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Isolation and Characterization of Carotenoid Pigments of *Micrococcus roseus*

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In addition to canthaxanthin, seven pigment fractions were isolated from *Micrococcus roseus*. They were purified by solvent partitioning and by column and thin-layer chromatography. Visible absorption spectra, chromatographic behavior, and partition coefficients of the pigments and derivatives prepared from the pigments were used in characterizing them. Both α- and β-carotene derivatives were present. The structure of one pigment was suggested as phoenicoxanthin (3-hydroxy-4,4'-diketo-β-carotene). Four other pigments were tentatively characterized as a dihydroxy-3,4-dehydro-α-carotene, a dihydroxy-α-carotene, a diketo-α-carotene, and a polyhydroxy-β-carotene. Two pigments were isolated in trace amounts and could not be characterized. All the pigments studied were isolated as mixtures of *cis-trans* isomers and all except the diketo-α-carotene were isolated as esters from *M. roseus*. Quantitation of the pigments showed that canthaxanthin (4,4'-diketo-β-carotene) represented 85% of the pigment recovered from extracts. Three of the other pigments contributed a significant proportion of the remaining pigments, whereas the other four were present in only small amounts. β-Carotene derivatives comprised 96% and α-carotene derivatives 4% of the pigments recovered from extracts.

Carotenoid pigments occur in a variety of microorganisms. *Micrococcus roseus* produces red-orange pigments when cultured on laboratory media. Like many other gram-positive cocci, *M. roseus* owes its color to carotenoids. The principal colored carotenoid of this organism has been identified as canthaxanthin (4,4'-diketo-β-carotene) (4).

The present investigation was undertaken to isolate the remaining pigments of *M. roseus* and subsequently to characterize them.

MATERIALS AND METHODS

M. roseus ATCC 516 was cultured, cells were harvested, and pigments were extracted from cells into methanol as described previously (4). Separation procedures are summarized in Fig. 1. Crude methanol extracts were partitioned against petroleum ether. The (ether) epiphase was dried, concentrated, and applied to columns of Silica Gel G. Fraction A was eluted with diethyl ether and fraction B was eluted with methanol. Details have been published elsewhere (4). The (methanol) hypophase was dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure with a rotary evaporator. The yellow residue was dissolved in a small quantity of methanol and the solution was subjected to thin-layer chromatography (Fig. 1).

Thin-layer chromatography of fractions A and B and of the hypophase yielded nine pigments (Fig. 1). Adsorbents and solvents were chosen after examining a number of systems. $CaCO_3$ was specified as "low in alkalinity." Pigments II and III had been identified as isomers of canthaxanthin (4) and were not examined in the present study except when used as a control for other pigments.

Visible absorption spectra were determined with a Spectronic 600 spectrophotometer (Bausch & Lomb, Rochester, N.Y.). Spectra were taken in hexane, carbon disulfide, and chloroform.

Partition coefficients were determined in a hexane-95% methanol system according to the method of Petracek and Zechmeister (15). Occasionally, a transient turbidity was encountered after partitioning the pigments. Therefore, partitioned solutions were allowed to stand for about 15 min prior to reading the absorbance.

Reduction of keto groups to hydroxyls was attempted by dissolving the pigment in 95% ethyl alcohol; a few crystals of $NaBH_4$ were added and the solution was allowed to stand overnight (11).

Saponification was carried out by dissolving the pigment in methanol; 6% (w/v) KOH was then added and the solution was held at 60 C for 30 min.

