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THE CAROTENOIDS OF *RHODOMICROBIUM VANNIELII* (RHODOSPIRILLACEAE) AND THE EFFECT OF DIPHENYLAMINE ON THE CAROTENOID COMPOSITION

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Key Word Index—*Rhodomicrobium vannielii*; Rhodospirillaceae; diphenylamine; rhodopin; phytoene; cyclic carotenoids; photosynthetic bacteria; 1′-methoxy-3′,4′-didehydro-1′,2′-dihydro-β,ψ-carotene.

Abstract—The presence of β-carotene, lycopene, rhodopin, rhodovibrin, anhydrorhodovibrin, spirilloxanthin, 'monodemethylated spirilloxanthin' and 'dihydroxylycopene' in anaerobic cultures of *Rhodomicrobium vannielii* has been confirmed and 3,4-didehydrorhodopin and mono- and dimethoxy derivatives of lycopene have been detected for the first time. In addition to β-carotene, two cyclic carotenoids, β-cryptoxanthin and the novel 1′-methoxy-3′,4′-didehydro-1′,2′-dihydro-β,ψ-carotene have been identified. Diphenylamine inhibits formation of the normal main carotenoid, rhodopin and phytoene (the new main carotenoid), phytofluene, 7,8,11,12-tetrahydrolycopene and neurosporene and their monohydroxy derivatives appear. The biosynthesis of carotenoids in *Rm. vannielii* is discussed.

INTRODUCTION

The carotenoids of the purple non-sulphur photosynthetic bacteria of the Rhodospirillaceae [1] (formerly Athiorhodaceae) are characteristically acyclic compounds with tertiary hydroxyl and methoxyl groups at C-1 and C-1′, e.g. rhodovibrin (1′-methoxy-3′,4′-didehydro-1,2,1′,2′-tetrahydro-ψ,ψ-caroten-1-ol, **5**) and spirilloxanthin (1,1′-dimethoxy-3,4,3′,4′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene, **7** [2]. Most investigations of the biosynthesis of carotenoids in the Rhodospirillaceae have used *Rhodospirillum rubrum* or *Rhodopseudomonas* species, and have relied heavily on the use of inhibitors, especially diphenylamine (DPA) [3–12] and, more recently, nicotine [13, 14]. Much of the available information about spirilloxanthin biosynthesis in *R. rubrum* has been obtained from experiments in which DPA was used. This substance inhibits the desaturation reactions of carotenoid biosynthesis, so that phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene, **14**) accumulates in place of the normal unsaturated carotenoids [3]. A study of the kinetics of disappearance of the more saturated carotenoids and formation of the normal carotenoids on removal of the DPA allowed a scheme to be proposed for the biosynthesis of spirilloxanthin from lycopene (ψ,ψ-carotene, 1) (Fig. 1) [4, 5].

After much confusion and argument about its taxonomic position, *Rhodomicrobium vannielii* is now included in the Rhodospirillaceae [1]. This organism is of interest because, although it produces spirilloxanthin and several possible intermediates in spirilloxanthin biosynthesis [15–17], its main carotenoid is the simple hydroxylated lycopene, rhodopin (1,2-dihydro-ψ,ψ-caroten-1-ol, **2**). It has been proposed [17] that spirilloxanthin is biosynthesized by *Rm. vannielii* by the route (Fig. 1) proposed [4] for *R. rubrum*, but no biochemical evidence has been presented. In addition to the range of acyclic carotenoids, a small amount of β-carotene (β,β-carotene, **8**) occurs in

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✠ Deceased.

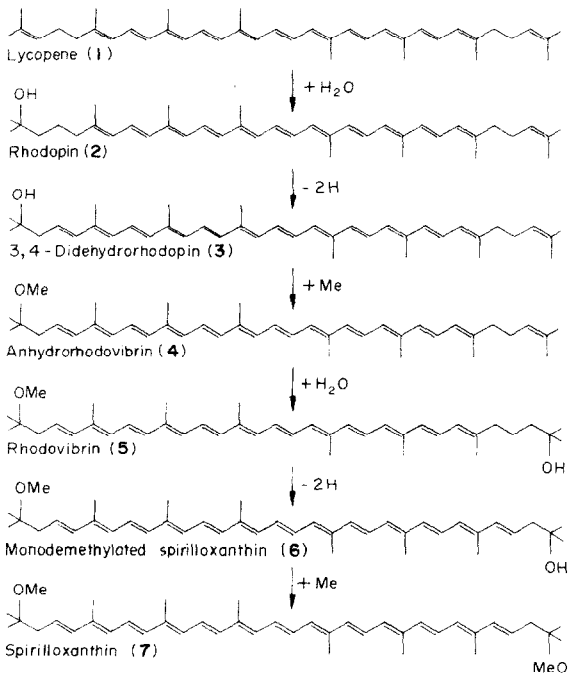
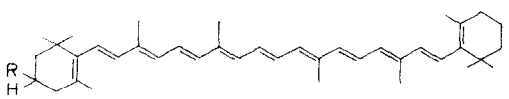


