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Bacterial Carotenoids

XVII. The Carotenoids of Photosynthetic Green Bacteria

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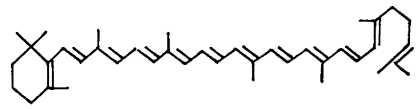
The present paper reports an investigation on the carotenoids of *Chloropseudomonas ethylicus*, and a re-examination of those of *Chlorobium thiosulfatophilum* and *Chlorobium limicola*.

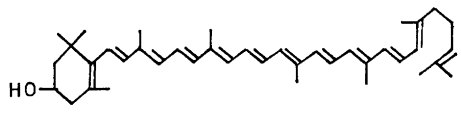
All three species produce as their major carotenoid a carotene, chlorobactene, very similar to, but not identical with γ-carotene (I). Chlorobactene has an aromatic end-group, and is shown to be (XII). In addition a monohydroxy-carotenoid with a tertiary hydroxyl group and the same chromophoric system was present in all species investigated. This carotenoid is not identical with rubixanthin (II) and possesses structure (XIV).

From *Chlps. ethylicus* small amounts of γ-carotene (I), lycopene (III) and rhodopin (IV) were also isolated.

An examination of the carotenoids of three *Chlorobium* spp. has been reported by Goodwin and Land.[1] They claimed that the major carotenoid of these bacteria was γ-carotene (I). *Chl. thiosulfatophilum* Larsen and *Chl. limicola* Nadson further contained a monohydroxy-derivative identified as rubixanthin (II). Both identifications were based on spectral comparison and co-chromatography tests with authentic carotenoids.



(I)



(II)

* Part XVI. *Acta Chem. Scand.* **18** (1964) 1562.

Since rubixanthin would represent the only mono-hydroxy carotenoid with a secondary hydroxyl group in 3-position present in photosynthetic bacteria (*cf.* Ref.[2]), a re-investigation was carried out. The monohydroxy carotenoid of green bacteria was found to be different from rubixanthin. To our surprise the main carotenoid of photosynthetic green bacteria, here called chlorobactene, proved to be different from synthetic γ-carotene. It should be mentioned that Frydman and Rapoport [2b] in a recent report state that the carotene of *Chl. thiosulfatophilum* strain L was found to be identical with authentic γ-carotene by visible light absorption spectra and co-chromatography on alumina paper, following the procedure of Shneour. We believe the latter procedure refer to that of Jensen [2c] whose system actually was used in the present investigation to prove the non-identity of chlorobactene and γ-carotene.

RESULTS AND DISCUSSION

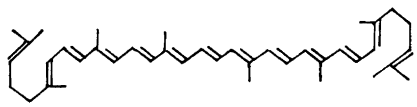
The carotenoid composition of photosynthetic green bacteria, as established in the present investigation is presented in Tables 3, 5, and 6, and is summarized in Table 1.

Table 1. Carotenoid composition of photosynthetic green bacteria.

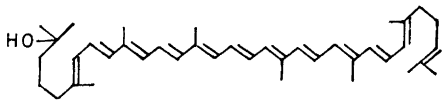
Carotenoid	*Chlps.ethylicus*	*Chl.thiosulfatophilum*	*Chl.limicola*
Comp.max. 410, 432, 455 mμ*	−	+	+
Pro-γ-carotene-like compound	+	+	−
γ-Carotene	++	−	−
Chlorobactene	++++	++++	++++
Lycopene	+	−	−
OH-Chlorobactene	++	++	++
Rhodopin	+	−	−

* In petroleum ether
++++ > 90 % of total carotenoid
++ 1 % < X < 5 % of total carotenoid
+ < 1 % of total carotenoid

Chlorobactene constituted more than 90 % of the total carotenoid in all three species investigated; the hydroxy compound was present to the extent of 4 % of the total carotenoid. From *Chlps. ethylicus* Shaposhnikov small



(III)



(IV)

Table 2. Adsorptive properties and absorption maxima in visible light of the *trans* carotenoids isolated from green bacteria.

Carotenoid in order of increasing adsorption	Eluant required from Woelm neutral alumina, activity Grade 2		Abs.max in mμ in pet.ether		
Phytoene		Pet.ether	274	284	296
Phytofluene		Pet.ether	332	347	367
ζ-Carotene	5 %	Ether*	376	396	418
Comp.max. 415, 432, 455 mμ*	5 %	Ether	415	432	455
Pro-γ-carotene-like compound	5—10 %	Ether (405)	430	453	(475)
γ-Carotene	10 %	Ether	435	461	491
Chlorobactene	3 %	Acetone	435	461	491
Lycopene	4 %	Acetone	445	472	504
OH-Phytofluene	5 %	Acetone	332	347	367
OH-ζ-Carotene	6 %	Acetone	376	396	415
OH-Chlorobactene	10 %	Acetone	435	461	491
Rhodopin	11 %	Acetone	445	472	504

* In pet. ether

Table 3. Carotenoid composition of *Chlps. ethylicus.*

Carotenoid	% of total carotenoid Batch I*	Batch II**
Pro-γ-carotene-like compound	0.3	—
γ-Carotene	—	*ca.* 4.0
Chlorobactene	96.8	91.0
Lycopene	—	0.9
OH-Chlorobactene	2.9	4.0
Rhodopin	—	0.2

* 0.5 l culture
** 27.5 l culture

amounts of γ-carotene (I), lycopene (III), and rhodopin (IV) were isolated and identified by comparison with authentic samples. The two *Chlorobium* spp. contained small amounts of a non-polar, unidentified carotenoid with absorption maxima at 410, 432, and 460 mμ in petroleum ether, spectroscopically equivalent to *ca.* 8.5 conjugated double bonds.

