Alternative Pathways of Spirilloxanthin Biosynthesis in *Rhodospirillum rubrum*

By B. H. DAVIES

Department of Biochemistry and Agricultural Biochemistry, University College of Wales, Penglais, Aberystwyth, Wales, U.K.

(Received 29 August 1969)

Detailed studies of the properties of carotenoids isolated from diphenylamine-inhibited cultures of *Rhodospirillum rubrum* have revealed a number of novel structures that indicate new features of carotenoid biosynthesis in the photosynthetic bacteria. Both neurosporene and 7,8,11,12-tetrahydrolycopene undergo hydration, methylation and dehydrogenation to yield spheroidene and 11',12'-dihydrospheroidene respectively; all the intermediates in these pathways have been identified. These pathways represent alternative routes of anhydrorhodovibrin and spirilloxanthin biosynthesis.

Mature cultures of the purple non-sulphur photosynthetic bacterium *Rhodospirillum rubrum* contain spirilloxanthin (VI) as the predominant carotenoid (van Niel & Smith, 1935; Polgár, van Niel & Zechmeister, 1944; Goodwin & Osman, 1953). When *R. rubrum* is cultured anaerobically in the presence of diphenylamine, the formation of spirilloxanthin is inhibited and other carotenoids accumulate (Goodwin & Osman, 1954). By studying the kinetics of the disappearance of these carotenoids on removing the diphenylamine inhibition, Jensen, Cohen-Bazire, Nakayama & Stanier (1958) and Jensen, Cohen-Bazire & Stanier (1961) established that they are precursors of spirilloxanthin and showed that lycopene (I) is converted into spirilloxanthin by a number of consecutive reactions, which operate first at one end of the molecule and then at the other (Scheme 1).

The hydration of an isopropylidene end group of lycopene, followed by a dehydrogenation to introduce a double bond between C-3 and C-4 at the hydroxylated end and, finally, methylation of the hydroxyl group, results in the formation of anhydrorhodovibrin (IV). The operation of the same sequence of three reactions at the remaining isopropylidene end group converts anhydrorhodovibrin into spirilloxanthin.

The same series of three reactions (Scheme 2) is used to convert neurosporene (VII) into spheroidene (X) in anaerobic cultures of *Rhodopseudomonas spheroides* (Jensen *et al.* 1961; Jensen, 1963).

Eimhjellen & Jensen (1964) demonstrated the existence of alternative pathways of spirilloxanthin formation in anaerobic cultures of *Rhodopseudomonas gelatinosa* (Scheme 3). These operate from neurosporene through spheroidene, with spheroidene being

either dehydrogenated to anhydrorhodovibrin or hydrated to 1'-hydroxy-1',2'-dihydrospheroidene (XI), which is then dehydrogenated to yield rhodovibrin (V); this is then converted into spirilloxanthin by the same reactions that operate in *R. rubrum*. In each case, the biosynthesis of anhydrorhodovibrin from lycopene is by-passed.

The present work, some aspects of which have been reported briefly (Davies & Holmes, 1969; Davies, Holmes, Loeber, Toubé & Weedon, 1969), shows that the pathways of methoxycarotenoid formation that operate in *Rps. spheroides* and *Rps. gelatinosa*, together with a novel pathway, also operate in *R. rubrum*. In addition, the structures of some of the novel carotenoid intermediates isolated from diphenylamine-inhibited cultures of *R. rubrum* necessitate a reappraisal of the exact sequence of the hydration, methylation and dehydrogenation reactions that are responsible for the biosynthesis of the methoxycarotenoids in photosynthetic bacteria.

MATERIALS AND METHODS

Methods for the determination of u.v.- and visible-absorption spectra and for the assignment of stereochemical configuration of the carotenoids, together with details of the source and purification of the solvents, have been described in detail in the preceding paper (Davies, 1970).

Culture and extraction of bacteria. As in previous studies (Davies, 1970), *R. rubrum* (strain C) was cultured in 201 batches in the light for 7 days at 29°C on a glutamate-malate medium to which diphenylamine had been added at inoculation (65 µM). The bacteria were harvested and extracted and the lipid was saponified before chromatography by methods already described in detail (Davies, 1970).

