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PHYTOENE, PHYTOFLUENE AND ζ-CAROTENE ISOMERS FROM A *SCENEDESMUS OBLIQUUS* MUTANT

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Abstract—A number of mutant strains of the green alga, *Scenedesmus obliquus*, when grown in the dark, accumulated ζ-carotene as their major carotenoid together with appreciable concentrations of phytoene and phytofluene. The phytoene was almost entirely the 15-*cis* isomer, and phytofluene was also present mainly as the 15-*cis* form, whereas the ζ-carotene could be separated into three isomers, predominantly all-trans ζ-carotene accompanied by the 15-*cis* and an unidentified *cis* isomer. All the ζ-carotene isomers, when illuminated in the presence of iodine, gave the same equilibrium mixture of stereo-isomers, including a product with unusual spectroscopic and chromatographic properties, which may be a cyclic compound. The pathway of carotenoid biosynthesis in *S. obliquus* is discussed. On illumination, most of the ζ-carotenic strains were killed, but PG1 strain survived, due to the production of cyclic carotenoids with chromophores long enough to protect chlorophyll from photosensitized oxidation.

INTRODUCTION

ζ-Carotenic strains of several higher plants and algae have been described [1–4]. Such strains are characterized by the accumulation of ζ-carotene (7,8,7′,8′-tetrahydro-ψ,ψ-carotene) in place of the normal range of chloroplast carotenoids, and are usually killed by light. After treatment of the green alga, *Scenedesmus obliquus*, with the mutagen, *N*-methyl-*N*-nitroso-*N*′-nitroguanidine [5], a number of strains have been isolated which are affected in their ability to synthesize chloroplast pigments. Amongst these were several pale yellow–green strains which synthesized only low concentrations of chlorophyll and produced ζ-carotene as their major carotenoid, together with some phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene) and phytofluene (7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene) [6, 7].

There is now increasing evidence, summarized by Davies and Taylor [8] that the 15-*cis* isomers of phytoene, phytofluene and ζ-carotene may be important in carotenoid biosynthesis by plants and micro-organisms. We have examined the carotenes accumulated by some of the ζ-carotenic strains of *S. obliquus* in detail, by using a column chromatographic method to separate the isomers of phytoene, phytofluene and ζ-carotene present in the algal extracts. The properties of the two phytoene, two phytofluene and three ζ-carotene isomers that were isolated have been compared with those of similar isomers reported from other sources. The possible biosynthetic significance of the findings is discussed.

The effect of light on the ζ-carotenic strains of *S. obliquus* is compared with that reported for the ζ-carotenic mutants of other algae and higher plants.

RESULTS AND DISCUSSION

For the characterization of the carotenes the 1K strain of *S. obliquus*, a pale yellow–green mutant strain previously shown to accumulate ζ-carotene (480 nmol ml^{-1} packed cell volume), phytofluene (66 nmol ml^{-1}) and phytoene (200 nmol ml^{-1}) as its major carotenoids when grown in the dark [6], was used. The carotene fraction of an extract of 1K was subjected to column chromatography on activated neutral alumina and the carotene isomers were eluted with increasing concentrations of ether in petrol. Each eluted carotene was identified by its light absorption and mass spectra, which showed clearly that there were two isomers of phytoene, two of phytofluene and three of ζ-carotene.

Chromatography on activated alumina columns has been used to separate and identify the 15-*cis* and all-*trans* isomers of phytoene isolated from a number of organisms [9]; 15-*cis*-phytoene was eluted with 2% ether in petrol, whereas a more polar solvent mixture (4% ether in petrol) was required to elute the all-*trans* isomer. In the present work, the major phytoene fraction was eluted with 2% ether and the minor isomer with 4% ether in petrol, suggesting that the phytoene was present mainly as the 15-*cis* isomer accompanied by smaller amounts of all-*trans*-phytoene. The absorption spectra of the isomers were identical to those illustrated by Porter [10] for 15-*cis*- and all-*trans*-phytoene respectively. It would appear that this strain of *S. obliquus* like many other organisms [9] accumulates predominantly 15-*cis*-phytoene (at least 90% of the total) accompanied by small amounts of the all-*trans*- isomer.

