

Bacterial Carotenoids XIV ***The Carotenoids of *Rhodomicrobium vannielii***

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The carotenoid composition of *Rhodomicrobium vannielii* Duchow and Douglas, has been re-examined in a quantitative manner.

It was possible to isolate all, except one, of the compounds of the so-called normal spirilloxanthin series. The distribution pattern thus indicates a pathway of biosynthesis of these carotenoids analogous to that established elsewhere. Rhodopin is the major carotenoid. The structure previously suggested for rhodopin (III) is supported by its NMR-spectrum.

β -Carotene constitutes a minor component, not invariably present. The taxonomic position of this organism, based partly on its content of β -carotene, is evaluated.

In addition, two dihydroxy-carotenoids were isolated in small amounts. The major one is a 1,2,1',2'-tetrahydro-1,1'-dihydroxylycopene (IX).

Rhodomicrobium vannielii, first isolated by Duchow and Douglas,¹ is an obligately anaerobic photosynthetic bacterium, containing bacteriochlorophyll and carotenoids.^{2,3} It occupies a unique taxonomic position, being the only photosynthetic bacterium placed outside the order *Pseudomonadales*, sub-order *Rhodobacteriineae*. Thus *Rm. vannielii* is placed in the order *Hyphomicrobiales*, family *Hyphomicrobiaceae*, comprising two monotypic genera, viz. *Hyphomicrobium* and *Rhodomicrobium*. *H. vulgare* Stutzer and Hartleb, is a non-photosynthetic, non-pigmented bacterium.⁴

This classification is based on the distinct morphological characteristics.⁴ In addition the photosynthetic pigments are located in a peripheral lamellar system⁵ differing from that of other photosynthetic bacteria⁶ and resembling that of blue-green algae.⁷ The presence of β -carotene (I), a bicyclic carotenoid typical of algae and higher plants and so far not found in other photosynthetic bacteria, has been considered as further support for the taxonomic position of *Rm. vannielii*.³

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Two previous reports are available on the carotenoid composition of this organism.^{2,3} These investigations have been carried out in a qualitative manner, and except for β -carotene, the identifications have been tentative, involving no direct comparison with authentic carotenoids.

RESULTS AND DISCUSSION

The present work is concerned with the quantitative composition of the carotenoids of *Rm. vannielii* and a detailed examination of the individual components.

A certain fluctuation of the carotenoid composition was observed (see Table 4), but systematic studies of this phenomenon were not carried out. As is pointed out later, most of the carotenoids present belong to the same biosynthetic sequence. Some quantitative variation between the individual carotenoids in various batches of cells, is therefore not unexpected.

The total carotenoid content of the bacteria used (see Table 2) was found to be about 0.70 % by weight of the acetone-extracted cells — a value considerably higher than those found in *Athiorhodaceae* spp.^{8,9} but lower than those of some of the giant *Thiorhodaceae* spp.¹⁰

The carotenoids claimed to be present in *Rm. vannielii* in the previous and present investigations are presented in Table 1, and the quantitative composition of the carotenoid complex, determined for 3 different cultivation batches in the present investigation, is set out in Table 4. The carotenoid components were separated on a column of deactivated alumina (see Table 3) and are mentioned below in the order of elution from the chromatographic column.

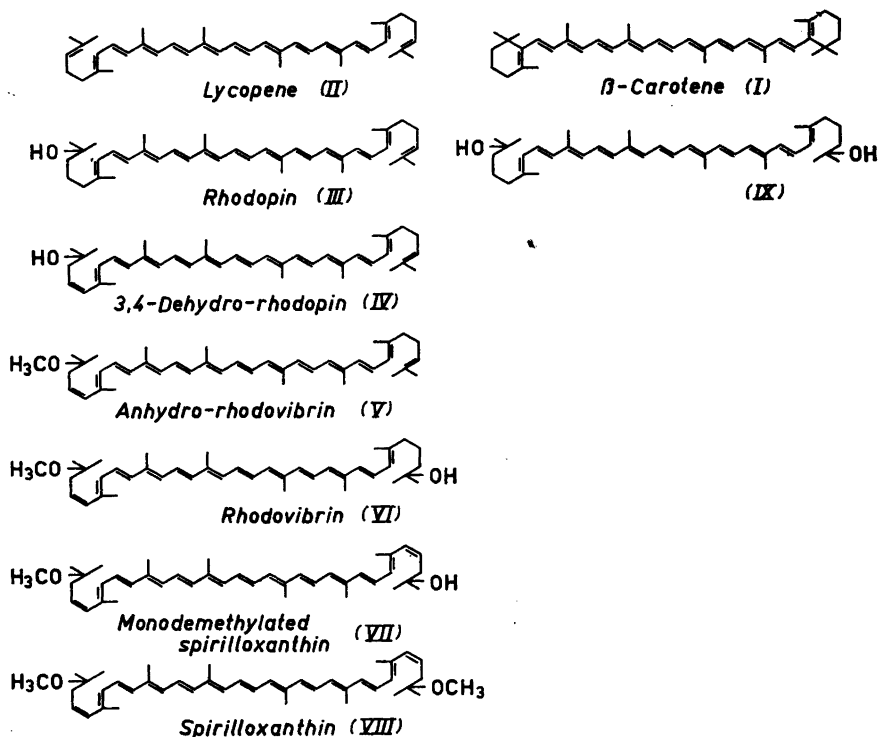
β -Carotene (I) was identified from its absorption spectrum in visible light, co-chromatography tests with synthetic β -carotene and a quantitative comparison of the iodine catalyzed equilibrium mixtures. The aromatic carotenoid