Scheme 1. Biosynthesis of spirilloxanthin from lycopene in *R. rubrum* (Jensen *et al.* 1961).

Preparative chromatography of carotenoids. Each batch of unsaponifiable material (approx. 100mg) from the bacteria was chromatographed on a column (30 cm × 5 cm) of alumina (Woelm neutral, Brockmann activity grade III) developed with light petroleum (b.p. 40–60°C) containing increasing concentrations of diethyl ether (up to 50%). Although some pigmented material still remained adsorbed on the column, a large number of carotenoids were eluted by this procedure, and individual bands from the column were, as far as possible, collected as individual fractions. The individual carotenoids were isolated by rechromatography of the fractions from the first column on separate columns (20 cm × 2 cm) of alumina (neutral, Brockmann activity grade III) with increasing concentrations of

diethyl ether (up to 50%, v/v) in light petroleum as the developing solvent. The eluate fractions were examined spectrophotometrically, and samples from successive columns that had identical absorption spectra and chromatographic behaviour were combined. These individual carotenoids were subjected to a final purification by chromatography on columns (20 cm × 2 cm) of alumina (neutral, Brockmann activity grade II or III, depending on the adsorption affinity of the carotenoid) developed with the appropriate mixture of light petroleum and diethyl ether (Table 1).

Thin-layer chromatography. Comparisons of the adsorption characteristics of certain carotenoids were carried out on thin layers (250 μm) of alumina (Woelm

Scheme 2. Biosynthesis of spheroidene from neurosporene in *Rps. spheroides* (Jensen *et al.* 1961; Jensen, 1963).

Scheme 3. Biosynthesis of spirilloxanthin from neurosporene in *Rps. gelatinosa* (Eimhjellen & Jensen, 1964).

neutral for t.l.c.) with 20% (v/v) benzene in light petroleum (b.p. 40–60°C) as the developing solvent. Chromatograms were developed in the dark in a saturation chamber (Davies, 1963, 1965).

Paper chromatography. Adsorption chromatography

on paper was used for the comparison of the chromatographic behaviour of certain pure carotenoids and for the preparation of a pure sample of spheroidene (see below). The carotenoids were separated on silica gel-loaded paper (Whatman SG 81) by ascending development with 5%

Fractions 11 and 12 were isolated by chromatography of the unsaponifiable bacterial extract on alumina (Brockmann activity grade III) and were eluted with 10–20% diethyl ether in light petroleum. Fraction 12 had an absorption spectrum in light petroleum that had an inflexion at 425nm and maxima at 460, 489 and 524nm and that corresponded to a chromophore of 13 conjugated double bonds. It was identical in its chromatographic and spectroscopic properties with the main red carotenoid isolated from an anaerobic culture of *R. rubrum* that had not been grown in the presence of diphenylamine. This carotenoid was therefore spirilloxanthin (Jensen, 1959; Jensen *et al.* 1958).

The minor carotenoid (fraction 11) that preceded spirilloxanthin in order of elution from the alumina column had an absorption spectrum in light petroleum, which indicated a chromophore of 12 conjugated double bonds (an inflexion at 422nm and maxima at 456, 482 and 515nm). These properties are consistent with its identification as anhydorrhodovibrin (Jensen *et al.* 1958, 1961).

Identification of fraction 9 as spheroidene (X). This carotenoid was eluted from the alumina column (Brockmann activity grade II) with 20% diethyl ether in light petroleum. It had an absorption spectrum in light petroleum (inflexion at 405nm and maxima at 427, 453 and 484nm) consistent with a chromophore of ten conjugated double bonds and is therefore probably identical with the carotenoid described by Goodwin & Osman (1954) as 'fraction G' and by Jensen *et al.* (1958) as 'P 450'. It was compared with a sample of spheroidene isolated from an anaerobic culture of *Rps. spheroides*; the absorption spectra were identical and the two samples failed to separate on chromatography either on an alumina column (Brockmann activity grade II developed with 20% diethyl ether in light petroleum) or on silica gel-loaded paper (Whatman SG81, developed with 5% acetone in light petroleum; R_F 0.32). Both samples had all-*trans* configurations since, in each case, iodine-catalysed photoisomerization resulted in hypsochromic and hypochromic shifts in the absorption spectrum, together with an increase in extinction in the 'cis-peak' region (340nm).