The total carotenoid content of the green bacteria investigated was of the order 0.3 % of the dry, acetone-extracted residue. Since *Chlps. ethylicus* could be cultivated with much higher cell yield than the *Chlorobium* spp., this bacterium was used as the major source of chlorobactene.

Chlorobactene crystallized as red needles, forming rosettes from acetone-petroleum ether, m.p. 147—148°C. The absorption spectrum in visible light, presented in Fig. 1, was indistinguishable from that of synthetic γ-carotene (I). Neither the IR-spectra of the two compounds, presented in Fig. 2, nor mixed melting point determination gave conclusive evidence of any structural

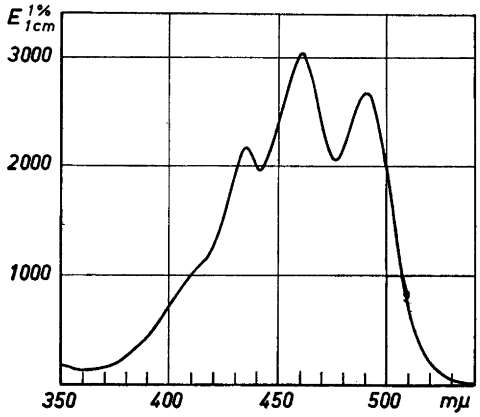


Fig. 1. Absorption spectrum in visible light of chlorobactene in petroleum ether.

deviations. Also, the spectral shift produced on iodine catalysis of chlorobactene was in good agreement with that reported by Pinckard [3] for γ-carotene (I).

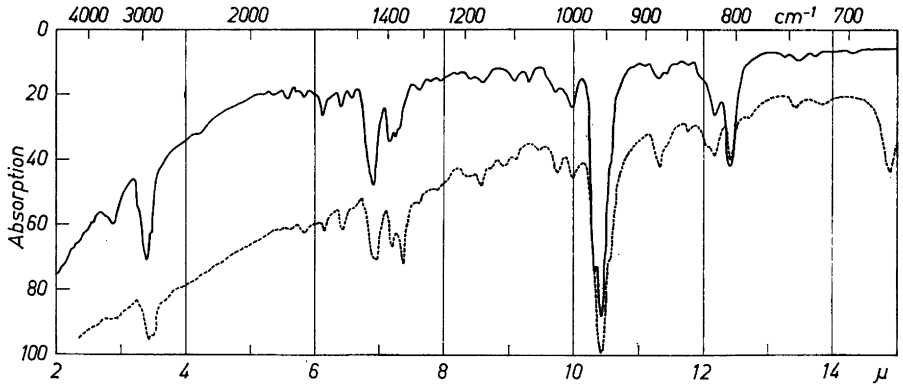


Fig. 2. IR-spectrum of — chlorobactene (0.3 mg) in KBr (0.2 g) and --- synthetic γ-carotene (1.3 mg) in KBr (0.6 g).

Mixtures of partly stereoisomerized synthetic γ-carotene and chlorobactene could not be separated on a deactivated alumina column. However, *trans* chlorobactene was easily separated chromatographically from *trans* γ-carotene on aluminium oxide-paper, and, on separate iodine catalysis of the two compounds, two independent stereoisomeric sets were formed, as shown in Table 4. Elementary analysis of chlorobactene unequivocally established the hydrocarbon nature of this compound, and was in best agreement with a molecular formula $C_{40}H_{52-54}$.

Table 4. Composition of iodine catalyzed equilibrium mixtures of natural chlorobactene, synthetic chlorobactene and synthetic γ-carotene.

Carotene	Member of the set	R_F-value Pet.ether	Abs. max. in mμ in acetone				% Of total carotenoid
Natural chlorobactene	Neo B	0.82	350	(435)	458	486	28
	Neo A	0.70	350	(435)	458	488	*ca.* 28
	Trans	0.62		440	465	495	*ca.* 45
Synthetic chlorobactene	Neo B	0.78	350	(433)	455	483	30
	Neo A	0.70	350	(435)	458	488	27
	Trans	0.63	350	440	463	494	47
Synthetic γ-carotene	Neo-forms	0.92	351	(437)	458	487	40
	Trans	0.83		440	465	495	60

Table 5. Carotenoid composition of *Chl. limicola.*

Carotenoid in % of total	Batch I	Batch II
Compound max. 410, 432, and 455 mμ in pet.ether	0.9	—
Chlorobactene	96.1	95.7
OH-Chlorobactene	3.0	4.3
Carotenoids in mg/l culture		0.62

The NMR-spectrum of chlorobactene is presented in Fig. 3. The bands at 8.39 and 8.33 (τ-values), each of which is equivalent to three protons, are characteristic of the lycopene end group, and, in addition, the spectrum contains a singlet at 8.19 (3 protons) typical of the "end-of-chain" methyl groups in lycopene.[4] Thus one of the end groups of chlorobactene is of the lycopene type. The most striking features of the spectrum are the sharp singlets at 7.77 (3 protons), 7.75 (6 protons) and 3.04 (2 protons). The first two absorptions clearly arise from three methyl groups and in hydrocarbons only methyl groups attached to aromatic nuclei absorb at such low τ-values. Similarly, absorption at 3.04 is too far down field for olefinic protons in an aliphatic hydrocarbon, but is in the expected position for aromatic protons of a polyalkylated benzene ring. This information shows that the second end-group of chlorobactene is a trimethylphenyl group. On biogenetic grounds it is expected that the orientation of substituents would be as in (V) or (VI) since these end-groups are known to be present in the naturally occurring carotenoids isorenieratene [5] (IX) and renierapurpurin [6] (X), discovered some years ago by Yamaguchi in a sea-sponge *Reniera japonica.* In view of the equivalence of the two aromatic protons in chlorobactene it is unlikely that one