The presence of allylic hydroxyl groups was deter-

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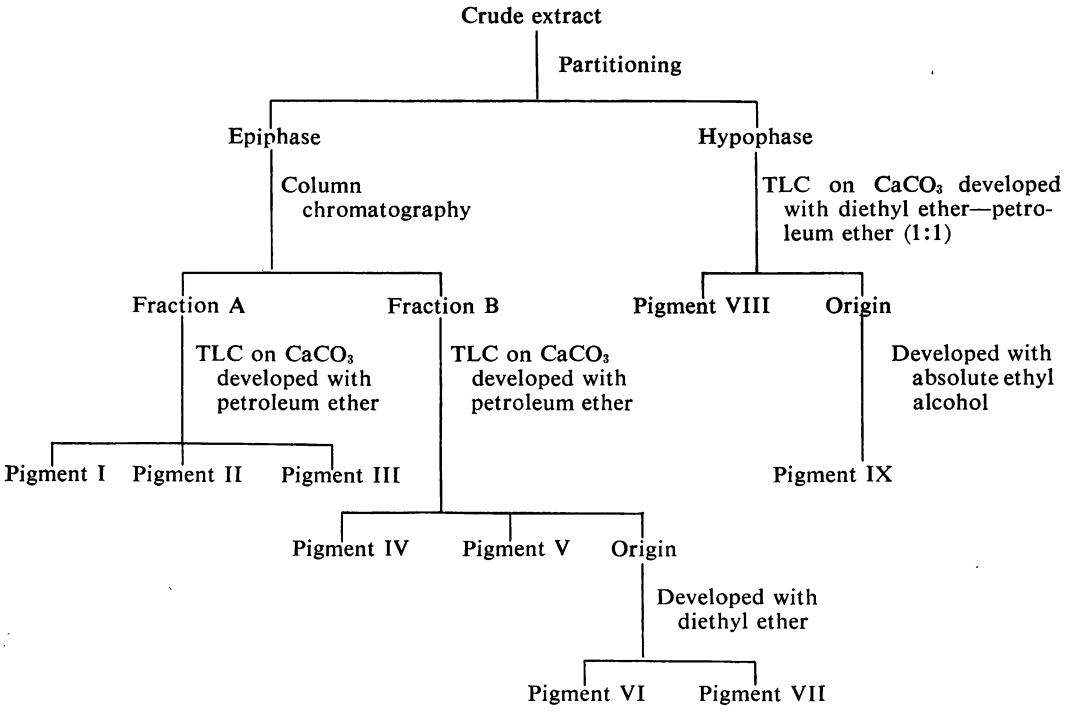

FIG. 1. *Methods used to separate pigments extracted from Micrococcus roseus. Pigments II and III were previously identified (4) and were not examined in the present study. TLC, thin-layer chromatography.*

mined by treating the pigment with acid alcohol (3). Allylic hydroxyls form less polar alkoxyl derivatives. Acetylation of any secondary hydroxyl groups present was carried out by dissolving the pigment in pyridine, adding acetic anhydride (0.01 ml/ml), and incubating the solution in the dark at ambient temperature for 2 hr (3).

Each pigment, dissolved in petroleum ether, was treated with a drop of ethanolic 0.05 N HCl to determine whether epoxide groups were present (10). Each pigment (in hexane) was also treated with 0.001% iodine in hexane to determine its isomeric configuration (10).

The concentration of each pigment was determined as described by Davies (5). An extinction coefficient of 2,505, that of β-carotene, was used at the wavelength maximum for each pigment.

RESULTS AND DISCUSSION

None of the pigments isolated from *M. roseus* had epoxide groups. All pigments studied in the present investigation were isolated as mixtures of *cis* and *trans* isomers.

Pigment I had a slightly asymmetrical absorption spectrum in the visible range (Fig. 2). Wavelength maxima in three solvents are shown in Table 1. The maxima suggested that the pigment had the same number of conjugated double bonds as β-carotene (Fig. 3); however, the lack of fine structure in the absorption spectrum suggested that the pigment had an α-carotene structure (Fig. 3) with the conjugated double bond system extending into the β-ionone ring. Such a 3,4-dehydro structure (Fig. 4) would account for the absorption maxima of pigment I and the reduced fine structure of its spectrum (1, 5).

Reduction yielded no change in polarity (Table 2) or in absorption spectrum, indicating that keto groups were not present. Saponification resulted in a more polar pigment, as indicated by a decreased R_F value and partition coefficient (Table 2). This saponified product was acetylated and the hydroxyl groups were liberated again by saponification. Changes in polarity of the molecule after such treatments (Table 2) indicated the presence of two ester groups on the original molecule.

