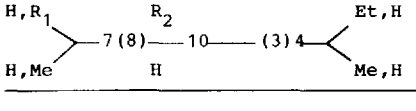
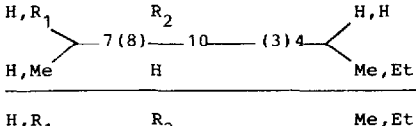
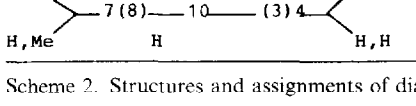


Fig. 4. RP-C_{18} HPLC traces of (A) pure BPHEO *ap* (Nos. 1–3) and (B) a mixture of pure BPHEO *agg* and *ap*. Mobile phase: acetonitrile–water (96:4).

Fig. 5. RP-C_{18} HPLC traces of BCHL *agg* (Nos. 1–8) and BCHL *ap* diastereomers (Nos. 9–15). Mobile phase: acetonitrile–water (85:15). Detection: 365 nm.

compound, it is very difficult to obtain an HPLC trace without degradation. At present it cannot be decided whether the peaks surrounding the main BCHL *b* peak are diastereomers of this compound or degradation products. BCHL *b* and its derivatives have been analysed by HPLC on RP-8 columns (Knauer) using methanol–water (95:5) containing sodium ascorbate as the mobile phase[21].

The HPLC traces of pure BPHEO *agg* and BPHEO *ap* (BPHEO *a* = BCHL *a* − Mg + 2H, see Fig. 1) are shown in Figs. 4 and 7. The synthesis of these compounds was achieved by shaking a carbon tetrachloride solution of BCHL *agg* or BCHL *ap* with 2 *M* hydrochloric acid about 10 min. In the chromatograms four peaks are recognizable which correspond to BPHEO *agg* diastereomers. Probably the low concentration of these compounds is the reason why seven peaks of each compound were not detectable analogous to the BCHL *a* diastereomers themselves. It is notable that the elution conditions of these compounds are different from those of the BCHL

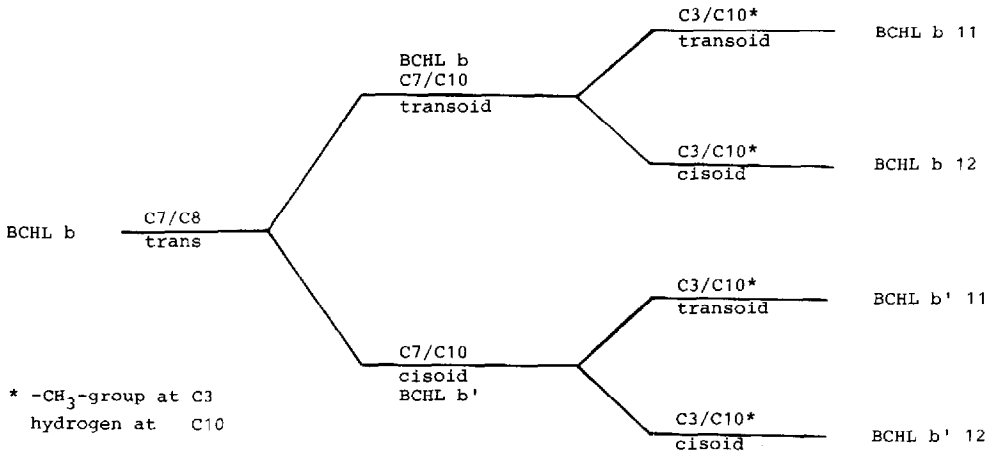
Structure R_1 = $CH_2CH_2COOC_{20}H_{39}$; R_2 = $COOCH_3$ Me = CH_3; Et = CH_2-CH_3	BCHL Diastereomer	Partition TLC Spot (assigned)	RP-HPLC Peak (assigned)
	a11	1	1
	a12	2	5
	a21	1	3
	a22	3	8
	a'11	1	2
	a'12	3	7
	a'21	n.d.	(4)
	a'22	n.d.	n.d.

Scheme 2. Structures and assignments of diastereomers.

a diastereomers. For this reason these two sets of compounds do not overlap in BCHL *a* diastereomer separations.