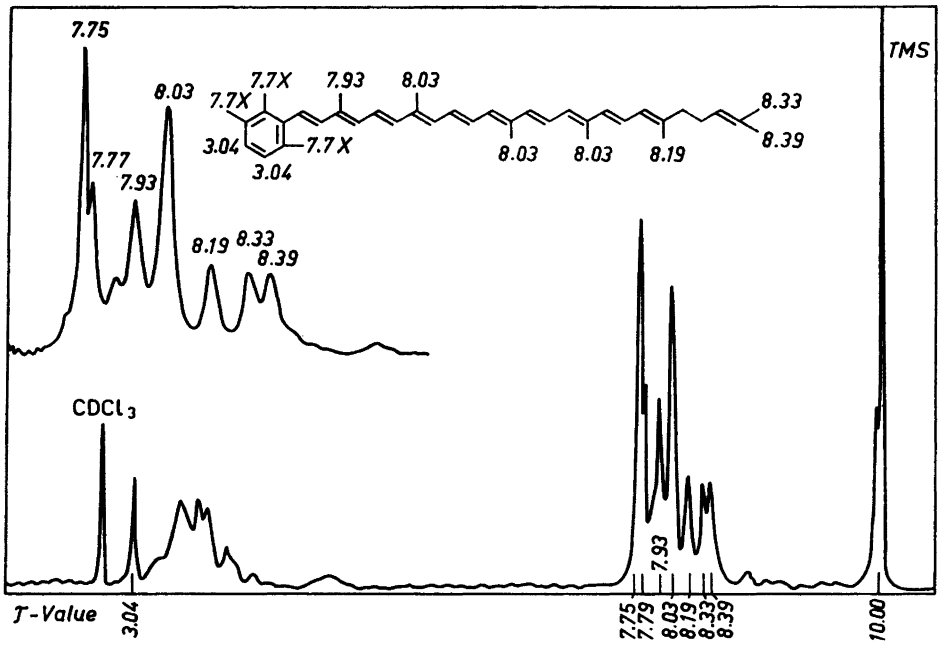
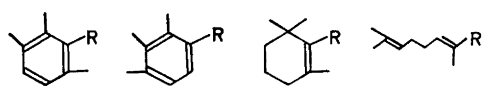


Fig. 3. Proton magnetic resonance spectrum of natural chlorobactene (4.7 mg in 0.1 ml $CDCl_3$) at 60 Mc/sec.

of them is *ortho* to the polyene system. Thus, the orientation (V) is favoured. One anomalous feature of the spectrum is the absorption at 7.93 by an "in-chain" methyl group. "In-chain" methyl groups generally absorb at 8.03 (in $CDCl_3$) [4] and the observed shift to a lower τ-value indicates that the protons of the "in-chain" methyl group nearest to the aromatic ring lie approximate in the plane of that ring. As these methyl protons lie close to the axis of rotation of the benzene-ring about the bond between the ring and the polyene chain, their observed deshielding gives no indication of the relative orientation of the planes of the aromatic ring and the polyene chain.

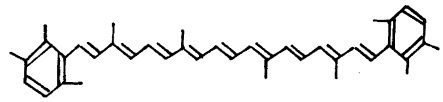
On the basis of the NMR-spectrum the structure of chlorobactene can be formulated as (XII), although the precise orientation of the substituents of the benzene ring is not rigorously established.



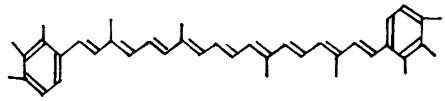
(V) (VI) (VII) (VIII)

Comparison of the absorption spectra in visible light for isorenieratene [5] (IX) and renierapurpurin [6] (X) with those of β-carotene (XI) and lycopene (III), reveal that the end-groups (V) and (VII) give the same spectral contri-

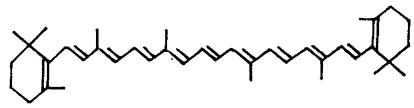
bution, whereas the spectral contribution of the end-group (VI), because of the absence of steric conflict with the hydrogen atom at carbon atom 8, is equivalent to that of the aliphatic end-group (VIII).



(IX)

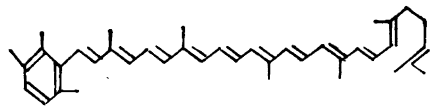


(X)



(XI)

These considerations and the observed superimposability of the spectra of γ-carotene and chlorobactene, show that the end-group (V) must be present in chlorobactene, thus proving the structure (XII) for this carotenoid.



(XII)

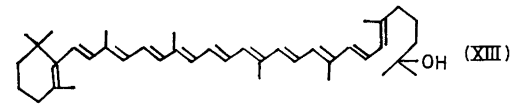
At a later stage compound (XII) was synthesized in Weedons [7] laboratory (see the following communication). The synthetic compound was found to be identical with the natural chlorobactene by a quantitative comparison of the iodine catalyzed equilibrium mixtures (see Table 4), and by identity of the NMR-spectra (see Fig. 3).

Because of the limited quantity available, the hydroxy compound could not be isolated in the crystalline state. It exhibited the same absorption spectrum in visible light, was more strongly adsorbed than γ-carotene (I) and chlorobactene (XII), and slightly more weakly retained than rhodopin (IV). The partition ratio was similar to that of rhodopin (IV), strongly indicating the presence of one hydroxyl group. Repeated attempts to acetylate this carotenoid, under conditions where lutein diacetate was formed in quantitative yield, gave no acetate. This result strongly suggested that the hydroxyl group had tertiary character. Indeed, direct chromatographic comparison with rubixanthin (II), isolated from *Rosa rubigenosa*, resulted in an easy separation of the two compounds. Rubixanthin was more strongly adsorbed than the bacterial pigment on aluminium oxide-paper as well as on deactivated alumina. Our results are contradictory to these of Goodwin and Land,[1] who stated that the bacterial pigment co-chromatographed with rubixanthin on deactivated alumina.