Table 1. Distribution pattern of carotenoids in *Rm. vannielii* according to various investigations.

Carotenoid	Volk and Pennington ²	Conti and Benedict ³	Present investigation
β -Carotene	+	+	+
Lycopene	—	+	+
Anhydro-rhodovibrin (P481)	—	+	+
Spirilloxanthin	+	+	+
Rhodopin	+	+*	+
Rhodovibrin	+	+	+
Monodemethylated spirilloxanthin	—	+	+
Di-OH-432	—	—	+
1,2,1',2'-Tetrahydro-1,1'-di-hydroxy-lycopene	—	+**	+

* Erroneously identified as lycioxanthin.

** Erroneously identified as lycophyll.



hydrocarbon isorenieratene¹¹ is known to exhibit the same absorption spectrum as β -carotene in visible light.¹² However, a direct comparison with isorenieratene in the system here used, revealed different adsorptive properties. Isorenieratene was not detected in *Rm. vanniellii*, and the result supports the previously claimed presence of β -carotene.^{2,3} It is, however, a minor constituent (0–2 % of the total carotenoid).

Lycopene (II) was isolated for the first time in the crystalline state from a photosynthetic bacterium. Its identity was proved by mixed melting point determinations, comparison of absorption spectra in visible light and IR (see Fig. 1) and co-chromatography tests with synthetic lycopene.

Anhydro-rhodovibrin (P481) (V)⁸ was identified from its absorption spectrum and co-chromatography tests of the main stereoisomers with those of authentic anhydro-rhodovibrin.

Crystalline spirilloxanthin (VIII)^{8,13,14} was obtained. Partition ratio, mixed melting point determination, absorption spectra in visible light and IR and co-chromatography tests revealed identity with synthetic spirilloxanthin.

The mono-hydroxy derivative of lycopene was identified with rhodopin^{14b} by co-chromatography tests, and by comparison of partition ratios and absorption spectra in visible light with those previously reported for rhodopin

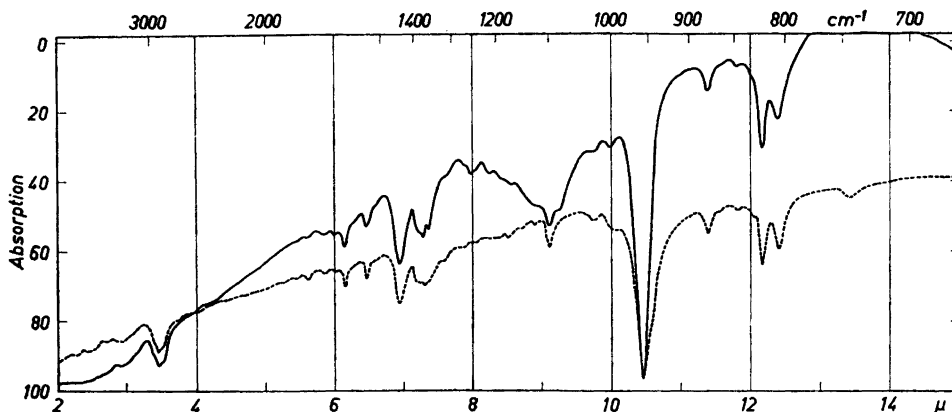


Fig 1. IR-spectrum of — lycopene from *Rh. vannielii* (1.17 mg in 0.6 g KBr) and --- synthetic lycopene (1.18 mg in 0.6 g KBr)