Of the two phytofluene isomers, that eluted first from the column (with 10% ether in petrol) was present in larger amounts than the second isomer (eluted with 40% ether in petrol). Porter's group have reported that 15-*cis*-phytofluene is eluted from a 1% water-deactivated alumina column with 5% ether in petrol whereas the all-*trans* isomer requires 15% ether–petrol [11]. The spectroscopic properties of the *S. obliquus* phytofluene isomers were in agreement with their identification as

the 15-*cis* and all-*trans* isomers respectively, the spectra being very similar to those reported by Davis *et al.* [12].

The three ζ-carotene isomers, I, II, and III, eluted respectively with 15%, 25% and 80% ether in petrol, had identical mass spectra, each having a strong fragment ion at M-137 due to cleavage of the 'bis-allylic' C-7,8 and C-7',8' bonds, but no M-205 fragment ion that would be produced by cleavage of the C-11,12 bond of the unsymmetrical isomer 7,8,11,12-tetrahydro-ψ,ψ-carotene [13]. ζ-Carotene-III, the isomer present in the highest concentration, had an absorption spectrum identical to that reported by Davis *et al.* [12] for all-*trans*-ζ-carotene. In particular, there was virtually no absorption in the '*cis*-peak' region at 286, 296 nm, and the ratio of the extinction at 401 nm to that at 420 nm was 0.98 (Davis *et al.* [12] report $E_{401}/E_{420} = 0.97$).

The possibility was considered that one or both of the minor ζ-carotene isomers could be a cyclic compound. The only cyclic isomer of ζ-carotene reliably characterized to date is 7', 8', 11', 12'-tetrahydro-β,ψ-carotene isolated from diphenylamine-inhibited cultures of a *Phycomyces blakesleeanus* mutant [14]. This compound, however, can readily be distinguished from ζ-carotene by its absorption spectrum (λ_{max} 358, 378, 397 nm). A bicyclic structure, previously suggested as a possible structure for 'η-carotene' isolated from berries of honeysuckle (*Lonicera japonica*) [15] would have absorption and mass spectra very similar to those of the acylic ζ-carotene; moreover such cyclic derivatives would be expected to be less strongly adsorbed on alumina than would all-*trans*-ζ-carotene. The possibility that one of the ζ-carotene isomers could be such a cyclic derivative was examined by hydrogenating samples of all three ζ-carotene isomers and analysing the products by GLC. [16]. In all cases the product chromatographed with perhydrolycopene and was clearly separated from perhydro-γ-carotene and perhydro-β-carotene, showing all the ζ-carotene isomers to be acyclic compounds.

On iodine-catalyzed photoisomerization, all three ζ-carotene isomers gave equilibrium mixtures with the same absorption spectrum (λ_{max} 376, 395, 424 nm) confirming that they were all geometrical isomers of ζ-carotene. In the case of all *trans*-ζ-carotene (isomer III), the absorption maxima shifted, as expected, to slightly lower wavelengths, and '*cis*-peaks' appeared at 286 and 296 nm. The spectra of the two minor ζ-carotenes exhibited strong absorption in the '*cis*-peak' region, and the intensity of this absorption was decreased by the iodine isomerization, behaviour characteristic of *cis* isomers. From its absorption spectrum, ζ-carotene II is considered to be 15-*cis*-ζ-carotene. The positions of the absorption maxima (286, 296, 377, 397, 422 nm) and the ratios of the extinction values at these wavelengths all agree with the values reported by Davis *et al.* [12] for synthetic 15-*cis*-ζ-carotene. The third isomer, ζ-carotene I (λ_{max} 286, 296, 379, 400, 425) appears also to be a *cis* isomer of ζ-carotene. It is not the 15-*cis* isomer, and it differs from all the other ζ-carotene isomers reported previously, e.g. poly-*cis*-ζ-carotene of tangerine tomatoes [17, 18] and *neo*-A-ζ-carotene [12]. Column chromatographic examination showed that ζ-carotene I was not present in the pseudoequilibrium mixture of isomers formed by iodine-catalyzed photoisomerization of any of the *S. obliquus* ζ-carotenes. This suggests that ζ-carotene I is unlikely to have been formed artificially by isomerization of one of the other isomers.