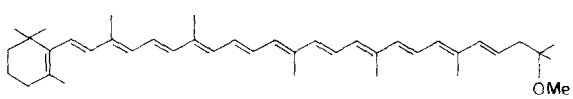
Fig. 1. Postulated pathway for the biosynthesis of spirilloxanthin from lycopene in *Rhodospirillum rubrum*.

Rm. vannielii, the only reported case of the occurrence of an alicyclic carotenoid in a member of the Rhodospirillaceae.

In the present work, which is part of a detailed study of the biosynthesis of carotenoids in the Rhodospirillaceae, the carotenoids of *Rm. vannielii* have been re-examined, and the effect of diphenylamine on the carotenoid composition has been investigated, in an attempt to obtain information about carotenoid biosynthesis in this organism.



(8) R=H (9) R = OH



(10)

RESULTS

The first stage of the present work was a re-examination of the carotenoids of *Rhodomicrobium vannielii* grown anaerobically in the light.

Carotenoid hydrocarbons

Two carotenoid hydrocarbons were isolated. These were identified as β-carotene and lycopene by comparison of their chromatographic properties and absorption and mass spectra with those of authentic samples, and by co-chromatography. The presence of β-carotene and lycopene in extracts of *Rm. vannielii* has been reported by other workers [15–17].

Carotenoids of the spirilloxanthin series

Several members of the spirilloxanthin series that had previously been isolated from *Rm. vannielii* [17] were identified. The main pigment had an absorption spectrum similar to that of lycopene, but had the chromatographic polarity of a monohydroxy compound. From this and its mass spectrum, this compound was identified as rhodopin. Other pigments present were spirilloxanthin, identical (absorption spectrum, MS, chromatographic properties, co-chromatography) to a sample of spirilloxanthin from *Rhodospirillum rubrum*, and smaller amounts of 3,4-didehydrorhodopin (3,4-didehydro-1,2-dihydro-ψ,ψ-caroten-1-ol, **3**), rhodovibrin, anhydrorhodovibrin (1-methoxy-3,4-didehydro-1,2-dihydro-ψ,ψ-carotene, **4**) and 'monodemethylated spirilloxanthin' (1′-methoxy-3,4,3′,4′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-caroten-1-ol, **6**). The mass spectra of rhodopin, rhodovibrin, anhydrorhodovibrin and monodemethylated spirilloxanthin were essentially identical to those illustrated by Enzell *et al.* [18]. An additional carotenoid with an absorption spectrum like that of lycopene, but with the chromatographic polarity of a diol was presumed to be 1,2,1′,2′-tetrahydro-ψ,ψ-carotene-1,1′-diol (**11**), a compound previously detected in extracts of *Rm. vannielii*. [17]. Very little of this substance was available and this gave only a very weak mass spectrum, with the parent ion M^+ at *m/e* 572 ($C_{40}H_{60}O_2$) but no well-defined major fragment ions.

"*Dihydroxy*-434"

In their analysis of the carotenoids of *Rm. vannielii*, Ryvarden and Liaaen-Jensen [17] investigated a compound which they designated "dihydroxy-434" on the basis of its chromatographic and spectroscopic properties (λ_{max} in ethanol at 412, 434 and 462 nm). This substance was also

detected in the present work and was shown by mass spectroscopy to have molecular weight 570, but it has not been characterized.

Methoxylycopenes

Two other carotenoids, isolated in small amounts, were tentatively identified from their chromatographic and spectroscopic properties as mono- and dimethoxy derivatives of lycopene (1-methoxy-1,2-dihydro-ψ,ψ-carotene, **12**, and 1,1′-dimethoxy-1,2,1′,2′-tetrahydro-ψ,ψ-carotene, **13**). Insufficient of the dimethoxy compound was available for MS analysis. The MS of the monomethoxy derivative had the parent ion M^+ at *m/e* 568 ($C_{41}H_{60}O$) but was too weak to permit detailed analysis. These compounds have previously been detected in small amounts in DPA-inhibited cultures of *R. rubrum* [12], and the dimethoxylycopene was identified as the main carotenoid of a photolithotropic bacterium, RG3 [19].