(III).⁸ The melting point and extinction coefficient observed indicate a higher purity than that previously obtained for this carotenoid. The NMR-spectrum recorded at 100 Mc/sec, presented in Fig. 2, shows a doublet at 8.32–8.39 τ (ca. 6 protons) characteristic of an isopropylidene end-group and a sharp

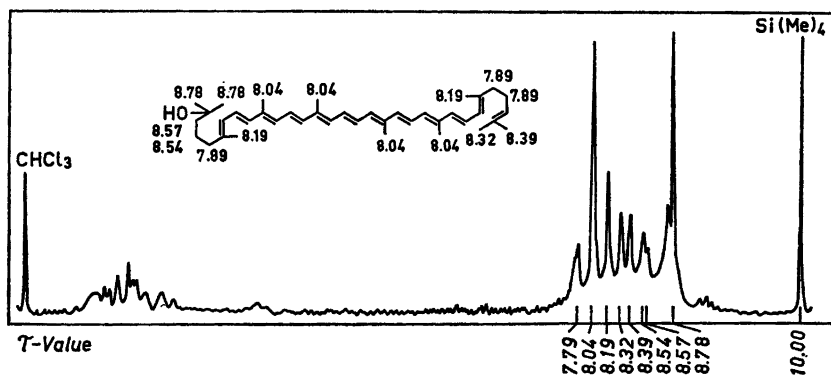


Fig. 2. Proton resonance spectrum of 2.5 mg rhodopin in 0.15 ml CDCl_3 , recorded at 100 Mc/sec.

signal at 8.78 τ expected for the 6 methyl protons of the hydroxylated end-group. There are in chain methyl groups (8.04 τ), end-of-chain methyl groups (ca. 6 protons at 8.19 τ) allylic methylene groups (ca. 6 protons at 7.89 τ) and finally two other methylene groups (ca. 4 protons at 8.54–8.57 τ).^{15,16} These data confirm the structure (III) suggested for rhodopin elsewhere.⁸ The claimed presence of lycoxanthin³ (3-hydroxy-lycopene),¹⁷ could not be confirmed. The carotenoid isolated by Conti and Benedict was no doubt rhodopin (III).

The OH-P481 compound present proved to be rhodovibrin^{8b} by co-chromatography tests with the *trans*, neo A and neo B stereoisomers of authentic rhodovibrin (VI).^{17b,8} 3,4-Dehydro-rhodopin (IV)¹⁸ was not detected in our material.

Monodemethylated spirilloxanthin was also isolated in small amount and identified by its absorption spectrum in visible light, partition ratio and adsorptive properties, comprising co-chromatography tests with an authentic sample.

Two di-hydroxy-carotenoids were present in small quantity. The minor one, referred to as di-OH-434 from the position of the main absorption band in petroleum ether, is characterized by absorption spectra in two solvents, partition ratios and R_F -values. An acetylation test was negative, indicating the presence of only tertiary hydroxyl groups.

The major di-hydroxy-derivative (1–6% of the total carotenoid) was obtained in mixed crystals with a colourless contaminant. The absorption spectrum of the *trans* compound in petroleum ether presented in Fig. 3, conforms that

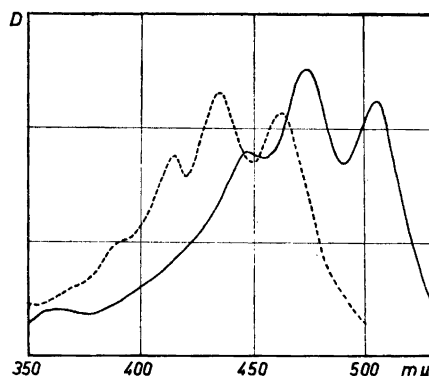


Fig. 3. Absorption spectrum in visible light of — natural *trans* (IX) and of — — Pigment X in pet.ether

of lycopene, indicating the presence of 11 conjugated double bonds in an aliphatic chain. The adsorptive properties and partition ratios compared to those of other di-hydroxy-carotenoids⁸ reveal the presence of 2 hydroxyl groups, none of which seemed to be allylic as judged by the behaviour towards acid chloroform. Repeated attempts of acetylation under conditions where lutein yielded a diacetate in quantitative yield, gave no acetates, strongly suggesting the presence of two tertiary hydroxyl groups. This is supported by the weaker adsorptive properties of this carotenoid compared with those of Pigment 3 from *Chromatium warmingii*,¹⁹ assumed to contain 11 conjugated double bonds, one tertiary and one secondary hydroxyl group.