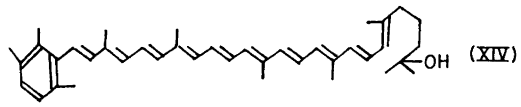
From the data of comparison presented in Table 7 there can be no doubt that the bacterial carotenoid is not identical with rubixanthin (II). The present compound being less strongly adsorbed on alumina than rubixanthin, supports the assumption that it contains a tertiary hydroxyl group. Treatment of the bacterial monohydroxy-carotenoid with acid chloroform according to the method of Karrer and Leumann,[8] did not yield a product with longer chromophore. The tertiary hydroxyl group is therefore presumably non-allylic.

Rubixanthin showed IR-absorption at 1040 cm^{-1} in KBr at a similar position as zeaxanthin.[9] On acetylation rubixanthin acetate was readily obtained. Because of a negative response towards treatment with acid chloroform,[8] hydroxyl in 4-position can be excluded. These results support the previous tentative allocation of the hydroxyl group of the vitamin A non-active rubixanthin (II) to 3-position.[10]

The evidence so far presented for the bacterial hydroxy carotenoid does not permit a differentiation between the structures (XIII) and (XIV), and since it occurs with both chlorobactene (XII) and γ-carotene (I) no biogenetic preference is possible.



(XIII)

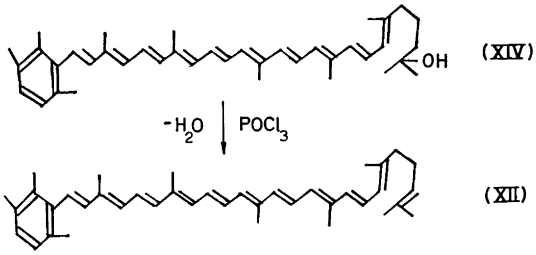


(XIV)

Surmatis and Ofner[11] have recently reported the dehydration of 1,2,1′,2′-tetrahydro-1,1′-dihydroxy-lycopene to lycopene (III) by means of phosphorus oxychloride in pyridine with high yield. The reaction was tested on a micro scale with rhodopin (IV) which yielded lycopene (III) in satisfactory yield. The hydroxy-carotenoid from green bacteria was easily dehydrated according to the same procedure. The reaction mixture consisted of 12 % of the unreacted compound and 88 % of chlorobactene (XII). The latter carotenoid was identified from its visible light absorption spectrum and co-chromatography tests of the three stereoisomers present in the iodine catalyzed equilibrium mixture with those of authentic, natural chlorobactene (XII). γ-Carotene (I) was not present in the reaction mixture.

On the basis of these data, the OH-γ-carotene-like compound from photosynthetic green bacteria is formulated as 1′,2′-dihydro-1′-hydroxy-chlorobactene (XV). We suggest the preliminary name OH-chlorobactene for this carotenoid until a suitable prefix to denote the 1,2-dihydro-1-OH-grouping can be found.

At a later stage the two compounds (XIII) and (XIV) were synthesized in Weedons laboratory (see the following communication).[7] As expected



a chromatographic comparison revealed identity of OH-chlorobactene with (XIV). 1′,2′-Dihydro-1′-hydroxy-γ-carotene (XIII) was less strongly adsorbed on aluminium oxide-paper.

Chlorobactene and OH-chlorobactene represent two new, monocyclic aromatic carotenoids which form a group with the already known bicyclic aromatic carotenes, isorenieratene [5] (IX), renierapurpurin [6] (X), and renieratene [12] with end-groups (V) and (VI). The occurrence of aromatic carotenoids in as remotely related organisms as sea sponges [5,6,12] and photosynthetic bacteria may suggest a more broad distribution of this group of carotenoids.

The isolation of chlorobactene (XII) and OH-chlorobactene (XIV) with properties similar to γ-carotene (I) and rubixanthin (II) makes a reinvestigation of certain cases of the reported occurrence of the two latter compounds desirable.

Weedon and co-workers [13] have suggested that the biosynthesis of aromatic carotenes may proceed by dehydrogenation and Wagner-rearrangements of β-type end-groups (VII). The occurrence together of γ-carotene (I) and chlorobactene (XII) in *Chlps. ethylicus* may support such a biosynthetic relationship. If this is the case, the biosynthesis of aromatic carotenoids may imply a

Table 6. Carotenoid composition of *Chl. thiosulfatophilum.*

Carotenoid in % of total Tentative identification	Batch I Normal cells	Batch II Normal cells	Batch III DPA-grown cells 6.1×10^{-5} M DPA
Phytoene	—	—	+*
Phytofluene	—	0.3	50.0
ζ-Carotene	—	—	15.5
Comp. max. 415, 432, 455 mμ in pet.ether	—	0.7	—
Pro-γ-carotene-like compound	2.0	—	—
Chlorobactene (*cis* + *trans*)	97.6	97.3	22.7
OH-Chlorobactene	0.4	1.7	11.8
Carotenoid in % of acetone-extracted residue		0.33	0.02**

* contaminated with DPA
** except phytoene

biosynthetic route to benzene derivatives via mevalonic acid. Such speculations, however, need confirmation by biosynthetic experiments.

The quantitative effect of diphenylamine (DPA) on *Chl. thiosulfatophilum* was investigated. The carotenoid composition of DPA-grown cells are given in Table 6. In 6.1×10^{-5} M DPA-grown cells the total carotenoid content (except phytoene) was reduced a factor of approximately 15 relative to normally grown cells. Due to this reduced carotenoid content the system was not well suited for studies of endogenous synthesis in washed suspensions. Under conditions for endogenous synthesis no transformation to more coloured carotenoids was recorded, whereas some hydroxylation of the carotenes present in DPA-grown cells seemed to take place. Hence the cyclisation or aromatization reactions could not be successfully studied.