Other cyclic carotenoids

The two most interesting pigments isolated were both identified as cyclic carotenoids. A monohydroxycarotenoid, closely associated with rhodopin, had an absorption spectrum like that of β-carotene but was indistinguishable chromatographically from β-cryptoxanthin (β,β-caroten-1-ol, **9**). The mass spectrum also was identical to that of β-cryptoxanthin, in particular the ratio of the intensities of the M-92 and M-106 peaks was typical of a bicyclic carotenoid [20], and no fragment ions were observed that could be ascribed to losses of 56 or 69 m.u., confirming the absence of ϵ-ring or acyclic (lycopene) end groups. The identification of this compound as β-cryptoxanthin was confirmed by the formation of an acetate which was indistinguishable chromatographically from β-cryptoxanthin acetate, and had a mass spectrum virtually identical to that of β-cryptoxanthin acetate.

A compound which chromatographed with "monomethoxylycopene" on silica gel, indicating that it was also a monomethoxy compound, was much less strongly adsorbed than the lycopene derivative on magnesium oxide. This property and its absorption spectrum which had λ_{max} close to those of lycopene and its derivatives, but with a rather less well-defined fine structure, suggested that this compound was monocyclic. The mass spectrum had the parent ion M^+ at *m/e* 566 ($C_{41}H_{58}O$) and the base peak at *m/e* 73. A strong fragment ion at *m/e* 493 (M-73) showed the presence of a 1-methoxy-1,2-dihydro-3,4-didehydro end group as in spirilloxanthin rather than the end group of "methoxylycopene" with the C-3, 4 bond saturated [21]. The absence of major fragment ions due to losses of 69 or 137 m.u. indicated the absence of an unsubstituted acylic end group as in lycopene or neurosporene (7,8-dihydro-ψ,ψ-carotene, **17**). The available evidence was in agreement with structure **10**, 1′-methoxy-3′,4′-didehydro-1′,2′-dihydro-β,ψ-carotene, but the amount of material available was too small to allow confirmation of the monocyclic structure by other methods, e.g. NMR. The structure proposed is intermediate between β-carotene and spirilloxanthin, both of which were present in the extract.

Carotenoids of Rhodomicrobium vannielii *grown anaerobically in the presence of diphenylamine*

Cultures of *Rm. vannielii* grown anaerobically in the light in the presence of DPA were greenish in colour, in contrast to the normal purple-brown. Chromatographic examination of the extract of DPA-grown cultures revealed that very little of the normal unsaturated carotenoids had been synthesized, but large amounts of phytoene accumulated. Phytoene and other carotenoid hydrocarbons were purified and identified by absorption and mass spectroscopy. The absorption and mass spectra of phytoene, phytofluene (7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene, **15**) 7,8,11,12-tetrahydrolycopene(7,8,11,12-tetrahydro-ψ,ψ-carotene, **16**), and neurosporene were identical to those of authentic samples, and in each case this identity was confirmed by co-chromatography. Chromatography of a phytoene sample on a column of activated (Brockmann grade I) neutral alumina showed that the 15-*cis* and all-*trans* isomers were present in a ratio of approximately 2:1 (M. K. Anderson and G. Britton, unpublished results).

Of the more polar carotenoids present, anhydrorhodovibrin, rhodopin, rhodovibrin and spirilloxanthin were found in very small quantities together with other compounds not detected in extracts of normal cultures. In particular a series of monohydroxy compounds chromatographed