These data and the occurrence together with lycopene (I) and rhodopin (III), lead to the suggestion of structure (IX) for this carotenoid as 1,2,1',2'-tetrahydro-1,1'-dihydroxy-lycopene. This pigment is presumably identical with the compound erroneously identified by Conti and Benedict³ as lycophyll (3,3'-dihydroxy-lycopene).¹⁷ Lycophyll was not present. Natural (IX) was further characterized by the quantitative composition of the iodine catalyzed equilibrium mixture, see Table 5.

Natural (IX) yielded easily and in high yield a yellow decomposition product, here referred to as Pigment X. The production of Pigment X from *trans* (IX) seemed to be acid-catalyzed. The absorption spectrum of Pigment X, presented in Fig. 3, with absorption maxima at 415, 434, and 462 $m\mu$ in petroleum ether, is indicative of the presence of 9 conjugated double bonds. Partition tests and co-chromatography tests with neurosporene and chloroxanthin revealed the absence of hydroxyl groups in Pigment X, and the presence of some slightly polar substituent(s), possibly oxygen-containing rings.

At this stage Surmatis and Ofner¹⁴ reported a total synthesis of 1,2,1',2'-tetrahydro-1,1'-di-OH-lycopene (IX), and a direct comparison became possible through the courtesy of Dr. J. Surmatis. The *trans* isomers of the natural and synthetic samples could not be separated by chromatography on various papers, and the composition of the iodine catalyzed equilibrium mixtures was found to be identical, as seen from Table 5. Both samples showed the same partition ratios, and on treatment with acid chloroform the synthetic specimen also yielded Pigment X, described above, in high yield. These identity criteria definitely prove the structure (IX) for the bacterial carotenoid.

Di-OH-lycopene-like carotenoids have been reported as minor constituents of the carotenoid complex of *Rhodospirillum rubrum*.²⁰ *Rhodopseudomonas palustris*,⁸ *Chromatium* strain D²¹ and *Chromatium vinosum*.¹⁰ Structure (IX) has been tentatively suggested for these carotenoids.⁸ Owing to lack of material, no direct comparison has so far been carried out, but this structure is plausible in view of the similarity of the accompanying carotenoids in *Rm. vannielii* and the photosynthetic purple bacteria mentioned above.

In summary, it can be concluded that *Rm. vannielii* synthesizes mainly the carotenoids of the so-called normal spirilloxanthin series²² (comprising lycopene, rhodopin, 3,4-dehydro-rhodopin, anhydro-rhodovibrin, rhodovibrin, monodemethylated spirilloxanthin and spirilloxanthin). This pigment complex is typical of several photosynthetic bacteria²² and suggests a biosynthetic pathway analogous to that established for these carotenoids in *Rhodospirillum rubrum*^{20,23,8} and *Chromatium* strain D.²¹ *Rm. vannielii* is characterized by a high content of hydroxylated carotenoids, and the major components exhibit the lycopene chromophore.

In addition, *Rm. vannielii* is capable of a very limited synthesis of the bicyclic β -carotene. It would be of interest to establish whether this occurs *via* the same biosynthetic route as in algae and higher plants, or by an alternative pathway.

It should perhaps be mentioned that the green photosynthetic bacteria (family *Chlorobacteriaceae*) synthesize as their major carotenoid an aromatic carotenoid hydrocarbon,²⁴ and are from a carotenoid and chlorophyll²⁵ standpoint more different from the other photosynthetic bacteria than is *Rm. vannielii*.*

* Note added in proof. Based on metabolic studies van Niel (in Gest, H., San Pietro, A. and Vernon, L. P.: *Bacterial Photosynthesis*, Antioch Press, Yellow Springs, Ohio 1963, p. 461) has recently advanced arguments in favour of *Rm. vannielii* belonging to the family *Thiorhodaceae*.

EXPERIMENTAL

Materials and methods have been described in an earlier paper of this series.²⁶ Circular paper chromatography was performed on Schleicher and Schüll No. 287 paper (kieselguhr paper)²⁷ or No. 288 (aluminium oxide-paper).²⁸ For co-chromatography tests the 3-divided-paper technique was used.²⁹ Partition tests were performed according to the procedure of Petracek and Zechmeister.³⁰ Iodine catalyzed stereoisomerization was carried out as described elsewhere,⁸ in petroleum ether or benzene solution. The terms % III/II and % D_B/D_{II} used for the description of spectral characteristics have been defined previously.⁸

Culture. *Rhodocrobium vannielii*, obtained from Dr. H.C. Douglas, Department of Microbiology, University of Washington, Seattle, Washington, was used.