Oxidation and thermal isomerization of BCHL agg diastereomers and separation of the products by RP-HPLC

The oxidation of the BCHL *a* diastereomers with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) has recently been described[9]. The products of the oxidation by DDQ are 2-desvinyl-2-acetylprotochlorophyll *agg*, a porphyrin derivative (1) (Fig. 8), and 2-desvinyl-2-acetylchlorophyll *agg*, a dihydroporphyrin derivative (2) (Fig. 8). The reaction leading to the dihydroporphyrin (2) is very rapid, whereas the reaction from the dihydroporphyrin to the unreduced porphyrin derivative (1) is slow. Oxidation of the side-chain at C-7 (see Fig. 1) does not occur, otherwise more than two oxidation products of each bacteriochlorophyll *a* would be detected.



Scheme 3. Theoretically possible BCHL diastereomers.

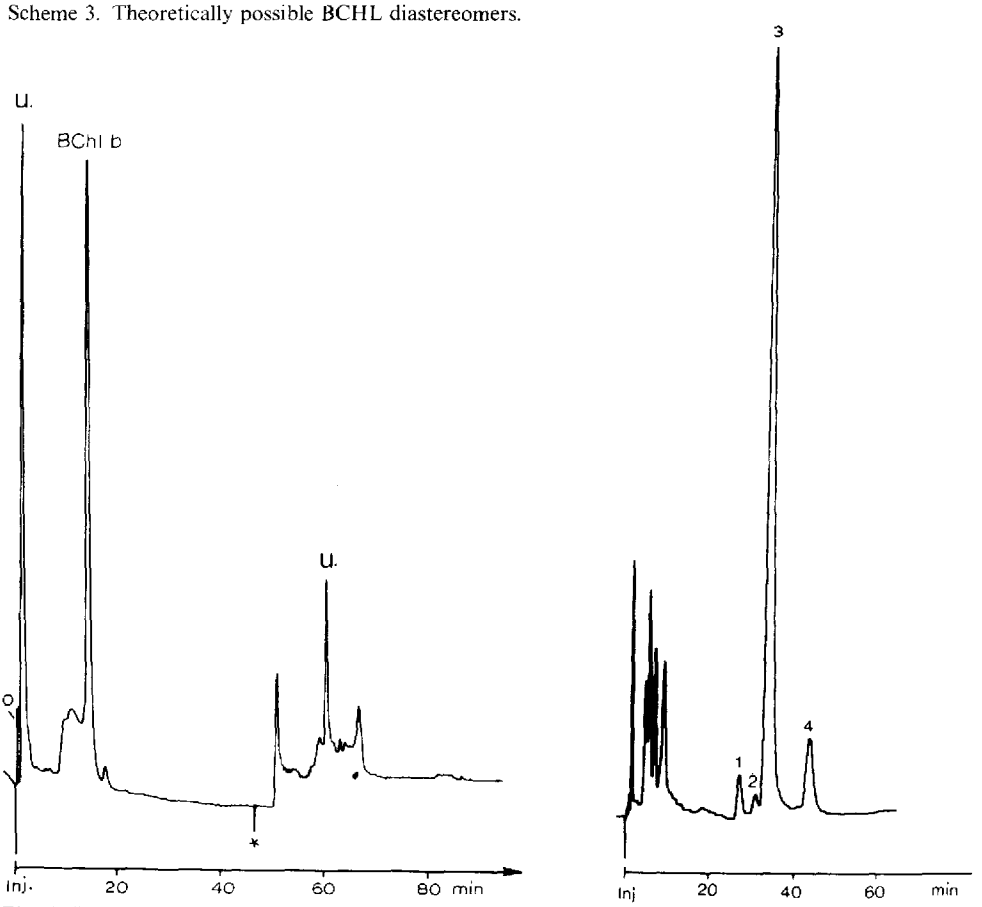


Fig. 6. RP-C_{18} HPLC trace of a propanol-2-extract of BCHL *b*. For separation conditions see Table V. Mobile phase: acetonitrile–water (90:10).

Fig. 7. RP-C_{18} HPLC trace of BPhRO *agg* (Nos. 1–4). For separation conditions see Table V. Mobile phase: acetonitrile–water (92:8).