with rhodopin on silica gel, but were separated on magnesium oxide. These compounds were identified as monohydroxy derivatives of phytoene (**18**), phytofluene (**19**),7,8,11,12-tetrahydrolycopene and neurosporene from their absorption spectra (identical to those of the parent hydrocarbons) and chromatographic properties. In addition the structures of hydroxy-7,8,11,12-tetrahydrolycopene and hydroxyneurosporene were confirmed by mass spectroscopy. Hydroxytetrahydrolycopene had the parent ion M^+ at *m/e* 558 ($C_{40}H_{62}O$) and fragment ions at *m/e* 540 (M-H_2O) confirming the presence of the hydroxyl group, and at *m/e* 353 (M-205) and 335 (M-H_2O-205) due to the cleavage of the bis-allylic C-11′ single bond, showing that the hydroxyl group was located in the more unsaturated half of the molecule, and confirming the structure as 1,2,7′,8′,11′,12′-hexahydro-ψ,ψ-caroten-1-ol (**20**). Similarly the mass spectrum of hydroxyneurosporene (≡chloroxanthin) had the parent ion M^+ at *m/e* 556 ($C_{40}H_{60}O$) and fragment ions at *m/e* 538 (M-H_2O), 419 (M-137) and 401 (M-H_2O-137) in agreement with structure (**21**) (1,2,7′,8′-tetrahydro-ψ,ψ-caroten-1-ol). These hydroxy derivatives have also been isolated in small amounts from cultures of *R. rubrum* grown in the presence of DPA [9,11].

Quantitative analysis

Results obtained for the quantitative analysis of the carotenoids of *Rm. vannielii* grown anaerobically in the light were generally similar to those reported by Ryvarden and Liaaen-Jensen [17]. Rhodopin constituted approximately 60% of the total carotenoid, with lycopene and spirilloxanthin each about 10%, anhydrorhodovibrin 3% and β-carotene 2%, and other carotenoids usually less than 1%. In the DPA cultures, the total carotenoid content was similar to that of the normal culture, but phytoene (65%) became the main carotenoid with rhodopin forming only 16%, lycopene 3%, spirilloxanthin 4% and hydroxyneurosporene 4% of the total carotenoid, and traces (1% or less) of the other components.

DISCUSSION

This work has confirmed that rhodopin is the main carotenoid of *Rhodomicrobium vannieli*. In addition, the presence of spirilloxanthin, anhydrorhodovibrin, rhodovibrin, 3,4-didehydrorhodopin and "monodemethylated spirilloxanthin" is in agreement with the operation of the pathway proposed [4,5] for spirilloxanthin biosynthesis in *Rhodospirillum rubrum* (Fig. 1). The presence of small amounts of mono- and dimethoxy derivatives of lycopene, however, indicates a possible alternative sequence (Fig. 2).

DPA inhibits production of the normal carotenoids of *Rm. vannielii* and, as in the case of *R. rubrum*, phytoene becomes the main carotenoid present. The occurrence of small amounts of phytofluene, 7,8,11,12-tetrahydrolycopene, neurosporene and lycopene shows that the hydrocarbon desaturation sequence is the same as that described by Davies [6] for *R. rubrum* (Fig. 3). The presence of hydroxy derivatives of these hydrocarbons shows that in *Rm. vannielii* as in *R. rubrum* hydroxylation can take place at any level of desaturation, even as early as the phytoene level, though it is apparently only the more desaturated

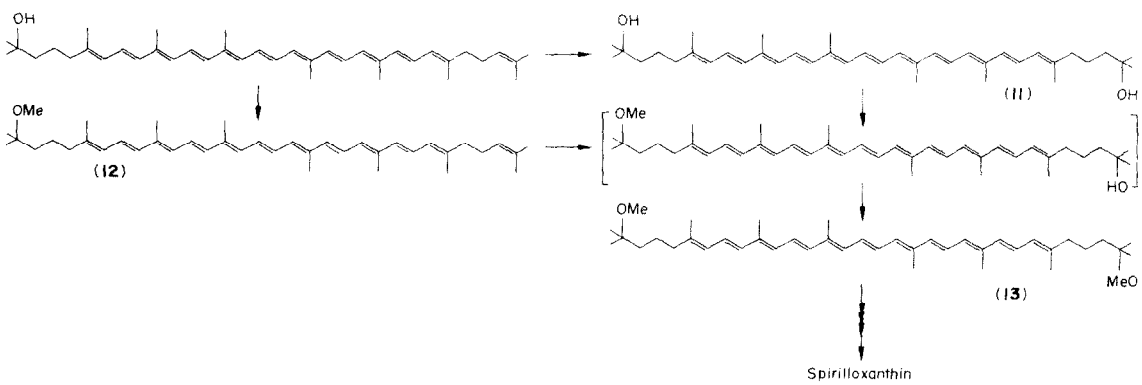


Fig. 2. Alternative routes for the formation of spirilloxanthin from lycopene in *Rhodomicrobium vannielii*.