The identification of this carotenoid as spheroidene was confirmed by its mass spectrum, which showed a molecular ion at m/e 568.4657 (Calc. for spheroidene, $C_{41}H_{60}O$: 568.4644) and ions at m/e 536 ($M - MeOH$) and m/e 495 ($M - 73$). This fragmentation was consistent with the sample being a methoxycarotenoid and was identical with that shown by authentic spheroidene (Davies *et al.* 1969). Since this work was completed, a report by Malhotra, Britton & Goodwin (1969) has confirmed the presence of spheroidene in diphenylamine-inhibited cultures of *R. rubrum*.

Identification of fraction 6 as 11',12'-dihydrospheroidene (XVI). This carotenoid, which was eluted

from an alumina column (Brockmann activity grade II) with 5–10% diethyl ether in light petroleum, had an absorption spectrum in light petroleum that showed an inflexion at 370 nm and maxima at 392, 414 and 440nm and that was consistent with the carotenoid having a chromophore of eight conjugated double bonds. This carotenoid has not been identified before, but has been described by Goodwin & Osman (1954) as 'fraction I' and by Jensen *et al.* (1958) as 'P 412'. Hypsochromic and hypochromic shifts in this spectrum on iodine-catalysed photoisomerization, together with the appearance of a 'cis-peak' at 300nm, indicated that this carotenoid had an all-*trans* chromophore. The other carotenoids in *R. rubrum* that have even numbers of conjugated double bonds in the chromophore are both monomethoxycarotenoids; anhydorrhodovibrin, with 12 conjugated double bonds, is formed from lycopene (11 conjugated double bonds), and spheroidene, with ten conjugated double bonds, is presumably formed from neurosporene (nine conjugated double bonds) by the same pathway that operates in *Rps. spheroides* (Jensen, 1963). By analogy, the 'fraction 6' carotenoid could be a monomethoxycarotenoid formed in the same way, but from a conjugated heptane. The formation of such a carotenoid from ζ -carotene (7,8,7',8'-tetrahydrolycopene) would be impossible, but the *R. rubrum* conjugated heptaene, 7,8,11,12-tetrahydrolycopene (Davies, 1970), could be converted into a monomethoxycarotenoid with a chromophore of eight conjugated double bonds, namely 11',12'-dihydrospheroidene.

The chromatographic properties of the 'fraction 6 carotenoid' were consistent with this structure. Paper chromatography of 7,8,11,12-tetrahydrolycopene, neurosporene, lycopene, the 'fraction 6 carotenoid', spheroidene and anhydorrhodovibrin on silica gel-loaded paper (Whatman SG81) with 5% acetone in light petroleum showed that the R_F values of the

Table 2. Comparison of the chromatographic and structural characteristics of some carotenoids from *R. rubrum*

R_F values are for chromatography on Whatman SG81 paper developed with 5% acetone in light petroleum.

Carotenoid	R_F	No. of conjugated double bonds	No. of methoxy groups
7,8,11,12-Tetrahydrolycopene	0.64	7	0
11',12'-Dihydrospheroidene (fraction 6)	0.44	8	1
Neurosporene	0.42	9	0
Spheroidene	0.32	10	1
Lycopene	0.35	11	0
Anhydorrhodovibrin	0.10	12	1

carotenoids by no means corresponded to the numbers of conjugated double bonds (Table 2). The increased adsorption affinities of anhydro-rhodovibrin and spheroidene are due to their methoxy groups, so the fact that the 'fraction 6 carotenoid' has an increased adsorption affinity of the same order indicates the presence of a methoxy group and supports the structure of 11',12'-dihydrospheroidene for this carotenoid.

The high-resolution measurement of the mass of the molecular ion (570.4816) corresponded closely to that required for the proposed structure (Calc. for $C_{41}H_{62}O$: 570.4801). The presence of a methoxy group was confirmed by the appearance in the mass spectrum of ions corresponding to ($M - MeOH$) and ($M - 73$); an ion at m/e 73 was also observed (Davies *et al.* 1969). Fission of the 'bis-allylic' single bonds was indicated by ions at m/e 501 ($M - 69$) and m/e 433 ($M - 137$). The '11',12'-dihydro' structure was confirmed by the presence of an ion at m/e 365 ($M - 205$) and a metastable peak at m/e 234 ($365^2/570 = 233.7$). The 'fraction 6 carotenoid' is therefore 11',12'-dihydrospheroidene.