EXPERIMENTAL

Materials and methods have been described in an earlier paper of this series.[14] When not otherwise stated, paper chromatography was carried out on Schleicher and Schüll No. 288 paper (aluminium oxide-paper); and in some cases, specially stated in the text, on Schleicher and Schüll No. 287 paper (kieselguhr-paper). The determination of the composition of iodine catalyzed equilibrium mixtures has been described elsewhere.[9] For co-chromatography tests the 3-divided-paper technique was employed.[15] Partition ratios were determined according to the procedure of Petracek and Zechmeister.[16] All procedures were carried out as far as possible under nitrogen and in subdued light. Analytical grade reagents were used for all reactions.

Harvesting of the cells. Unless otherwise stated the bacterial cells were harvested by centrifugation on a Servall Centrifuge at about 5000 *g*.

Pigment extraction was carried out with acetone and methanol as previously described.[9]

Saponification of the pigments was carried out in 7 % KOH-methanol solution. The mixture was kept for 1 h at room temperature, and the unsaponifiable matter transferred to pet.ether in the usual manner.[9]

Chromatographic separation was performed on the circular papers mentioned above and on columns of Woelm neutral deactivated alumina, activity Grade 2.[17] Size and preparation of the columns have been described elsewhere.[9] In Table 2 are listed the abs. max. and the eluants required for the carotenoids isolated from the three following bacteria.

Chlps. ethylicus Shaposhnikov

Culture. Chlps. ethylicus, obtained from Dr. V. V. Shaposhnikov, Biological Department, Moscow State University, was used.

Medium and cultural conditions. The medium recommended by Shaposhnikov, Kondratieva and Fedoron,[18] modified by Pfennig,[19] was used. Mass cultures were grown for 5 days in 5 × 5.5 l carboys, filled to the neck with medium, in a cultivation device similar to that described elsewhere.[9] The temperature was maintained at 28—29°C. A slow stream of pure nitrogen secured anaerobic conditions. The pH remained at 7.1—7.3.

250 ml of a 4 days old culture, grown in a glass-stoppered bottle of 2 l capacity in a light cabinet at 27—28°C, was used as inoculum for each carboy. As sub-inoculum was used 100 ml of a 5 days old culture.

Harvesting of the cells. To 5 l dark green culture was added 500 ml of a saturated, aqueous $MgSO_4$-solution with stirring, following by 5 l of acetone. The mixture was allowed to settle, and the supernatant discarded. The green, hypophasic slurry was further treated with an equal volume of acetone, and the colourless supernatant again discarded. The green suspension was poured into a separatory funnel, and the colourless hypophase and supernatant were discarded. The green, middle layer was centrifuged

in the Servall centrifuge, and the pigments extracted with acetone — methanol; see above.

Carotenoid composition. After saponification and subsequent column chromatography a total carotenoid content of 5.62 mg/l culture was established spectrophotometrically, using $E^{1\%}_{1cm} = 3000$ and $E^{1\%}_{1cm} = 3400$ for pigments with γ-carotene and lycopene spectra, respectively. The composition of the carotenoid mixture is given in Table 3. The pro-γ-carotene-like compound exhibited the characteristic visible light absorption spectrum of pro-γ-carotene,[3] abs.max. (405), 430, 453 and (475) mμ in pet.ether. Iodine catalysis for identification of the parent *trans* carotenoid was not carried out.

γ-Carotene

The chromatographic fractions of *cis*-chlorobactene from the alumina column contained a minor component presumably identical with γ-carotene, as judged from its absorption spectrum (abs. max. at 440, 464, and 494 mμ, % III/II = 37 in acetone) and adsorptive properties on paper comprising co-chromatography tests with synthetic *trans* γ-carotene ($R_F = 0.32$ with pet.ether as developer).

Trans γ-carotene was obtained in an enriched fraction after re-chromatography on $Ca(OH)_2$. The chromatogram was developed with 3 % acetone-pet.ether. The column was extruded and the coloured zones cut out and eluted with ether. *Trans* γ-carotene exhibited the same R_F-value on paper as neo-chlorobactene B, and was obtained in a relatively pure state by repeated paper-chromatographic purification, allowing stereoisomerization to take place in diffuse daylight between each chromatographic analysis, thereby gradually removing neochlorobactene B by isomerization to its neo A and *trans* isomers (*cf.* Table 7).

Chlorobactene

Crystallization from pet.ether-dry acetone afforded red needles forming rosettes. The crystalline material was collected by centrifugation, washed with cold pet.ether on a platinum Gooch crucible and dried at 0.1 mm Hg and room temperature over Dehydrite and paraffin wax in a hand desiccator; yield 33.2 mg. After two recrystallizations a m.p. of 147—148°C was obtained.

Solubility. The crystalline material was fairly soluble in pet.ether and readily soluble in acetone.

Abs. spectrum in visible light. Abs.max. in pet.ether were located at 434, 460, and 491 mμ, % D_B/D_{II}[9] = 4.5, % III/II[9] = 61, $E^{1\%}_{1cm} = 3040$ at 460 mμ. The extinction curve, presented in Fig. 1, was superimposable with that of synthetic γ-carotene.

IR-Spectrum of 0.30 mg chlorobactene in 0.2 g KBr is presented in Fig. 2. together with that of synthetic γ-carotene (1.3 mg in 0.6 g KBr).

Partition ratio found: Pet.ether/95 % methanol 96:4.

Elementary analysis. Found H 9.93; C 89.90. Calc. for $C_{40}H_{52}$: H 9.84; C 90.16; for $C_{40}H_{54}$: H 10.18, C 90.05 and for $C_{40}H_{56}$: H 10.51; C 89.60.