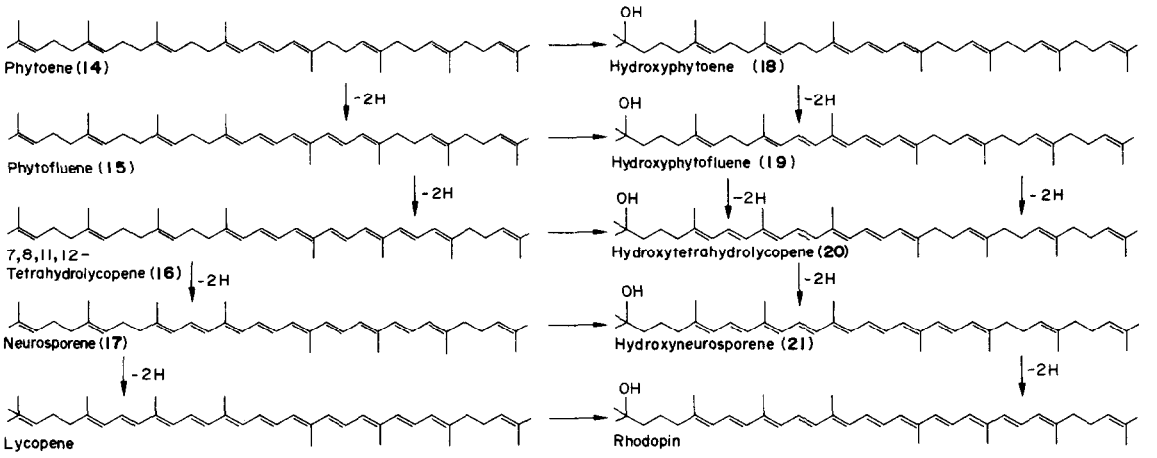


Fig. 3. Alternative pathways for the biosynthesis of rhodopin in *Rhodomicrobium vannielii* grown in the presence of diphenylamine.

half of the molecule that is hydroxylated. The overall biosynthetic scheme can thus be modified to include several alternative sequences (Fig. 3). This may however, simply reflect the fact that the enzymes responsible for desaturation, hydroxylation and methylation do not show absolute substrate specificity, although under normal conditions these reactions will take place in a particular sequence (Fig. 4). Under abnormal conditions, such as DPA inhibition, the phytoene etc. that accumulate can be hydroxylated even though they are not the normal substrates for the hydroxylating enzyme.

Rm. vannielii appears to be unique in the Rhodospirillaceae in its ability to form carotenoids with alicyclic (β-ring) end groups, although it has been proposed [2] that the mechanisms of cyclization and C-1 hydroxylation are likely to be similar. In addition to β-carotene, previously detected in this organism [15–17], the present work has revealed the occurrence of two other carotenoids containing this structural feature. The interesting monocyclic pigment (**10**), the natural occurrence of which has not previously been reported, is intermediate in structure between β-carotene and spirilloxanthin.

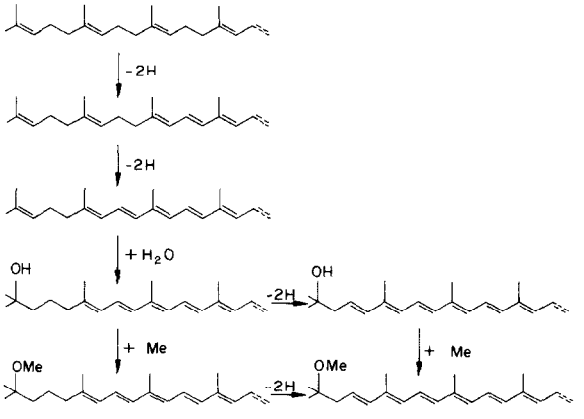


Fig. 4. Sequence of reactions in the biosynthesis of carotenoids in photosynthetic bacteria.

The other cyclic carotenoid identified was β-cryptoxanthin. In other systems, e.g. higher plants, algae and non-photosynthetic *Flavobacteria*, the introduction of hydroxyl groups at C-3 of cyclic carotenoids is normally an aerobic process, the hydroxyl groups arising from molecular oxygen by a mixed-function oxidase reaction [22–24]. The presence of β-cryptoxanthin in anaerobic cultures of *Rm. vannielii* is thus especially surprising. Analysis of a new batch of anaerobic *Rm. vannielii* grown some 3 yr after the first investigation confirmed the presence of this pigment.