Identification of 'fraction 5' as 3,4,11',12'-tetrahydrospheroidene (XV). This carotenoid was eluted from an alumina column (Brockmann activity grade II) with 5% diethyl ether in light petroleum and had an absorption spectrum in light petroleum that was identical with that of 7,8,11,12-tetrahydrolycopene, showing an inflexion at 354nm and maxima at 374, 394.5 and 418.5nm. Hypsochromic and hypochromic shifts in the spectrum on iodine-catalysed photoisomerization showed its configuration to be all-*trans*, so that it could not be a stereoisomer of all-*trans*-7,8,11,12-tetrahydrolycopene. The 'fraction 5 carotenoid' and 7,8,11,12-tetrahydrolycopene had markedly different chromatographic characteristics (R_F values 0.35 and 0.85 respectively on a thin layer of aluminium oxide developed with 20% benzene in light petroleum).

The mass spectrum of this carotenoid exhibited a strong molecular ion (m/e 572.4966; 56%) that corresponded to a tetrahydrospheroidene structure (Calc. for $C_{41}H_{64}O$: 572.4957). Weak ions of m/e 503 ($M - 69$) and m/e 435 ($M - 137$), together with an appreciable loss of 205 mass units (m/e 367, 6%; metastable m/e 235.5; $367^2/572 = 235.5$), indicated the presence of the 3,7,11-trimethyldodeca-2,6,10-trienyl unit, as found in phytoene, 7,8,11,12-tetrahydrolycopene and 11',12'-dihydrospheroidene. A loss of methanol from both the molecular ion and m/e 367 was indicated by ions at m/e 540 and m/e 335 respectively; these fragmentations were confirmed by metastable peaks at m/e 510 ($540^2/572 = 509.8$) and m/e 305.8 ($335^2/367 = 305.8$). The mass-spectral, chromatographic and spectroscopic properties of this carotenoid are consistent with the structure 3,4,11',12'-tetrahydrospheroidene.

This carotenoid has also been isolated from anaerobic cultures of *Rps. spheroides* (B. H. Davies, unpublished work). The specimen from *Rps. spheroides* had an absorption spectrum identical with that of the *R. rubrum* carotenoid and the two samples failed to separate on t.l.c. (aluminium oxide layer, developed with 20% benzene in light petroleum, R_F 0.35).

Identification of fraction 7 as 3,4-dihydrospheroidene (XII). This carotenoid was slightly more strongly adsorbed on an alumina column (Brockmann activity grade II) than 11',12'-dihydrospheroidene, yet, like neurosporene, it had a chromophore of nine conjugated double bonds (the absorption spectrum in light petroleum showed an inflexion at 394nm and maxima at 414, 439 and 469nm). As the properties of this carotenoid bore the same relationship to those of neurosporene as the properties of 3,4,11',12'-tetrahydrospheroidene did to those of 7,8,11,12-tetrahydrolycopene, its structure was probably 3,4-dihydrospheroidene. The mass spectrum showed a molecular ion (m/e 570.479) that was consistent with this structure (Calc. for $C_{41}H_{62}O$: 570.480). Iodine-catalysed photoisomerization produced changes that indicated an all-*trans* configuration. 3,4-Dihydrospheroidene is also present in extracts from anaerobic cultures of *Rps. spheroides* (B. H. Davies, unpublished work).

Identification of fraction 10 as 3,4-dihydroanhydro-rhodovibrin. This carotenoid, like lycopene (Davies, 1970), was present at a very low concentration in the diphenylamine-inhibited cultures of *R. rubrum*. It was eluted from the alumina column (Brockmann activity grade II), between spheroidene and anhydro-rhodovibrin, with 20–50% diethyl ether in light petroleum and had an absorption spectrum in light petroleum that was identical with that of lycopene, having an inflexion at 414nm and maxima at 446, 473 and 504nm. Insufficient material was available for mass spectrometry, but, by analogy with 3,4,11',12'-tetrahydrospheroidene and 3,4-dihydrospheroidene, the structure 3,4-dihydroanhydro-rhodovibrin is proposed.