Table 1. Concentrations of ζ-carotene isomers in some ζ-carotenic strains of *Scenedesmus obliquus*, cultured heterotrophically in the dark.

S. obliquus strain	Carotene concentration (nmoles ml^{-1} packed cell vol) ζ-Carotene I	ζ-Carotene-II (15-*cis*)	ζ-Carotene-III (all-*trans*)
1K	23	84	363
11	18	100	343
PG1	21	24	352
C51	33	36	330
1E	22	33	182

The iodine isomerization mixture formed from all three isomers invariably contained at least one component that was very weakly adsorbed on activated alumina (eluted with 15% ether–petrol) and had an unusual absorption spectrum with λ_{max} at 354, 374, 396 nm suggestive of a conjugated hexaene rather than a heptaene chromophore. The mass spectrum of this compound showed it to be an isomer of ζ-carotene, with MW 540, and, like ζ-carotene, to produce a characteristic fragment ion at M − 137. The adsorption properties on alumina, and on Si gel and MgO TLC systems suggested that it might contain some form of cyclic end-group. The nature of this product, however, was not investigated further.

The carotenes accumulated by several other ζ-carotenic strains of *S. obliquus* were examined (Table 1). The same isomers were also present in all these strains, and in all cases the main carotenoid was all-*trans*-ζ-carotene, phytoene was present almost entirely as the 15-*cis* isomer, and phytofluene predominantly as the 15-*cis* isomer. Quantitative variations in the concentrations of the ζ-carotene isomers between the different strains were relatively small.

There is considerable confusion about the roles of *cis* and *trans* isomers of phytoene, phytofluene and ζ-carotene in carotene biosynthesis. With the exception of some non-photosynthetic bacteria [19], all plants and micro-organisms so far examined accumulate 15-*cis*-phytoene, together with, in most cases, only small amounts of the all-*trans* isomer [9]. Gregonis and Rilling [20] have shown that the stereochemistry of hydrogen loss differs in the formation of these two phytoene isomers. On the question of which isomers of phytoene, phytofluene and ζ-carotene may be biosynthetic intermediates, different workers have arrived at different conclusions. Porter [21] suggests that in higher plants (particularly tomato fruit) the sequence 15-*cis*-phytoene → 15-*cis*-phytofluene → all *trans*-phytofluene → all-*trans*-ζ-carotene is the main route, whereas in *Halobacterium cutirubrum* only the *trans* isomers are involved [22]. In the case of *Phycomyces blakesleeanus* [23] and *Flavobacterium* R1560 [24] cell-free preparations, 15-*cis* to all-*trans* isomerization at the level of phytoene has been suggested. The relative amounts of the various isomers of phytoene, phytofluene and ζ-carotene isolated from the ζ-carotenic strains of *S. obliquus* suggest that the situation in these strains may be similar to that seen in the higher plant system of Porter [21].

The carotenoid compositions described above were those of dark heterotrophically-grown cultures of the *S. obliquus* mutant strains. With the exception of PG1 all these ζ-carotenic strains were killed when illuminated

under aerobic conditions. Krinsky [25] has discussed the possible role of carotenoids in protecting both photosynthetic and non-photosynthetic organisms against the potentially harmful or lethal effects of photosensitized oxidation. In the bacterial systems studied most extensively, protection against photosensitized oxidation is only afforded by those carotenoids that have a chromophore of more than seven conjugated double bonds and hence absorb light of visible wavelengths; ζ-carotene has been found not to be effective. Thus the sensitivity to light and oxidation displayed by most strains of *S. obliquus* in which ζ-carotene is the main carotenoid and only trace amounts of any carotenoid with a chromophore longer than seven conjugated double bonds are present, is in agreement with the conclusions reached by Krinsky [25]. The PG1 strain, however, responded in a completely different way to illumination. This strain was not killed on transfer to the light, but was able to form a normal chloroplast with pigment composition similar to that of the wild type strain. These changes in pigment composition occurring when the dark grown *S. obliquus* PG1 is illuminated have been examined [7].