Tests of the saponified pigment for allylic hydroxyl groups were negative, eliminating carbons 2 and 3′ as the positions of the esters. The most probable locations are carbons 3, 4, and 4′. Pigment I was therefore characterized as a mixture of *cis-trans* isomers of a dihydroxy-3,4, dehydro-α-carotene which was isolated as a diester.

Pigments IV and VI were isolated in such small amounts that no data were obtained other than chromatographic behavior.

Pigment V had a smooth and symmetrical absorption spectrum (Fig. 5); the absorption maxima in three solvents are shown in Table 1. After treatment with $NaBH_4$, the absorption spectrum of the reduced pigment had three peaks (Fig. 5) and the wavelength of the principal peak was 16 mμ lower than the absorption maximum before reduction (Table 3), suggesting that two conjugated keto groups had been reduced. Partition coefficients (Table 3) also indicated the presence of two keto groups on the untreated pigment and two hydroxyl groups on the reduced pigment. Saponification of the reduction product did not change the pigment's polarity (Table 3), indicating that additional (esterified) hydroxyls were lacking.

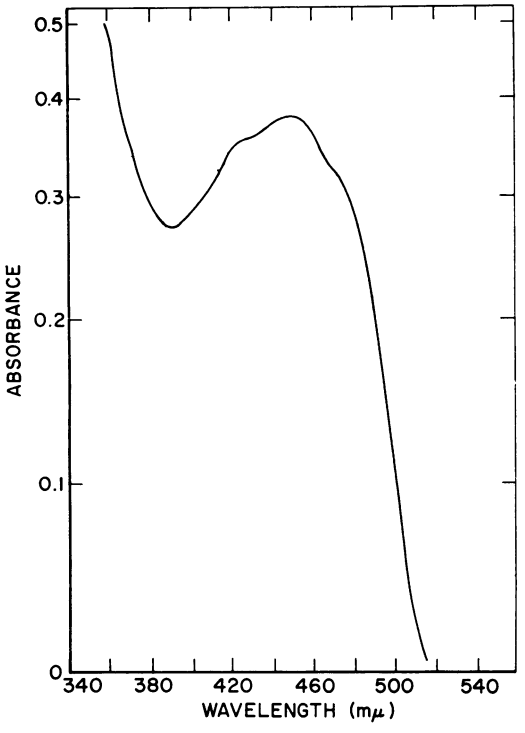

FIG. 2. *Visible absorption spectrum of pigment I from Micrococcus roseus, in hexane.*

After reduction the absorption maximum in hexane was 446 mμ, suggesting that the molecule was an α-carotene derivative. The two keto groups of the untreated pigment must be arranged to extend the conjugated double bond system in such a way as to account for its absorption maxima (Table 1). Therefore, the structure suggested for pigment V is that of 3,4,diketo-α-carotene (Fig. 6), in which both keto groups are in conjugation with the double bond system through the β-ionone ring.

The absorption spectrum of pigment VII was similar in shape to that of pigment I (Fig. 2). Its absorption maxima (Table 1) suggested an α-carotene molecule. The partition coefficient for this pigment (Table 4) indicated the presence of one hydroxyl group plus another less polar functional group. Treatment with $NaBH_4$ did not alter the polarity indicating that keto groups were absent. Saponification greatly increased the polarity of this pigment (Table 4), and the partition coefficient indicated that the product contained two hydroxyl groups (Table 4). Thus, the second functional group was an ester.

No attempt was made to locate these hydroxyl groups due to the small amount of pigment present. Pigment VII was characterized as a mixture of *cis-trans* isomers of a dihydroxy-α-carotene. The pigment was isolated as a monoester from *M. roseus*.

Pigment VIII had a symmetrical spectrum (Fig. 7); the absorption maxima in three solvents are shown in Table 1. The absorption maximum was the same as that of canthaxanthin in CS_2 (4), suggesting that both contain the same number of conjugated double bonds.

TABLE 1. *Absorption maxima of pigment fractions isolated from Micrococcus roseus*

Pigment	Absorption maxima (mμ) in		
	CS_2	Hexane	$CHCl_3$
I	(436–462),[a] 484, (501–520)	(418–430), 451, (469–484)	(420–445), 465, (480–490)
V	500	462	476
VII	(430–450), 475, 512	(420–430), 444, 474	(430–440), 453, (478–493)
VIII	494	467	483
IX	(435–450), 471, (485–502)	(423–434), 451, (471–490)	(423–441), 457, (482)

[a] In this table and in subsequent tables, figures in parentheses indicate a shoulder rather than a distinct peak.