NMR-Spectrum is presented in Fig. 3. The spectrum was obtained with a Varian Associates HR 60 instrument using a solution of 4.7 mg in 0.1 ml of $CDCl_3$. The spectrum was calibrated by the side band technique. The NMR-spectrum of synthetic (XII) was recorded for comparison.

Stereochemical studies. Paper-chromatographic purity tests revealed that chlorobactene crystallized as the pure *trans* isomer.

Iodine catalysis, carried out in diffuse daylight in pet.ether solution according to the method of Zechmeister and Polgàr,[20] resulted in a hypsochromic shift to 350, 435, 456, and 485 mμ, % III/II = 34, % D_B/D_{II} = 12, accompanied by 18 % drop in extinction value of the main absorption peak. The equilibrium was reached after 2 h.

The composition of the iodine catalyzed equilibrium mixture, established by paper chromatography and subsequent spectrophotometric determination of the eluted isomers, is presented in Table 4. The true nature of the isomers (or group of isomers) neo A and neo B as members of the same stereoisomeric set was proved by reversible isomerization on standing for 2 h in diffuse daylight, followed by paper-chromatographic examination, whereupon the *trans* isomer was re-formed.

A similar study was performed for synthetic γ-carotene as well as for synthetic chlorobactene. The result is summarized in Table 4.

Mixed m. p. determination with synthetic γ-carotene. Found: synthetic γ-carotene m.p. 130–132°C, chlorobactene m.p. 145–146°C and mixed m.p. 130–132°C.

Co-chromatography test with synthetic γ-carotene carried out with the pure *trans* isomers on circular paper gave a clear separation, synthetic γ-carotene had $R_F = 0.83$ and chlorobactene $R_F = 0.62$ when pet.ether was used as developer. The separation obtained on co-chromatography of the iodine catalyzed equilibrium mixtures of the two compounds is apparent from the R_F-values given in Table 4.

Co-chromatography test with synthetic (XII). The *trans* isomer of natural chlorobactene co-chromatographed on paper with that of synthetic (XII). No separation was achieved of the corresponding stereoisomers obtained from the two compounds on separate iodine catalysis, as may be seen from the results presented in Table 4.

Lycopene

was adsorbed above chlorobactene on the alumina column as well as on paper and, after paper-chromatographic purification, had abs.max. at 364, 446, 474, and 506 mμ in acetone; $R_F = 0.58$ with 2 % acetone in pet.ether as eluant. On standing, the less strongly adsorbed *cis*-isomers neo a and neo b[9] were produced. Co-chromatography tests on paper with a similarly stereoisomerized synthetic lycopene revealed identity of the two stereoisomeric sets. When 2 % acetone in pet.ether was used as developer the following R_F-values were found: *trans* (0.58), neo a (0.69), and neo b (0.83).

OH-Chlorobactene

could not be isolated in the crystalline state due to oily contaminants, which could not be removed by re-chromatography on deactivated alumina or sucrose columns, nor by acetylation, The small amounts available of OH-chlorobactene did not allow further purification experiments.

Abs.max. of the *trans* isomer were located at (440), 464 and 494 mμ in acetone. Identity with OH-chlorobactene from *Chl. limicola* was proved by co-chromatography, on paper ($R_F = 0.61$ using 10 % acetone in pet.ether as developer).

Rhodopin

was isolated as a semicrystalline precipitate, yield 0.1 mg. Abs. max. in acetone were located at 363, (450), 475, and 508 mμ. Upon co-chromatography on kieselguhr-paper with rhodopin from *Rhodomicrobium vannielii* no separation was obtained; $R_F = 0.39$ using 10 % acetone in pet.ether as developer.

Chl. limicola Nadson

Culture. Chl. limicola strain DSIR, obtained from Dr. S. Holt, Division of Applied Biology, National Research Council, Ottawa, Canada, was used.

Medium and cultural conditions. The medium described by Larsen [21] was used. The cultures were grown under anaerobic conditions in glass-stoppered bottles, filled to the neck with medium, and unless otherwise stated, in a light cabinet maintained at *ca.* 27°C for a period of one week.

The carotenoid content, determined after column chromatography of the saponified pigment extract is presented in Table 5.

Compound abs.max. 410, 432, and 455 mμ in pet.ether was present only in traces, not sufficient for further examination. The spectrum showed moderate fine-structure % III/II = *ca.* 40.

Chlorobactene

crystallized from acetone-pet.ether as tiny, red needles, abs. max. 435, 460, and 491 mμ in pet.ether. This compound co-chromatographed on circular paper ($R_F = 0.62$ in pet.ether) with chlorobactene from *Chlps. ethylicus*, and was more strongly adsorbed than synthetic γ-carotene in the same chromatographic system.

OH-Chlorobactene

Abs.spectrum in visible light. Abs.max. of the paper-chromatographically purified *trans* isomer were located at 435, 460, and 491 mμ, % III/II = 46 in pet.ether.

Partition ratio found: Pet.ether/95 % methanol 75:25.

Acetylation test was carried out with 16 μg (spectrophotometrically determined) OH-chlorobactene in 1 ml of dry pyridine and 0.15 ml of acetic anhydride. After 2 days at room temperature the reaction mixture was worked up as described elsewhere,[9] pigment recovery 90 %. Paper-chromatographic examination revealed that 90 % of the recovered pigment consisted of unchanged OH-chlorobactene, the remainder being yellow and more strongly adsorbed decomposition products. Other experiments gave a similar result.

A parallel experiment with lutein (*cf.* Ref.[9]) gave lutein diacetate in quantitative yield.