Medium and cultural conditions. Sterilized medium, prepared as reported by Duchow and Douglas¹ was used. The subinoculum was grown in a glass-stoppered bottle of 150 ml capacity, completely filled with medium, and after inoculation from an agar culture incubated in a light cabinet at 25°C for 6 days. The inoculum was grown for 4 days in the same manner in a bottle of 1 l capacity after inoculation with 50 ml of the subinoculum.

Mass cultures were grown in 5 l carboys using 250 ml of inoculum for each carboy. The cultivation device has been described elsewhere.⁸ In total, three batches of 25 l were grown. For batch I, stirring and anaerobic conditions were maintained by a stream of pure nitrogen; the pH was occasionally adjusted to 7.0 with sterile phosphoric acid. In batches II and III, the carboys were completely filled with medium, and no stirring of the culture was provided. The pH did not rise beyond 7.2 during the cultivation. Further details are specified in Table 2. For batch II a parallel determination of the growth curve indicated that the stationary phase of growth was reached after 9 days.

Harvesting of the cells. Batches I and II were harvested by centrifugation in a Sharples centrifuge (ca. 10 000 g). Batch III was harvested by a modification of the procedure previously described for *Rhodospseudomonas gelatinosa*.²⁸ On treatment with aqueous, saturated MgSO₄-solution and acetone 1.5 l of cell concentrate was obtained. This slurry was centrifuged on a Servall centrifuge (5800 g).

Pigment extraction, saponification and chromatographic separation. The pigments were extracted at room temperature with acetone-methanol (1:1) followed by successive portions of pure acetone and a final treatment with CS₂. After application of the solvent, the suspension was stirred well and centrifuged on the Servall centrifuge.

The saponification and chromatographic procedures were carried out as previously described,^{8,9} except that the unsaponifiable matter was transferred to peroxide-free ether. Particulars of the deactivated alumina chromatogram are given in Table 3. The total carotenoid content of the cells is reported in Table 2, and the composition of the carotenoid mixture in Table 4.

β-Carotene (I)

from zone a (batch II), exhibited after re-chromatography on deactivated alumina abs. max. at 450 and 480 mμ in pet.ether. The spectral curve was indistinguishable from that of synthetic β-carotene. Upon co-chromatography on aluminium oxide-paper a single zone, $R_F=0.53$, was obtained, using pet.ether as developer.

On separate iodine catalyses in pet.ether⁸ of the two β-carotene samples a similar spectral change was produced with 15 % extinction drop of the main maximum and shift of abs. max. to 449.5 and 475 mμ. Subsequent paper-chromatographic examination of the iodine catalyzed equilibrium mixtures on aluminium oxide-paper showed a separation into two zones ($R_F=0.60$ and $R_F=0.53$) in each case, using pet.ether as developer. The former zone contained 17 % and 19 %, and the latter 83 % and 81 % of the total carotenoid, for β-carotene from *Rm. vannielii* and synthetic β-carotene, respectively.

Chromatographic separation of synthetic samples of *trans* β-carotene and *trans* isorenieratene was easily achieved on aluminium oxide-paper using pet.ether as developer, the latter carotenoid being more strongly retained. On iodine catalysis in pet.ether solution similar spectral shifts were observed. However, co-chromatography on circular paper, of the iodine catalyzed equilibrium mixtures in the above system gave satisfactory separation of the two stereoisomerization mixtures.

Table 2. Mass cultivation of *Rm. vannielii*.

	Batch		
	I	II	III
Age of the culture in days	8	12	8
Yield of acetone-extracted cells in grams	17.7	8.7	—
Carotenoids in % of acetone-extracted cells	0.72	0.67	—
Carotenoids in mg/l culture	6.1	2.2	2.7

Table 3. Chromatographic separation of the carotenoids of *Rm. vannielii* on deactivated alumina.

Zone in order of increasing adsorption	Colour of the zone	Required eluant in petroleum ether	Identification
a	yellow	0 % acetone	β -carotene
b	orange	2–3 % acetone	lycopene
c	orange-red	4 % acetone	anhydro-rhodovibrin
d	purple	5 % acetone	spirilloxanthin
e	orange-yellow	10–12 % acetone	rhodopin
f	orange-red	12–14 % acetone	rhodovibrin
g	orange	25 % acetone	monodemethylated spirilloxanthin di-OH-434
h	orange-yellow	35 % acetone	1,2,1',2'-tetrahydro-1,1'-dihydroxylycopene

Table 4. Composition of the carotenoid mixture in three batches of *Rm. vannielii*.