Monohydroxycarotenoids. Fractions 13–16 were eluted from the alumina column (Brockmann activity grade III) with higher concentrations (20–50%) of diethyl ether in light petroleum. The high polarity and the absorption spectra of the impure carotenoids suggested that they were 1-hydroxy-1,2-dihydro derivatives of phytofluene, 7,8,11,12-tetrahydrolycopene (XIV), neurosporene (VIII) and spheroidene (XI) (Table 1). A corresponding derivative of lycopene (rhodopin, II) was present in trace amounts, but was not investigated in detail. The impure carotenoids were recombined and subjected to a silylation procedure. That they were hydroxycarotenoids was confirmed by the fact that this procedure resulted in the formation of a set of

considerably less-polar trimethylsilyl ethers, which could be separated and purified by chromatography on a column of alumina (Brockmann activity grade II) with low concentrations (0–20%) of diethyl ether in light petroleum as the developing solvent.

The presence of the 1-hydroxy-1,2-dihydro derivatives of the hydrocarbon polyenes in diphenylamine-inhibited cultures of *R. rubrum* has been described by Jensen *et al.* (1958). Cultures of *Rhodospseudomonas gelatinosa* have been shown to form 1'-hydroxy-1',2'-dihydrospheroidene (XI, Eimhjellen & Jensen, 1964), but, before the present work, this pigment had not been observed in *R. rubrum*. A report by Malhotra *et al.* (1969), however, has also indicated the presence of this carotenoid in diphenylamine-inhibited cultures of *R. rubrum*.

The trimethylsilyl ether of 1'-hydroxy-1',2'-dihydrospheroidene was purified by chromatography on an alumina column (Brockmann activity grade III) developed with 5% diethyl ether in light petroleum. It had an absorption spectrum in light petroleum that was identical with that of spheroidene, showing an inflexion at 405nm and maxima at 427, 453 and 484nm. Its mass spectrum showed a molecular ion at *m/e* 658.5135 (Calc. for $C_{44}H_{70}O_2Si$: 658.5145) and losses of 32 and 73 mass units confirmed the presence of a methoxy group.

DISCUSSION

The identification of spheroidene and 11',12'-dihydrospheroidene in diphenylamine-inhibited cul-

tures of *R. rubrum* means that parallel pathways of methoxycarotenoid formation must be operating in these bacteria. Spheroidene must be formed from neurosporene, the biosynthetic precursor of lycopene, and 11',12'-dihydrospheroidene is formed from 7,8,11,12-tetrahydrolycopene, which is the biosynthetic intermediate between phytofluene and neurosporene in the dehydrogenation of phytoene to lycopene in *R. rubrum* (Davies, 1970).

The pathway of spheroidene formation in *R. rubrum* and *Rps. spheroides* (Scheme 4) appears to differ slightly from that described for *Rps. spheroides* by Jensen (1963). Although neurosporene (VII) is hydrated to 1-hydroxy-1,2-dihydroneurosporene (chloroxanthin; VIII), the next step appears to be a methylation to form 3,4-dihydrospheroidene (XII), which has been isolated from both *Rps. spheroides* (B. H. Davies, unpublished work) and *R. rubrum*, rather than a dehydrogenation to yield the hypothetical demethylated spheroidene (IX; Jensen, 1963). Dehydrogenation of 3,4-dihydrospheroidene leads to the formation of spheroidene (X).

The formation of 11',12'-dihydrospheroidene (XVI) from 7,8,11,12-tetrahydrolycopene (XIII) in *R. rubrum* takes place by a similar sequence of three reactions (Scheme 5). 7,8,11,12-Tetrahydrolycopene is hydrated to yield 1-hydroxy-1,2,7',8',11',12'-hexahydrolycopene [(XIV); described by Jensen *et al.* (1958) as hydroxy- ζ -carotene] and then this is methylated to yield 3,4,11',12'-tetrahydrospheroidene (XV). Dehydrogenation converts this intermediate into 11',12'-dihydrospheroidene. The isolation of

Scheme 4. Biosynthesis of spheroidene from neurosporene in *R. rubrum* and *Rps. spheroides*.