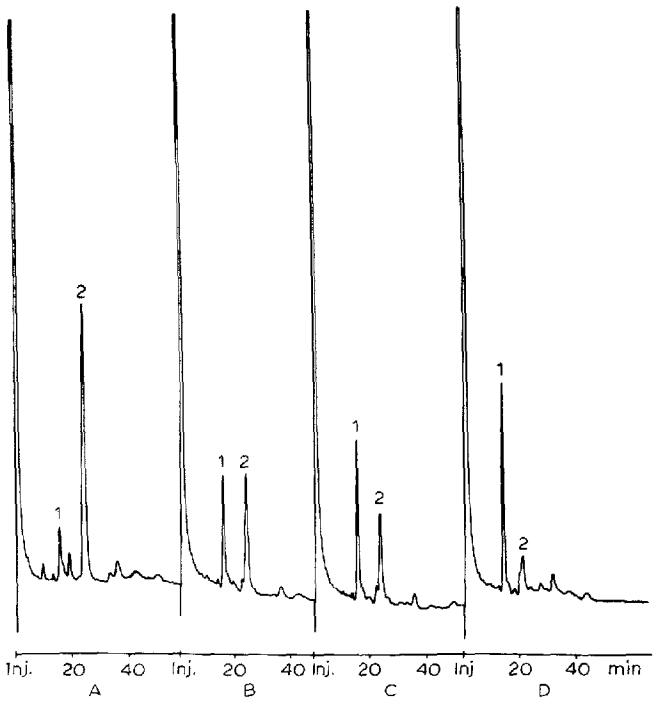


Fig. 8. RP-C_{18} HPLC traces of BCHL *agg* oxidation products formed by DDQ after different reaction times. (1) 2-Desvinyl-3-acetylprotochlorophyll *agg*; (2) 2-desvinyl-2-acetylchlorophyll *agg*. (A) after 10 min; (B) 30 min; (C) 60 min; (D) 120 min. Mobile phase: acetonitrile–water (85:15).

The reaction of BCHL *b* with DDQ results, in agreement with the structure of the former compound, in the same products as from the reaction of BCHL *a* with DDQ, plus 2-desvinyl-2-acetyl-4-(α-hydroxy)ethylchlorophyll *ap*[13].

The thermal isomerization at C-10 of the BCHL *a* diastereomers to the corresponding C-10 diastereomers did not take place under the reaction conditions described for CHL *a* and *b*[20] and the only detectable product was 2-desvinyl-2-acetyl-CHL *a*. Because oxygen was not excluded during these experiments, it is possible that the formation of this compound was the result of a reaction between BCHL *a* diastereomers and oxygen.

Absorption spectra of bacteriochlorophylls

The absorption spectra of BCHLs have been published[14,15]. To demonstrate the existence of the BCHL *agg* and BCHL *ap* diastereomers after separation by RP-HPLC, some of the fractions containing pure, individual compounds were collected and concentrated. The absorption spectra of the single BCHL *a* diastereomers were recorded with a Cary 219 spectrophotometer. The absorption maximum in the red region is at 780 nm in acetonitrile–water (90:10) as solvent. This agrees very well with the absorption maximum of the starting mixture (Fig. 9). The absorption maxima of BCHL *a*, BCHL *b*, BPHEO *agg* and BPHEO *ap* and the oxidation products are listed in Table V, and the respective absorption spectra are shown in Fig. 9. The band at 780 nm is of particular value in the identification of BCHL *a* diastereomers because no other BCHL or degradation product of these compounds absorb at this wavelength