Carotenoid	% of total carotenoid		
	I	II	III
β -Carotene	2.1	1.1	0
Lycopene	24.5	7.2	9.3
Anhydro-rhodovibrin	0	0	0.9
Spirilloxanthin	7.7	12.1	5.7
Rhodopin	65.0	80.6	75.2
Rhodovibrin	0	0	2.0
Monodemethylated spirilloxanthin	0	0	0.3
Di-OH-434	0	0	0.9
1,2,1',2'-Tetrahydro-1,1'-di-OH-lycopene	0.8	trace	5.7

Lycopene (II)

from zone b crystallized from pet.ether as the *trans* isomer as dark red needles, forming rosettes; yield 4.1 mg from batch I. Partition ratio found: Pet.ether/95 % methanol 100:0. The following melting points were observed: Synthetic lycopene 168°C, lycopene from the present source 163°C and mixed m.p. 166–167°C. Abs.max. in pet.ether were located at 445, 474 ($E_{1\text{ cm}}^{1\%} = 2940$) and 505 $m\mu$ and the shape of the absorption curve was indistinguishable from that of synthetic lycopene. The IR-spectrum recorded in KBr, is presented in Fig. 1 together with that of synthetic lycopene. On co-chromatography with synthetic lycopene on aluminium oxide-paper a single zone, $R_F = 0.72$ was obtained, using 2 % acetone-pet.ether as developer.

Anhydro-rhodovibrin (V)

from zone c, had after paper-chromatographic purification abs. max. at 460, 485 and 520 $m\mu$ in acetone. The spectrum exhibited pronounced fine-structure. On aluminium oxide-paper the main stereoisomers had the following R_F -values: *trans* (0.49), neo A (0.54) and neo B (0.76) using 2 % acetone-pet.ether as solvent. On a mixed chromatogram with stereoisomerized anhydro-rhodovibrin from *Chromatium vinosum*,¹⁰ the three stereoisomers could not be separated on the above paper using the same developer.

Spirilloxanthin (VIII)

from zone d crystallized as the *trans* isomer in violet flakes from 8 % acetone-pet.ether and as violet needles on further concentration of the mother liquor; yield 2.3 mg from batch I. Partition ratio found: Pet.ether/95 % methanol 84:16. The following melting points were observed: Spirilloxanthin from *Rm. vannielii* 213–215°C, synthetic spirilloxanthin 216°C and mixed m.p. 213–215°C. Abs.max. in acetone were located at 470, 497, and 530 $m\mu$. The spectrum showed complete agreement with that of synthetic spirilloxanthin. The IR-spectrum, recorded in a KBr-disc, had major absorption bands at 3400, 2900, 1620, 1540, 1440, 1390 – 1375 – 1360 (triplet), 1220, 1180, 1130, 1080 (OMe), 1025, 1000, 960, 890, 823, and 730 cm^{-1} , and conformed to that of synthetic spirilloxanthin. On co-chromatography with synthetic spirilloxanthin on aluminium oxide-paper a single zone $R_F = 0.73$ was obtained, using 10 % acetone-pet.ether as developer.

Rhodopin (III)

from zone e crystallized as the *trans* isomer from acetone-pet.ether; yield 28.7 mg from batch I, m.p. 172–174°C. Abs.max. in acetone were located at 445, 472 ($E_{1\text{ cm}}^{1\%} = 2980$) and 504 $m\mu$. The IR-spectrum had major absorption bands at 3450, 2900, 1625, 1590, 1440, 1375, 1360, 1140, 958, 905, 880, and 820 cm^{-1} as measured in a KBr-disc. The NMR-spectrum of 2.5 mg rhodopin in 0.15 ml CDCl_3 at 100 Mc/sec is presented in Fig. 2. Partition ratio found: Pet.ether/95 % methanol 76:24.

On co-chromatography with *trans* rhodopin from *Thiospirillum jenense*¹⁰ a single zone was obtained ($R_F = 0.80$ on kieselguhrpaper and $R_F = 0.54$ an aluminium oxide-paper) using 10 % acetone-pet.ether as the solvent.

Rhodovibrin (IV)

from zone f was resolved into three zones on aluminium oxide-paper with 10 % acetone-pet.ether as developer ($R_F = 0.40, 0.52$ and 0.60); these could not be separated from the three main stereoisomers (*trans*, neo A, and neo B) of rhodovibrin from *Rhodospseudomonas palustris* strain 5.⁸ The *trans* isomer had abs.max. at 460, 488, and 521 $m\mu$ in acetone.