Test of allylic hydroxyl groups. On treatment of 0.44 mg (spectrophotometrically determined amount) OH-chlorobactene with 0.003 N HCl in $CHCl_3$-solution for 20 min in diffuse daylight according to the procedure of Entschel and Karrer,[22] no darkening of the solution was observed. The recovered pigment (92 % of initial) consisted of *ca.* 75 % of *cis* and *trans* OH-chlorobactene beside a less polar compound ($R_F = 0.92$ on paper using 10 % acetone in pet.ether as developer) with abs.max. at 350, (435), 455, and (485) mμ in acetone, as revealed by paper-chromatographic and subsequent spectrophotometric examination. No product with a longer chromophore was produced.

Stereochemical studies. Whereas the column fractions of OH-chlorobactene gave a broad, diffuse zone on kieselguhr-paper resolution into two distinct zones was obtained on aluminium oxide-paper. Preliminary photochemical and iodine catalyzed isomerization experiments demonstrated the interconvertibility of the two stereoisomers, eluted from the latter paper. Consequently a column fraction containing *cis* and *trans* OH-chlorobactene could be used for the preparation of the iodine catalyzed equilibrium mixture.[20] The latter was characterized by abs.max. at 350, (435), 456, and 485 mμ in pet.ether. The composition of this iodine catalyzed equilibrium mixture, spectrophotometrically determined after paper-chromatographic separation is presented in Table 7. The true nature of both stereoisomers as members of the same stereoisomeric set was demonstrated in the usual way by reversible iodine catalyzed stereoisomerization. *Trans*-OH-chlorobactene was a minor component in all isomerization mixtures studied.

Conversion to chlorobactene (XII). The dehydration method of Surmatis and Ofner[11] was adopted. To 0.40 mg of OH-chlorobactene (spectrophotometrically determined) in 5 ml pyridine (previously dried over BaO) was added 0.05 ml $POCl_3$. The reaction mixture was mechanically stirred for 30 min at 50°C under nitrogen. The carotenoids were transferred to ether in a separatory funnel, and the ether extract was washed with water; pigment recovery 76 %. The reaction mixture was submitted to column chromatography, and found to contain 88 % chlorobactene (XII) and 12 % unreacted OH-chlorobactene. Chlorobactene (XII) was identified from its abs. spectrum in visible light (abs.max. 435, 460, and 490 mμ in pet.ether), adsorptive properties on deactivated alumina (required eluant 25 % diethylether in pet.ether, compared to 10 % acetone in pet.ether necessary for elution of OH-chlorobactene) and co-chromatography tests on paper of the *trans*, neo A and neo B isomers present in the iodine catalyzed equilibrium mixture, (*cf.* Table 4) with those of natural chlorobactene (XII). In the latter experiment no separation of the corresponding stereoisomers was achieved.

In a parallel experiment with rhodopin (IV) (0.49 mg isolated from *Rhodomicrobium vannielii*[23]) a pigment recovery of 75 % was determined after column chromatography. The recovered carotenoid consisted of 86 % *cis* and *trans* lycopene (III) and 14 % of unchanged rhodopin. The same criteria were used for identification of lycopene (III) as for chlorobactene (XIII) above. The lycopene produced had abs.max. at 440, 470, and 500 mμ in pet.ether, required eluant 3—5 % acetone in pet.ether from deactivated alumina (for rhodopin required 10 % acetone in pet.ether) and the *trans* and neo a isomers[9] present in the reaction mixture co-chromatographed with those of iodine catalyzed synthetic lycopene on kieselguhr paper when pet. ether was used as developer.

Co-chromatography with rubixanthin gave two separated zones of the trans isomers, OH-chlorobactene, $R_F = 0.62$, rubixanthin $R_F = 0.52$ on the circular paper, using 10 %

Table 7. The composition of the iodine catalyzed equilibrium mixtures of OH-chlorobactene from green bacteria and rubixanthin.

Carotenoid	Member of the stereoisomeric set	R_F-value S.&S. No. 287 2 % acetone*	R_F-value S.& S. No. 288 10 % acetone*	In acetone Abs.max. in mμ	$\%D_B/D_{II}$[9]	% Of total
OH-Chlorobactene	Neo A		0.80	(352) (436) 460 489	21	71
	Trans	0.20	0.62	(350) (440) 464 494	7	29
Rubixanthin	Neo B	0.46		350 (430) 455 480	21	25
	Neo A	0.39		350 (435) 458 486	12	29
	Trans	0.33	0.52	(350) (440) 465 495	7	46

* In pet. ether

acetone in pet.ether as developer. From a column of deactivated alumina OH-chlorobactene required 10 % and rubixanthin 20–25 % acetone in pet.ether for elution.

The non-identity of OH-chlorobactene and rubixanthin was further demonstrated by co-chromatography tests of the iodine catalyzed equilibrium mixtures of the two carotenoids on the two papers. The R_F-values observed are given in Table 7.

Co-chromatography with synthetic (XIII) and synthetic (XIV) on circular paper (developer 10 % acetone in pet.ether) revealed identity of *trans* OH-chlorobactene with synthetic *trans* (XIV). R_F-values found: *Trans* synthetic (XIII) 0.50, *trans* OH-chlorobactene 0.50, and *trans* synthetic (XIV) 0.60. Synthetic (XIV) had abs.max. at (435), 461, and 491 mμ in pet.ether; % III/II = 41.

Chl. thiosulfatophilum Larsen

Culture. Chl. thiosulfatophilum strain Ll, originally obtained from Dr. J. Lascelles, Microbiology Unit, Department of Biochemistry, University of Oxford, England, was used.

Medium and cultural conditions. Chl. thiosulfatophilum was grown in the same medium and under the same conditions as described for *Chl. limicola* in bottles of 1 l capacity for 5 days.