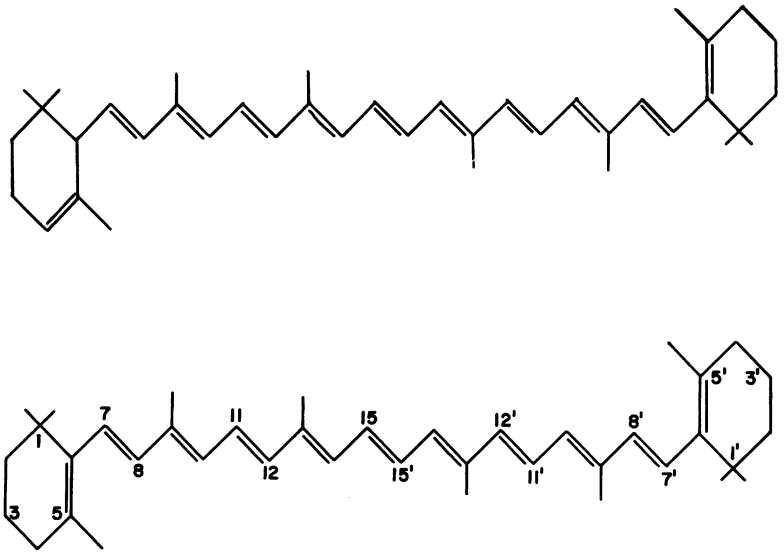

FIG. 3. *Structure of all-trans α-carotene (top) and of all-trans β-carotene (bottom).*

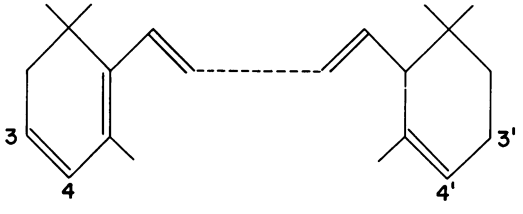

FIG. 4. *Structure of 3,4-dehydro-α-carotene. A suggested structure for pigment I places esterified hydroxyl groups on two of the three positions: 3,4 or 4'.*

TABLE 2. *R_F values and partition coefficients of pigment I and derivatives prepared from pigment I*

Treatment	R_F value[a]	Partition coefficient (% epiphasic)
Untreated	1.0	92–96
Reduction	1.0	92–94
Saponification	0.0	6–10
Acetylation after saponification	1.0	75–82
Saponification after acetylation	0.0	3–8
Acid alcohol after first saponification	0.0	5

[a] $CaCO_3$ plates, petroleum ether solvent.

The partition coefficient (Table 5) suggested that there were two carbonyl groups on the molecule and probably another functional group which contributed slightly to the polarity. Reduction of pigment VIII yielded the typical three-peaked spectrum (Fig. 7) characteristic of a molecule with 11 conjugated double bonds, a β-carotene chromophore. The 18-mμ decrease in wavelength of the principal peak upon reduction suggested that two conjugated double bonds were eliminated by reduction and the product was more polar (Table 5). Treatment of the reduced product of pigment VIII with acid alcohol confirmed that the carbonyl groups which had been reduced to hydroxyl groups were allylic. The reduced molecule could also be acetylated (Table 5), giving further evidence of hydroxyl groups.

Acetylation did not alter the polarity of pigment VIII (Table 5), indicating that free hydroxyl groups were absent. Saponification increased the polarity of the pigment by 25%; this suggested that one hydroxyl group had been liberated from an ester linkage. The position of the OH group liberated by saponification was rather limited: the 4 and 4' positions were occupied by carbonyl groups; the 2 and 2' positions of cyclic carotenoids have as yet not been found in nature to have hydroxyl groups (2); this left either the 3 or 3' position for the hydroxyl group.