It is unfortunate that the cyclic carotenoids are present in *Rm. vannielii* in amounts too small to permit proper investigation of their biosynthesis. The occurrence of rhodopin as the main pigment, however, suggests that *Rm. vannielii* should be a good organism with which to study the C-1 hydroxylation reaction characteristic of photosynthetic bacteria.

EXPERIMENTAL

Organism and culture conditions. Cultures of *Rhodomicrobium vannielii* were the kind gift of Dr. N. G. Carr of this

department. The organism was grown anaerobically in completely filled glass-stoppered Roux bottles (10 l. total) in the light (8 × 60 W tungsten bulbs at a distance of 40–100 cm) for 6 days at 28 ± 2° on a standard medium [25]. When required, DPA (2·5 mg/ml EtOH) was added at the time of inoculation, to bring the overall DPA concentration to $6·9 \times 10^{-5}$ M.

Pigment extraction and purification. Cells were harvested by centrifugation and the pigments were extracted with methanol and acetone and saponified in the normal way [26]. The saponified extract was chromatographed on a column of neutral alumina (100 g, Woelm, deactivated to Brockmann Grade III) [26], which was developed with increasing amounts of Et_2O (*E*) in light petrol (bp 40–60°, *P*). Four fractions were collected (I–IV) eluted with 2% *E*/*P*, 25% *E*/*P*, 60% *E*/*P* and 5% EtOH/*E*. Fraction I was subjected to TLC on Si gel G with 5% C_6H_6 (*B*)/*P* as developing solvent and gave two bands. The higher R_f substance was further purified by TLC on MgO: Kieselguhr G (1:1) with 5% Me_2O (*A*)/*P* and on Si gel G (0·5% *E*/*P*) to give β-carotene [λ_{max} (petrol) at 425, 449, 473 nm; MS—M^+ at *m*/*e* 536 (32%, $C_{40}H_{56}$); fragment ions at *m*/*e* 444 (12%, M-92, toluene, *m** 368; $444^2/536$ = 367·8), 430 (1%, M-106, xylene), 69 (100%). TLC of the lower R_f band on MgO: Kieselguhr G (*A*/*B*/*P* 2:2:1) followed by Si gel G (0·5% *E*/*P*) gave lycopene [λ_{max} (petrol) at 443, 469, 501 nm. MS—M^+ at *m*/*e* 536 (8%, $C_{40}H_{56}$); fragment ions at *m*/*e* 467 (1%, M-69, *m** 407; $467^2/536$ = 406·8), 444 (1%, M-92, *m** 368; $444^2/536$ = 367·8), 430 (3%, M-106), 69 (100%)].