DPA-cultures were grown for 5 days in the presence of 6.1×10^{-5} M DPA, added to the growing culture one day after the inoculation, in alcoholic solution as described elsewhere.[24] The bottles were covered with a yellow cellophane sheet to prevent photodestruction of DPA.

The carotenoid content of normal cells, determined after column chromatography of the saponified extract of two cultures is presented in Table 6.

The major carotene and the hydroxy-compound were identical with those present in *Chl. limicola*, chlorobactene (XII) and OH-chlorobactene (XIV), as proved by co-chromatography on circular papers. The minor pro-γ-carotene-like compound and the carotenoid with abs.max. 410, 432, and 455 mμ were similar to those described above.

In a separate experiment 3 × 1 l cultures were grown in a water bath at 31.5°C as described elsewhere,[4] and aliquots were withdrawn for carotenoid analysis after 83, 152, and 215 h of growth. The carotenoids were extracted with acetone, transferred to pet.ether in a separatory funnel, and aliquots of *ca.* 50 μg carotenoid were submitted to paper-chromatographic examination in the usual manner. γ-Carotene was not detected in any samples. Chlorobactene was always the major carotenoid and OH-chlorobactene was present in less than 10 % of the total carotenoid.

The carotenoid content of DPA-grown cells, established after column chromatography of the saponified extract is also given in Table 6. Phytoene, phytofluene, and ζ-carotene

were tentatively identified from their absorption maxima in pet.ether and adsorptive properties (*cf.* Table 2). The extinction coefficients earlier used for these compounds [24] were employed. In this case the identification of chlorobactene and OH-chlorobactene was tentative since no direct comparison with (XII) and (XIV) was performed.

Rosa rubigenosa

Rubixanthin for comparison with OH-chlorobactene was isolated from *Rosa rubigenosa* according to the procedure of Kuhn and Grundmann.[10] The fruits were collected at Norges Tekniske Høgskole in October 1962, the seeds removed and the peels deep-frozen for storage. The peels (530 g) were dehydrated with 1500 ml methanol by standing at room temperature for 3 days, and dried at 35°C. The dry material was finely ground on a Micro Mill, yield 145 g of fruit powder.

The extraction and saponification of the pigments were carried out as described by Kuhn and Grundmann.[10] The unsaponifiable matter was transferred to pet.ether-benzene and the carotenoids were separated on a column of deactivated alumina. The eluants required and the spectrophotometrically determined composition of the carotenoid mixture is given in Table 8, total carotenoid content 0.1 % of the dry weight. All components were tentatively identified from their adsorptive properties and absorption spectra in visible light, *cf.* Ref.[10]

Rubixanthin

Crystallization. The column fraction of rubixanthin afforded on crystallization from acetone-pet.ether at −20°C two crops of mainly white substance. Thereafter was obtained on further concentration a dark red specimen with metallic sheen of *trans* rubixanthin, yield 3.3 mg, m.p. 133−134°C.

Abs. spectrum in visible light. Abs.max. in pet.ether were located at 435, 460, and 490 mμ, % III/II = 43, $E^{1\%}_{1cm}$ = 2750 at 460 mμ. Purity of sample: 92 %.

IR-Spectrum. The IR-spectrum, recorded in KBr, exhibited abs.max. at 3450 (OH), 2940 (CH), 1635, 1570 (conj. double bonds), 1455 (CH_3), 1360−1380 (CH_3, gemini CH_3), 1205, 1170, 1120, 1040 (sec.OH), 1008, 955 (*trans* double bond), 825 (tri-substituted double bond) and 805 cm^{-1}.

Partition ratio found: Pet.ether/95 % methanol 77:23.

Acetylation test. On acetylation of 0.77 mg (spectrophotometrically determined) *trans* rubixanthin in 2 ml of dry pyridine and 0.2 ml of acetic anhydride for 21 h at room temperature, a pigment recovery of 86 % was obtained. The reaction mixture consisted of 86 % rubixanthin acetate and 14 % of recovered rubixanthin, as established by column chromatography. *Trans* rubixanthin acetate, purified by chromatography on kieselguhr paper had abs.max. in acetone at (440), 463, and 493 mμ. Partition ratio found: Pet.ether/95 % methanol 94:6. *Trans* rubixanthin acetate required 3 % acetone

Table 8. Carotenoid composition of *Rosa rubigenosa.*

Carotenoid Tentative identification	Eluant required (in pet.ether)	% Of total carotenoid
β-Carotene	5 % ether	20
Unresolved carotene fraction	5 % ether	4
γ-Carotene	15 % ether	5
Lycopene	4 % acetone	21
cis-Rubixanthin	12−20 % acetone	12
trans-Rubixanthin	20−25 % acetone	24 } 36
Zeaxanthin	100 % acetone	14

in pet.ether for elution from deactivated alumina, and exhibited the following R_F-values: Aluminium oxide-paper (5 % acetone-pet.ether) 0.08, kieselguhr-paper (pet.ether) 0.46.

Test of allylic hydroxyl groups. Treatment of rubixanthin (0.40 mg, determined spectrophotometrically) with 0.003 N HCl-$CHCl_3$ solution (5 ml) as described above for OH-chlorobactene, gave a pigment recovery of 63 %. No product with longer chromophore was detected on paper-chromatographic examination of the reaction mixture using 10 % acetone-pet. ether as developer.

Stereochemical studies. Rubixanthin crystallized as the pure *trans* isomer, as revealed by paper chromatography tests. The composition of the iodine catalyzed equilibrium mixture, established after resolution on kieselguhr-paper, and R_F-values of its main stereoisomers are listed in Table 7. The true nature of the neo A and neo B isomers as members of the rubixanthin stereoisomeric set was proved by reversible isomerization on standing in acetone solution for 2 h in diffuse daylight and subsequent paper-chromatographic analysis.

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