The keto carotenoid 3-hydroxy-4,4'-diketo-β-carotene (Fig. 8) has recently been described independently by Egger (6) and Fox and Hopkins (7). Fox and Hopkins, who isolated this pigment from two flamingo species of *Phoenicoparrus* and one of *Phoenicopterus*, named this pigment phoenicoxanthin, whereas Egger, who isolated it from the angiosperm *Adonis annua* L., named it adonirubin. The absorption maxima of phoe-

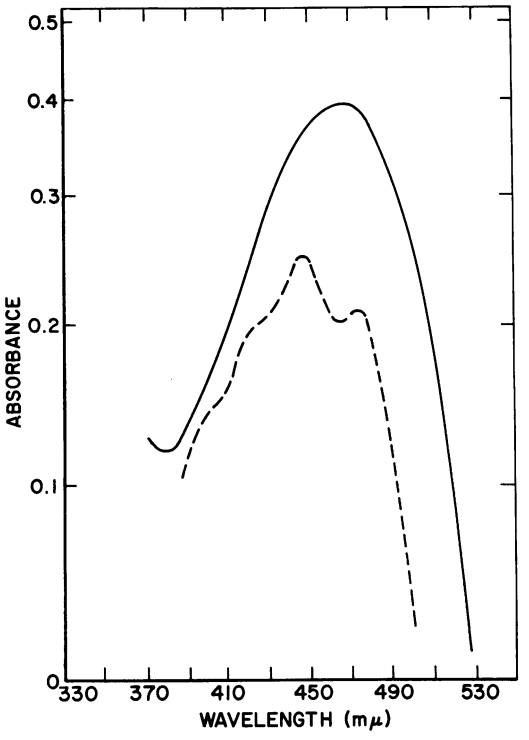

FIG. 5. *Visible absorption spectra of pigment V from Micrococcus roseus and of pigment V after reduction. Solid line, untreated pigment in hexane; dashed line, after reduction, in methanol.*

TABLE 3. *R_F values and partition coefficients of pigment V and derivatives prepared from pigment V*

Treatment	R_F value[a]	Absorption maxima (mμ)[b]	Partition coefficient (% epiphasic)
Untreated	0.2	462	51–57
Reduction	0.0	(418–425), 446, 475	12–17
Saponification after reduction	0.0	(418–425), 446, 475	16–19

[a] $CaCO_3$ plates, petroleum ether solvent.
[b] Taken in hexane.

nicoxanthin and pigment VIII are the same, as are their absorption maxima in hexane after reduction. Fox and Hopkins (7) obtained a partition coefficient of 29% epiphasic for phoenicoxanthin. The partition coefficient of the saponified product of pigment VIII (20 to 21% epiphasic) was close enough to their value to be within the limits of experimental error.

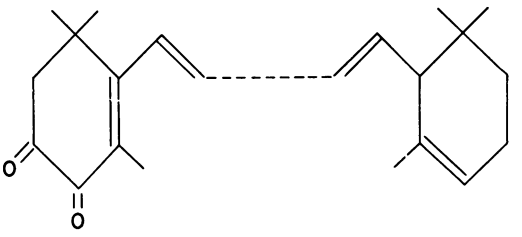

FIG. 6. *Structure of 3,4-diketo-α-carotene, suggested as the structure of pigment V.*

TABLE 4. *R_F values and partition coefficients of pigment VII and derivatives prepared from pigment VII*

Treatment	R_F values	Partition coefficient (% epiphasic)
Untreated	0.0[a]	72–74
	0.2[b]	
Reduction	0.2[b]	72–74
Saponification	0.0[b]	14–15
	1.0[c]	

[a] $CaCO_3$ plates, petroleum ether solvent.
[b] $CaCO_3$ plates, diethyl ether solvent.
[c] $CaCO_3$ plates, absolute ethyl alcohol solvent.

It is suggested that pigment VIII is a *cis-trans* mixture of 3-hydroxy-4,4′-diketo-β-carotene. This pigment is esterified in *M. roseus*. It appears that this is the first report of this pigment in a bacterium.

The most polar pigment found in *M. roseus*, pigment IX, had an absorption spectrum and wavelength maximum identical to β-carotene (Table 1, Fig. 9). The partition coefficient (Table 6) indicated that there were two hydroxyl groups on the molecule. Treatment with acid alcohol showed that these two hydroxyl groups were allylic.