TLC of column fraction II on Si gel G with 20% *E*/*P* gave 2 main coloured bands. The upper band was separated on MgO: Kieselguhr G with *A*/*B*/*P* (1:1:1) into 3 constituents which, after TLC on Si gel G (10% *E*/*P*) were identified as 1′-methoxy-3′,4′-didehydro-1′,2′-dihydro-β,ψ-carotene (10) [λ_{max} (petrol) at 447, 473, 505 nm, MS—M^+ at *m*/*e* 566 (10%, $C_{41}H_{58}O$); fragment ions at *m*/*e* 493 (2%, M-73, *m** 429; $493^2/566$ = 429·4), 474 (1%, M-92, *m** 397; $474^2/566$ = 397·0), 460 (9%, M-106), 401 (0·5%, M-92-73, *m** 339; $401^2/474$ = 339·2) 387 (4%, M-106-73, *m** 326; $387^2/460$ = 325·6), 73 (100%)], monomethoxylycopene [λ_{max} (petrol) at 443, 469, 501 nm, MS—M^+ at *m*/*e* 568 (1%, $C_{41}H_{60}O$)] and anhydrorhodovibrin [λ_{max} (petrol) at 455, 482, 516 nm, MS—M^+ at *m*/*e* 566 (45%, $C_{41}H_{58}O$), major fragment ions at *m*/*e* 534 (2%, M-MeOH, *m** 504; $534^2/566$ = 503·8), 493 (2·5%, M-73), 474 (5%, M-92, m* 397; $474^2/566$ = 396·9), 460 (87%, M-106), 387 (6%, M-106-73, *m** 326; $387^2/460$ = 325·5) and 73 (100%)]. TLC of the lower band on MgO: Kieselguhr G (*A*/*B*/*P* 2:1:2) gave 2 components which were further purified on Si gel G (20% *E*/*P*) and identified as "dimethoxylycopene" [λ_{max} (petrol) at 444, 469, 501 nm] and spirilloxanthin [λ_{max} (EtOH) at 465, 491, 526 nm; MS—M^+ at *m*/*e* 596 (5%, $C_{42}H_{60}O_2$), fragment ions at *m*/*e* 564 (1%, M-MeOH, *m** 534; $564^2/596$ = 533·7) 523 (2%, M-73, *m** 459; $523^2/596$ = 458·9), 504 (1%, M-92), 490 (13%, M-106), 458 (1%, M-106-MeOH, *m** 428; $458^2/490$ = 428·1), 417 (3%, M-106-73, *m** 355; $417^2/490$ = 354·9), 398 (3%, M-106-92, *m** 323; $398^2/490$ = 323·3), 384 (4%, M-106-106), 73 (100%)]. Column fraction III was separated by TLC on Si gel G (50% *E*/*P*) into 2 main coloured zones. Further TLC of the upper band on MgO: Kieselguhr G (*A*/*B*/*P*, 1:1:8) gave 3 carotenoids, which were rechromatographed on Silica gel G (40% *E*/*P*) and identified as β-cryptoxanthin [λ_{max} (EtOH) at 426, 451, 475 nm; MS—M^+ at *m*/*e* 552 (100%, $C_{40}H_{56}O$), fragment ions at *m*/*e* 534 (5%, M-H_2O, *m** 517; $534^2/552$ = 516·6) 460 (24%, M-92, *m** 383; $460^2/552$ = 383·4) and 446 (2%, M-106); acetate (Ac_2O–C_5H_5N [26]) λ_{max} (EtOH) at 425, 450, 475 nm, MS—M^+ at *m*/*e* 594 (100%, $C_{42}H_{58}O_2$) and fragment ions at *m*/*e* 534 (7%, M-$MeCO_2H$, *m** 480; $534^2/594$ = 480·0), 502 (20%, M-92, *m** 424; $502^2/594$ = 424·2), 488 (2%, M-106) and 442 (5%, M-92-$MeCO_2H$, *m** 389; $442^2/502$ = 389·2)], rhodopin [λ_{max} (EtOH) at 443, 470, 501 nm; MS—M^+ at *m*/*e* 554 (10%, $C_{40}H_{58}O$) and major fragment ions at *m*/*e* 536 (1%, M-H_2O, *m** 519; $536^2/554$ = 518·6), 485 (1%, M-69, *m** 425; $485^2/536$ = 424·6), 467 (2%, M-87, *m** 394; $467^2/554$ = 393·6 and M-H_2O-69, *m** 407; $467^2/536$ = 406·8), 462 (3%, M-92, *m** 385; $462^2/554$ = 385·2) and 448 (9%, M-106)], and 3,4-didehydrorhodopin [λ_{max} (EtOH) at 455, 482, 514 nm; MS—M^+ at *m*/*e* 552 (10%, $C_{40}H_{56}O$) and fragment ions at *m*/*e* 534 (0·5%, M-H_2O), 483 (0·5%, M-69), 460 (0·2%, M-92) 446 (15%, M-106) and 428 (1%, M-106-H_2O)]. From the lower band two carotenoids were obtained by TLC on MgO: Kieselguhr G (*A*/*B*/*P*, 2:2:1). These were further purified on Si gel G (60% *E*/*P*) and identified as rhodovibrin [λ_{max} (EtOH) at 455, 483 and 516 nm; MS—M^+ at *m*/*e* 584 (6%, $C_{41}H_{60}O_2$) and fragment ions at *m*/*e* 566 (1%, M-H_2O, *m** 548; $566^2/584$ = 548·5), 552 (0·5%, M-MeOH, *m** 522; $552^2/584$ = 521·7), 492 (0·5%, M-92), 478 (15%, M-106), 405 (2%, M-106-73, *m** 343; $405^2/478$ = 343·2) and 73 100%)] and monodemethylated spirilloxanthin [λ_{max} (EtOH) at 462, 494, 528 nm; MS—M^+ at *m*/*e* 582 (3%, $C_{41}H_{58}O_2$) and major fragment ions at *m*/*e* 564 (2%, M-H_2O, *m** 546; $564^2/582$ = 546·5), 550 (5%, M-MeOH, *m** 520; $550^2/582$ = 519·7), 524 (3%, M-Me_2CO), 509 (5%, M-73, *m** 445; $509^2/582$ = 445·1), 476 (7%, M-106), 403 (2%, M-106-73, *m** 341; $403^2/476$ = 341·1) and 73 (100%)].