Monodemethylated spirilloxanthin (VII)]

from zone g, was deposited from the chromatographic fraction and had after paper-chromatographic purification abs.max. at 468, 497, and 531 $m\mu$ in acetone. Partition ratio found: Pet.ether/95 % methanol 58:42.

On co-chromatography on aluminium oxide-paper of the three main stereoisomers of this pigment with those of monodemethylated spirilloxanthin from anaerobically grown *Rhodospseudomonas gelatinosa*,³¹ no separation of the corresponding stereoisomers was achieved using 10 % acetone-pet.ether as developer ($R_F=0.32, 0.40$ and 0.49).

Di-OH-434

from zone g, had after re-chromatography on deactivated alumina and paper-chromatographic purification abs.max. at 412, 434, and 462 $m\mu$ in pet.ether, and at 413, 435, and 464 $m\mu$ in acetone, % III/II=40 in both solvents. On kieselguhr-paper the *trans* compound had $R_F=0.55$ and on aluminium oxide-paper $R_F=0.50$ with 10 % acetone-pet.ether as developer. Partition ratio found; Pet.ether/95 % methanol 25:75 and pet.ether/85 % methanol 75:25.

On acetylation (55 μg di-OH-434, spectrophotometrically determined) according to the procedure described below, 70 % of the pigment was recovered. The reaction mixture contained unchanged di-OH-434 and some decomposition product, more strongly adsorbed on deactivated alumina.

Natural 1,2,1',2'-tetrahydro-1,1'-dihydroxy-lycopene (IX)

Crystallization. *Trans* (IX) from zone h co-crystallized with a colourless contaminant from dry acetone-pet.ether as needles, m.p. 180–182°C; yield 1.36 mg from batch III.

Solubility. The crystalline sample was insoluble in cold pet. ether, moderately soluble in benzene and fairly readily soluble in acetone and CS_2 .

Absorption spectrum in visible light of the *trans* carotenoid had abs.max. in pet.ether at 363, 446, 473, and 504 $m\mu$ in acetone at 365, 448, 474, and 506.5 $m\mu$ in benzene at 458, 485, and 520 $m\mu$ and in CS_2 at 479, 506, and 543 $m\mu$. The spectrum recorded in pet. ether is presented in Fig. 3. The mixed crystals above had $E_{1\text{cm}}^{1\%}=1585$ at 474 $m\mu$ in acetone.

Quantitative partition test. Found for pet.ether/95 % methanol 5:95 and for pet. ether/85 % methanol 63:37.

Test for allylic hydroxyl groups, (see Refs.^{8,29}) gave no product with prolonged chromophore. Pigment X (see below) was produced in varying amounts.

Stereochemical studies. The composition of the equilibrium mixture obtained on iodine catalysis in benzene solution was determined according to the method previously described.⁸ The result is presented in Table 5. The equilibrium was reached after 3 h in diffuse light. The maxima had then shifted from 458, 485, and 520 $m\mu$ to 352, 370, 455, 481,

Table 5. Composition of the iodine catalyzed equilibrium mixtures in benzene of natural and synthetic 1,2,1',2'-tetrahydro-1,1'-di-OH-lycopene (IX).

Sample	Member of the stereoisomeric set	R_F -value		Abs.max. in $m\mu$ in acetone				% of total	
		kieselguhr-paper	10 % acetone-pet.ether						
Natural (IX)	neo B	0.49		345	361	438	465	496	49
	neo A	0.32		345	361	440	467	498	12
	<i>trans</i>	0.28				445	474	506	38
Synthetic (IX)	neo B	0.51		345	360	440	464	494	52
	neo A	0.33		345	360	442	468	498	14
	<i>trans</i>	0.28				445	474	506	34

and 512 μ and a drop of 28 % in extinction coefficient for the main maximum was observed. Prolonged catalysis resulted in irreversible production of Pigment X (see below). The true nature of the neo A and neo B isomers as members of the stereoisomeric set of (IX) was proved by reversible isomerization in diffuse daylight for 2 h, followed by paper-chromatographic examination.

Acetylation. *Trans* (IX) (0.28 mg spectrophotometrically determined), was dissolved in dry pyridine (1 ml) and acetic anhydride (0.2 ml) added. The mixture was kept at room temperature for 18 h, and the pigments were transferred to ether in the usual manner;⁹ pigment recovery 98 %. The composition of the reaction mixture was 9 % Pigment X (see below) and 91 % *cis* and *trans* (IX); these values were established by column chromatography on deactivated alumina followed by paper-chromatographic examination of the column fractions.