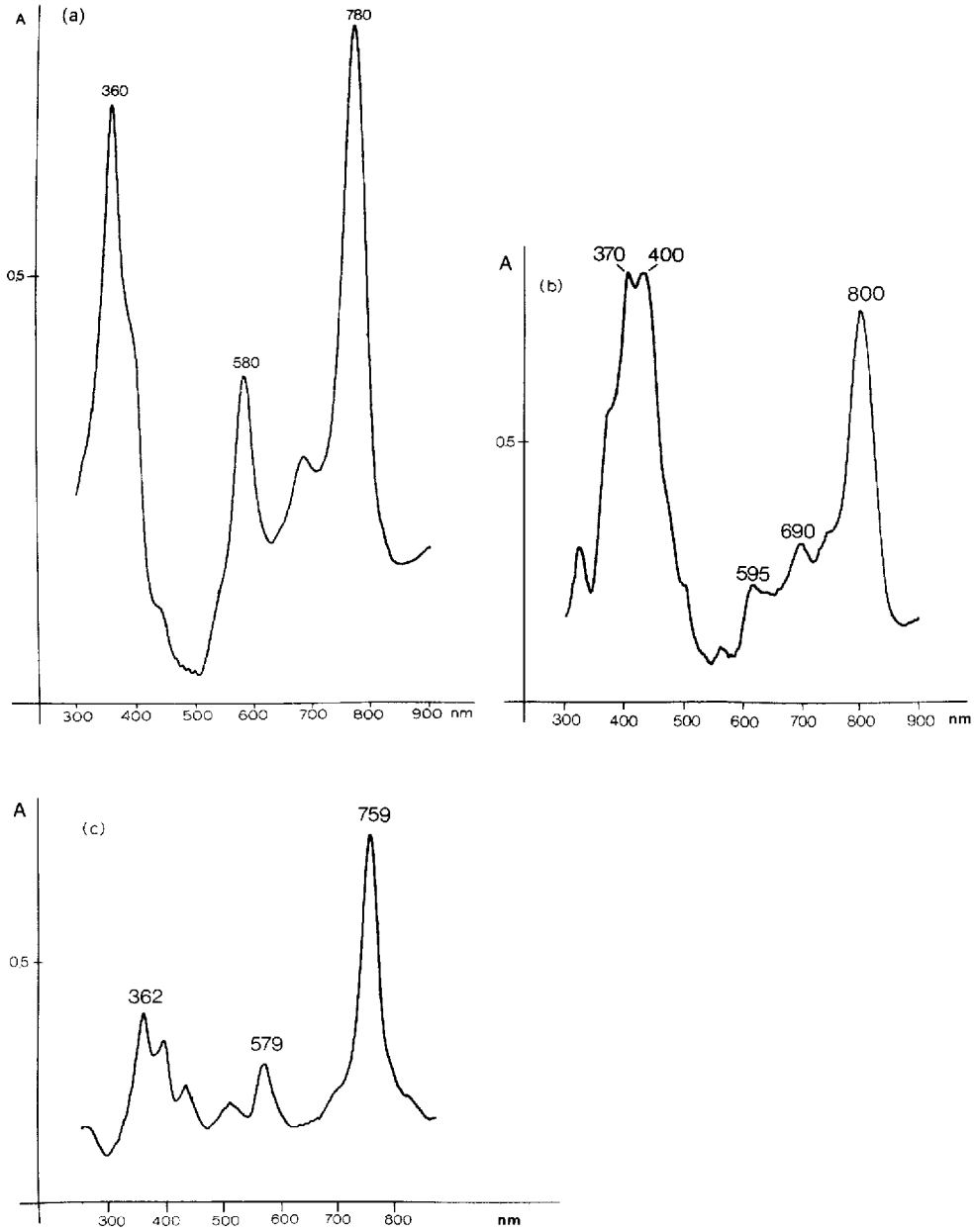


Fig. 9. (a) Absorption spectra of BCHL *agg* and BCHL *ap* in carbon tetrachloride; (b) absorption spectrum of BCHL *b* extract in 2-propanol; (c) absorption spectra of BPHEO *agg* and BPHEO *ap* in carbon tetrachloride.

(Table V). Absorption spectroscopy can therefore be used to elucidate the nature of chromatographically separated BCHL *a* compounds as diastereomers. Definitive proof will be given by nuclear magnetic resonance spectroscopy only[20].

TABLE V

ABSORPTION MAXIMA OF CHL *a*, CHL *b* IN DIETHYL ETHER, BCHL *ap*, BCHL *agg* IN CARBON TETRACHLORIDE AND BCHL *b* IN ISOPROPANOL

Compound	*Maxima in this work (nm)*	*Maxima in literature (nm)*	*Ref.*
CHL *a* (dried)	382(sh),409(sh) 429,492,530 577,617,661	410(sh),430 578,615,662	20
CHL *b* (dried)	477(sh),454 595,642	430(sh),453 594,642	20
BCHL *ap*	360,395(sh) 430(sh),580,780	358,390(sh),577 773 in acetone	14
BCHL *agg*	As BCHL *ap*		
BCHL *b*	370,400,450(sh) 595,690,800	368,407,675 794 in acetone	15
BPHEO *agg*	362,390(sh),533 579,680,759	357,385,523 675,745	14
BPHEO *ap*	As BPHEO *agg*		
2-Desvinyl-2-acetyl-CHL *a*	430,542,679	388,436,505 538,591,628,677 in acetone	16
BCHL *agg* diastereomers, peaks 1,2,3,7,8		All as BCHL *agg*	

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