Two carotenoids were isolated from column fraction IV by TLC on MgO: Kieselguhr G (*A*/*B*/*P*, 1:1:8) and further purified on Si gel G (80% *E*/*P*). These were tentatively identified as "dihydroxy-434" [λ_{max} (EtOH) at 412, 434, 462 nm; MS—M^+ at *m*/*e* 570 ($C_{40}H_{58}O_2$)] and "dihydroxylycopene" [λ_{max} (EtOH) at 443, 470, 500 nm; MS—M^+ at *m*/*e* 572 ($C_{40}H_{60}O_2$)].

Purification of pigments from DPA-inhibited cultures. The saponified extract was chromatographed on a column of neutral alumina as before. TLC of column fraction I on Si gel G (5% *B*/*P*) gave 3 bands which on MgO: Kieselguhr G (1% *A*/*P*, 5% *A*/*P*, *A*/*B*/*P*, 1:1:2 respectively) gave 5 carotenoids, phytoene, phytofluene, 7,8,11,12-tetrahydrolycopene, neurosporene and lycopene. Each of these was further purified on silica gel G (*P*) before determination of its absorption and mass spectra. Phytoene had λ_{max} (light petrol) at 275, 285, 296 nm and MS peaks at *m*/*e* 544 (M^+,$C_{40}H_{64}$) and 339 (M-205), phytofluene had λ_{max} (light petrol) at 331, 348, 367 nm and MS peaks at *m*/*e* 542 (M^+, $C_{40}H_{62}$), 405 (M-137) and 337 (M-205), 7,8,11,12-tetrahydrolycopene had λ_{max} (petrol) at 374, 395, 419 nm and MS peaks at *m*/*e* 540 (M^+, $C_{40}H_{60}$), 471 (M-69) and 335 (M-205) and neurosporene had λ_{max} (petrol) at 413, 439, 467 nm and MS peaks at *m*/*e* 538 (M^+, $C_{40}H_{58}$), 469 (M-69) and 401 (M-137). TLC of column fraction III on MgO: Kieselguhr G (*A*/*B*/*P*, 1:1:2) gave 5 bands which were re-chromatographed on Si gel G (40% *E*/*P*) to give hydroxyphytoene [λ_{max} (EtOH) at 275, 285, 296 nm], hydroxyphytofluene [λ_{max}(EtOH) at 330, 348, 366 nm], hydroxy-7,8,11,12-tetrahydrolycopene [λ_{max}(EtOH) at 373, 394, 418 nm; MS peaks at *m*/*e* 558 (50%, M^+, $C_{40}H_{62}O$), 540 (4%, M-H_2O, *m** 523; $540^2/558$ = 522·6), 466 (1%, M-92), 464 (1%, M-94, *m** 386: $464^2/558$ = 385·8) [9], 452 (1%, M-106), 353 (10%, M-205, *m** 223; $353^2/558$ = 223·3), 335 (2%, M-H_2O-205, *m** 318; $335^2/353$ = 317·9; *m** 208; $335^2/540$ = 207·8), and 69 (100%)], hydroxyneurosporene [λ_{max}(EtOH) at 416, 439, 468 nm; MS peaks at *m*/*e* 556 (35%, M^+, $C_{40}H_{60}O$), 538 (30%, M-H_2O, *m** 521; $538^2/556$ = 520·6), 464 (1%, M-92), 450 (10%, M-106), 446 (2%, M-H_2O-92, *m** 370; $446^2/538$ = 369·7), 419 (1·5%, M-137, *m** 316; $419^2/556$ = 315·8) 401 (3%, M-H_2O-137, *m** 299; $401^2/538$ = 298·9), 327 (M-92-137, *m** 230; $327^2/464$ = 230·5) and 69 (100%)], and rhodopin. Other carotenoids pres-

ent in trace amounts were purified as described for the normal cultures.

Absorption spectra. Quantitative analysis was performed by the spectrophotometric method described by Davies [27].

Mass spectra. Mass spectra were determined by Mrs. A. M. Ball and Mr. J. R. Ireland on an A.E.I. MS 12 instrument with the direct insertion probe (probe temp. 180–220°; ionizing potential 70 eV).

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