In two other experiments Pigment X constituted 28 % and 45 % respectively of the recovered carotenoid, the rest being *cis* and *trans* (IX).

The small amounts of compound (IX) recovered from the acetylation tests were pooled and crystallized; yield *ca.* 0.2 mg. As judged by the IR-spectrum of this specimen and that of the mixed crystals described above, the white contaminant was still present.

A parallel acetylation experiment with lutein (0.19 mg) gave 95 % pigment recovery and lutein diacetate in quantitative yield. Lutein diacetate had *abs.max.* at 425, 448, and 476 μ in *pet.ether* and partition ratio *pet.ether*/95 % *methanol* 98:2

*Co-chromatography with Pigment 3 from Chromatium warmingii*¹⁰ on kieselguhr-paper with 10 % *acetone-pet.ether* as developer gave a clear separation: *Trans* natural (IX) $R_F=0.30$ and *trans* Pigment 3 $R_F=0.20$.

Co-chromatography with synthetic (IX). There was no separation of the *trans* isomers on kieselguhr-paper ($R_F=0.28$; 10 % *acetone-pet.ether*) nor on aluminium oxide-paper ($R_F=0.37$; 20 % *acetone-pet.ether*). Also the neo A and neo B isomers from the iodine catalyzed mixtures were chromatographically inseparable as is evident from the data given in Table 5.

Pigment X was produced from *trans* (IX) during repeated chromatography on deactivated alumina, and formed as a byproduct during prolonged iodine catalysis, acetylation, and treatment with acid CHCl_3 , at times in considerable yield.

Pigment X had *abs.max.* at 415, 434, and 462 μ in *pet.ether*, and *abs.max.* 417, 439, and 468 μ in *acetone*. The spectrum is presented in Fig. 1. Pigment X required 2 % *acetone-pet.ether* for elution from deactivated alumina and exhibited on kieselguhr-paper $R_F=0.47$ (*pet.ether*), $R_F=0.78$ (1 % *acetone-pet.ether*) and on aluminium oxide-paper $R_F=0.77$ (2 % *acetone-pet.ether*). Pigment X produced from natural (IX) could not be separated from Pigment X obtained from synthetic (IX) (see below). On co-chromatography on kieselguhr-paper (1 % *acetone-pet.ether*) of the *trans* isomers one single lemon yellow zone $R_F=0.77$ was observed. On co-chromatography with (a) neurosporene and (b) chloroxanthin, from *Rhodospseudomonas gelatinosa*,³¹ on kieselguhr-paper (*pet.ether* alone and 2 % *acetone-pet.ether*), Pigment X showed intermediate adsorption properties.

The following partition ratio was found: *Pet.ether*/95 % *methanol* 95:5. As judged by the absorption spectrum. R_F -values and partition ratio, Pigment X was unaltered on acetylation by the procedure described above.

Synthetic 1,2,1',2'-tetrahydro-1,1'-di-OH-lycopene (IX)

m.p. 193°C, had *abs.max.* at 446, 472.5, and 504.5 μ in *pet.ether*, 446, 474, and 506 μ in *acetone*, 458, 485, and 518 μ in CHCl_3 , 458, 486, and 521 μ in *benzene* and 475, 503, and 541 μ in CS_2 .

The crystalline compound was poorly soluble in *pet.ether*, moderately soluble in *acetone* and *benzene* and fairly readily soluble in CHCl_3 and CS_2 .

In the system *pet.ether*/85 % *methanol* the partition ratio 65:35 was found.

On iodine catalysis of *trans* (IX) in *benzene* solution the *abs.max.* shifted to 352, 370, 455, 482, and 512 μ % $D_B/D_{II}=19$, % $III/II=38$. The extinction coefficient of the main maximum dropped to 73 % of the initial value. The composition of the iodine catalyzed equilibrium mixture, determined as for natural (IX) above, is presented in Table 5.

After treatment of 0.95 mg (IX) in 3 ml CHCl_3 with 0.3 ml of a saturated $\text{HCl}-\text{CHCl}_3$ solution for 15 min in diffuse daylight according to the procedure used for elimination of allylic hydroxyl groups,²⁶ a pigment recovery of 75 % was determined in the deacidified reaction mixture. The reaction mixture contained exclusively Pigment X; abs. max. 415, 439, and 469 μ in acetone, $R_F=0.79$ on kieselghur paper (1 % acetone-pet. ether).

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