Scheme 5. Biosynthesis of 11',12'-dihydrospheroidene from 7,8,11,12-tetrahydrolycopene in *R. rubrum*.

3,4,11',12'-tetrahydrospheroidene from anaerobic cultures of *Rps. spheroides* (B. H. Davies, unpublished work) suggests that this pathway also operates in this organism.

In these two pathways, the formation of 3,4-dihydrospheroidene and 3,4,11',12'-tetrahydrospheroidene indicates that methylation precedes the dehydrogenation step. This order is opposite to that which has been established for the pathway of anhydrorhodovibrin and spirilloxanthin formation in *R. rubrum* (Jensen *et al.* 1961) and which has been postulated for spheroidene biosynthesis in *Rps. spheroides* and *Rps. gelatinosa* (Jensen, 1963). As Jensen (1961) has pointed out, however, the apparent order of these two reactions is simply a measure of their relative reaction velocities and is indicated by which of two alternative intermediates appears. Thus, in spheroidene biosynthesis, the apparent intermediate is 3,4-dihydrospheroidene rather than demethylated spheroidene because the methylation is faster than the dehydrogenation. The same situation obviously exists in the pathway of 11',12'-dihydrospheroidene formation. It is conceivable that under different cultural conditions (e.g. a different diphenylamine concentration) or in different organisms the other intermediate, or even both, might appear. Indeed, the tentative identification of 3,4-dihydroanhydrorhodovibrin in the present work and the identification of 3,4-dehydrorhodopin (III; Jackman & Jensen, 1961) in *Rhodospseudomonas palustris* suggest that this latter situation holds for the biosynthesis of anhydrorhodovibrin from lyco-

pene in the photosynthetic bacteria (Scheme 6) and that the substrate specificity of the enzymes concerned may not be absolute.

The results obtained by Jensen *et al.* (1958) in their studies on the disappearance of the accumulated carotenoid precursors when inhibited cultures of *R. rubrum* are washed free of diphenylamine and reincubated indicate that the changes in the concentrations of two unidentified carotenoids, 'P412' and 'P450', are consistent with their being precursors, ultimately, of spirilloxanthin. Now that these two carotenoids have been identified as 11',12'-dihydrospheroidene (XVI) and spheroidene (X) respectively, it is clear that they take part in an alternative pathway of anhydrorhodovibrin (IV) biosynthesis, in which successive dehydrogenations convert 11',12'-dihydrospheroidene into spheroidene and then into anhydrorhodovibrin (Scheme 6).

The presence of 1'-hydroxy-1',2'-dihydrospheroidene (XI) in diphenylamine-inhibited cultures of *R. rubrum* suggests that the *Rps. gelatinosa* pathway of spirilloxanthin formation (Eimhjellen & Jensen, 1964; Scheme 3) may also operate in *R. rubrum*. In this pathway, spheroidene is hydrated to 1',2'-dihydro-1'-hydroxyspheroidene, which is then hydrogenated to yield rhodovibrin, a known precursor of spirilloxanthin.

All the alternative pathways of methoxycarotenoid formation in *R. rubrum* that can be deduced from the present studies and from those of Jensen *et al.* (1961) are summarized in Scheme 6. Although it is necessary to culture *R. rubrum* in the presence of diphenylamine

Scheme 6. Alternative pathways of spirilloxanthin biosynthesis in *R. rubrum*.

to isolate sufficient of many of the carotenoids for detailed analysis, the intermediates are not merely artifacts arising from the effect of diphenylamine inhibition since several are present in normal anaerobic cultures of *Rps. spheroides*. Thus all the pathways indicated in Scheme 6 are genuine biosynthetic sequences. Although it is not possible on the basis of the above results to assess the relative importance of each of these alternative pathways to spirilloxanthin biosynthesis, the quantitative results of Jensen *et al.* (1958) indicate that 11',12'-dihydrospheroidene, spheroidene and anhydrorhodovibrin (described as 'P412', 'P450' and 'P481' respectively) are present in roughly equimolar amounts in diphenylamine-inhibited cultures.