When pigment IX was saponified, its polarity increased. The partition coefficient (Table 6) suggested that at least one, perhaps two, hydroxyl groups had been liberated from ester linkages. Because the two free hydroxyl groups were in the allylic (4 and 4′) positions, the liberated hydroxyl group or groups are most likely located on the 3 or 3′, or both, carbons.

We suggest that pigment IX, which was isolated as an ester from *M. roseus*, is a mixture of *cis-trans* isomers of a polyhydroxy-β-carotene, either 3,4,4′-trihydroxy-β-carotene or 3,3′,4,4′-tetrahydroxy-β-carotene (Fig. 10). Leftwick and Weedon (13) reported the synthesis of the tetrol by reduction of astaxanthin.

Canthaxanthin comprised 85.2% of the pigment content (Table 7). Pigments I, VIII, and IX

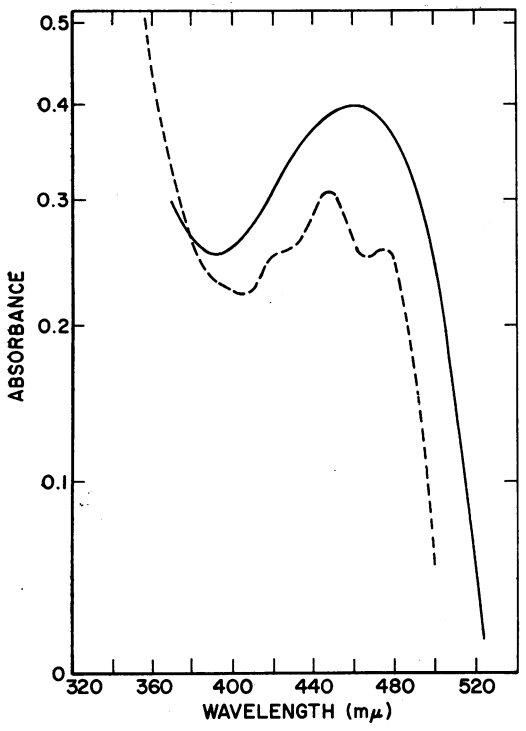

FIG. 7. *Visible absorption spectra of pigment VIII from Micrococcus roseus and of pigment VIII after reduction. Solid line, untreated pigment; dashed line, after reduction. Spectra taken in hexane.*

TABLE 5. *R_F values and partition coefficients of pigment VIII and derivatives prepared from pigment VIII*

Treatment	R_F values[a]	Partition coefficient (% epiphasic)
Untreated	1.0	41–45
Acetylation	1.0	46
Saponification	0.0	20–21
Reduction	0.0	34
Acetylation	1.0	51
Acid alcohol	1.0	86

[a] $CaCO_3$ plates, diethyl ether-petroleum ether (1:1) solvent.

were less prominent but were found in significant amounts. Pigments V and VII were present in small amounts, whereas pigments IV and VI were present in only trace amounts. β-Carotene derivatives comprised 96% of the pigments isolated and α-carotene comprised 4%. The percentages are skewed because the concentrations of pigments IV and VI were not obtained and because the same extinction coefficient was assumed for all pigments.

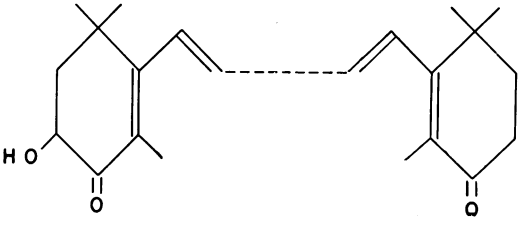

FIG. 8. *Structure of 3-hydroxy-4,4'-diketo-β-carotene, suggested as the structure of pigment VIII.*

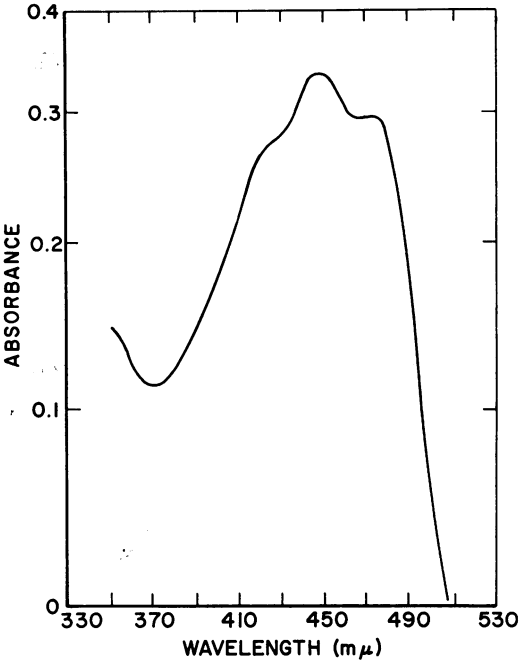

FIG. 9. *Visible absorption spectrum of pigment IX from Micrococcus roseus, in methanol.*

TABLE 6. *R_F values and partition coefficients of pigment IX and derivatives prepared from pigment IX*

Treatment	R_F values	Partition coefficient (% epiphasic)
Untreated	1.0[a]	3–11
Acid alcohol	1.0[b]	68
Saponification	0.0[a]	0

[a] $CaCO_3$ plates, absolute ethyl alcohol solvent.
[b] $CaCO_3$ plates, diethyl ether-petroleum ether (1:1) solvent.

The presence of both α- and β-carotene molecules in *M. roseus* can be easily accounted for by the fact that their biosynthetic pathways are identical until the formation of neurosporene

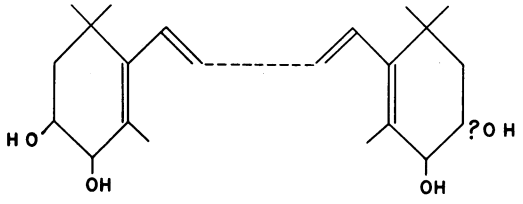

FIG. 10. *Structures(s) suggested for pigment IX, 3,4,4'-trihydroxy-β-carotene or 3,3', 4,4'-tetrahydroxy-β-carotene.*

TABLE 7. *Relative percentages of pigments isolated from Micrococcus roseus*

Pigment	Percentage (w/w) of total carotenoid isolated
I	3.1
II & III	85.2
IV	trace
V	0.6
VI	trace
VII	0.4
VIII	3.5
IX	7.0

(16). At this point, the pathways diverge to form the α- and β-molecules via α- and β-zeaxanthin, respectively (8, 16), although β-carotene could arise via lycopene (16, 18). β-Carotene is not derived from α-carotene (18) and the reverse synthesis has also been ruled out (9, 16). However, insertion of oxygen functions is the last sequence of steps in xanthophyll synthesis. Lee (12) proposed that hydroxyls are added first, followed by dehydrogenation to yield keto compounds. According to this proposal, a dihydroxy-β-carotene molecule would be the precursor for all of the pigments of *M. roseus* which were derivatives of β-carotene (II, III, VIII, IX) and a dihydroxy-α-carotene would be the precursor for all the pigments which are derivatives of α-carotene (I, V, VII).

Isozeaxanthin (4,4'-dihydroxy-β-carotene) would be the obvious precursor for pigments II, III, VIII, and IX, but it has not yet been isolated from *M. roseus*. It may be one of the pigments found in trace quantities (pigments IV or VI). Judged by their chromatographic behavior, these two pigments are of relative polarities to suggest that they may be dihydroxy compounds (5).

Although the characterization of these pigments and some of their derivatives probably describes their chromophores accurately and provides evidence regarding their functional groups, additional data are required to substantiate the proposed structures. Of particular importance will be comparison with authentic pigments via co-chromatography and preparation of iodine-catalyzed isomers. Infrared and nuclear magnetic resonance spectra may also be useful, particularly in determining hydroxyl functions. Moreover, Liaaen-Jensen et al. (14) and Thirkell et al. (17) reported that C-50 carotenoids are common in gram-positive nonphotosynthetic bacteria and can be distinguished from C-40 carotenoids only by mass spectrometry.

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