The dehydrogenation reactions occurring in these bacteria can be divided into two types, increasing the length of the carotenoid chromophore by either one or two conjugated double bonds, depending on whether the reaction takes place at C-3 and C-4 or at some other position in the molecule when a previously isolated double bond is also brought into conjugation. These two types of dehydrogenation reaction must be catalysed by different enzymes, for the latter type (indicated by horizontal arrows in Scheme 6) appears to be specifically inhibited by diphenylamine (Davies, 1970) whereas the reactions forming 3,4-double bonds are not. The existence of a mutant of *Rps.*

spheroides that forms predominantly neurosporene and chloroxanthin and only a trace of spheroidene (Nakayama, 1958) also suggests that the different types of dehydrogenation reaction require different enzymes.

Apart from the dehydrogenations, the other types of reaction involved in all the alternative pathways of spirilloxanthin biosynthesis in *R. rubrum* are only two in number, namely (a) hydration, converting the isopropylidene end group of a polyene hydrocarbon into a '1-hydroxy-1,2-dihydro' end group and (b) methylation of the hydroxyl group to form a methoxy carotenoid. It is likely that the apparent participation of so many alternative pathways of methoxy-carotenoid biosynthesis is simply a result of the enzymes catalysing the basic reactions having low substrate specificities. They are therefore able to use not only lycopene but also lycopene precursors, which accumulate because of the specific effect of diphenylamine, as substrates for the biosynthesis of methoxy-carotenoids.

The author thanks Mrs J. H. Warren for her skilled technical assistance and Miss E. A. Holmes for her collaboration in the studies on spheroidene and 11',12'-dihydrospheroidene. All the mass-spectral analyses were carried out in the Department of Chemistry, Queen Mary College, London, through the kind co-operation of Professor B. C. L. Weedon, Dr T. P. Toubé and Mr D. E. Loeber.

The author is particularly indebted to Professor Weedon for many useful discussions.

REFERENCES

- Davies, B. H. (1963). *J. Chromat.* **10**, 518.
- Davies, B. H. (1965). In *Chemistry and Biochemistry of Plant Pigments*, p. 489. Ed. by Goodwin, T. W. London and New York: Academic Press Inc.
- Davies, B. H. (1970). *Biochem. J.* **116**, 93.
- Davies, B. H. & Holmes, E. A. (1969). *Biochem. J.* **113**, 35 P.
- Davies, B. H., Holmes, E. A., Loeber, D. E., Toubé, T. P. & Weedon, B. C. L. (1969). *J. chem. Soc. C*, p. 1266.
- Eimhjellen, K. E. & Jensen, S. L. (1964). *Biochim. biophys. Acta*, **82**, 21.
- Goodwin, T. W. & Osman, H. G. (1953). *Biochem. J.* **53**, 541.
- Goodwin, T. W. & Osman, H. G. (1954). *Biochem. J.* **56**, 222.
- Jackman, L. M. & Jensen, S. L. (1961). *Acta chem. scand.* **15**, 2058.
- Jensen, S. L. (1959). *Acta chem. scand.* **13**, 381.
- Jensen, S. L. (1961). *Acta chem. scand.* **15**, 1182.
- Jensen, S. L. (1963). In *Bacterial Photosynthesis*, p. 19. Ed. by Gest, H., San Pietro, A. & Vernon, L. P. Yellow Springs, Ohio: Antioch Press.
- Jensen, S. L., Cohen-Bazire, G., Nakayama, T. O. M. & Stanier, R. Y. (1958). *Biochim. biophys. Acta*, **29**, 477.
- Jensen, S. L., Cohen-Bazire, G. & Stanier, R. Y. (1961). *Nature, Lond.*, **192**, 1168.
- McCormick, A. & Jensen, S. L. (1966). *Acta chem. scand.* **20**, 1989.
- Malhotra, H. C., Britton, G. & Goodwin, T. W. (1969). *Phytochemistry*, **8**, 1047.
- Nakayama, T. O. M. (1958). *Archs Biochem. Biophys.* **75**, 356.
- Polgár, A., van Niel, C. B. & Zechmeister, L. (1944). *Archs Biochem. Biophys.* **5**, 243.
- van Niel, C. B. & Smith, J. H. C. (1935). *Arch. Mikrobiol.* **6**, 219.