Several ζ-carotenic strains of other algae and higher plants have been described. Claes [3, 4] worked extensively with a series of X-ray-produced mutant strains of *Chlorella vulgaris*. Some of these strains, 5/515, 5/520 and 9a, were similar to the ζ-carotenic strains of *S. obliquus* in that, when cultured heterotrophically, they accumulated ζ-carotene as their major carotenoid. The 5/520 strain was remarkable because it also produced appreciable quantities of the poly-*cis* carotenoids, prolycopene and proneurosporene. ζ-Carotenic strains of maize and sunflower have also been studied extensively [1, 2].

In the *S. obliquus* mutant strains described in this paper, and the mutant PG6, a preliminary description of which has been published previously [26], the only chlorophyll detected in cultures grown in the dark was chlorophyll *a* and this appeared to be present, *in vivo*, in only one form with an absorption maximum at 672 nm. This is also the case with the *Chlorella* mutant 5/871 [27] and the ζ-carotenic mutant of maize [28].

EXPERIMENTAL

Strains of *S. obliquus*, defective in chloroplast pigment synthesis (PG1, 1E, 1I, 1K and C51), were isolated by chemical mutation of the wild type strain [5] and selected as a result of their difference in colour when compared with the wild type strain. All strains were cultured heterotrophically in the dark at 30° on NO_3 medium, supplemented with 0.5% glucose and 0.25% yeast extract [29]. The algal strains were harvested in the late log-phase of growth and the packed cell volume was determined by centrifugation of an aliquot. The lipid from approximately 13 ml packed cell volume of each of the strains was repeatedly extracted with hot MeOH until all the pigments were removed. The lipid was transferred to Et_2O and dried over anhydrous Na_2SO_4. The Et_2O extract was evapd to dryness *in vacuo*, made to 12 ml in petrol (bp 40–60°) and 4 ml of this soln was chromatographed on a column of neutral Al_2O_3 (10 g, activity Grade III). The carotenes were eluted with 2% Et_2O in petrol (E–P). After evaporation to dryness and dissolution in petrol, the individual carotenes were separated by further chromatography on a column of neutral Al_2O_3 (15 g, Activity Grade I). Carotenes were progressively eluted with increasing concentrations of Et_2O in petrol. The eluting solvents used were 2, 4, 10, 15, 25, 40, 60, 80% E–P (100 ml of each) and 25 ml fractions were collected. The carotenes present in each fraction were identified from their characteristic absorption spectra. All the fractions containing a specific carotene were bulked and the carotene concentrations were determined from their light absorption spectra. $E^{1\%}_{1\,cm}$ values used were: phytoene 1250 at 285 nm [30], phytofluene 1577 at 347 nm [12], ζ-carotene 2555 at 400 nm [12]. MS were determined by Mr. G. Harriman on an AEI MS12 instrument with direct insertion probe, at an ion source temperature of 180–220°. GLC and hydrogenation were performed as described by ref. [16]. I_2 catalyzed photoisomerization was as described by ref. [31]. I_2 was removed by washing the soln with 2% (w/v) aq. Na thiosulphate.

REFERENCES

1. Faludi-Dániel, A., Frovalszky, L. and Gyurján, I. (1968) *Planta* **78**, 184.
2. Walles, B. (1965) in *Biochemistry of Chloroplasts* (Goodwin, T. W. ed.) Vol. 2, p. 633. Academic Press, New York.
3. Claes, H. (1954) *Z. Naturforsch.* **9b**, 461.
4. Claes, H. (1957) *Z. Naturforsch.* **12b**, 401.
5. Bishop, N. I. (1971) *Methods Enzymol.* **23**, 130.
6. Powls, R. and Britton, G. (1977) *Arch. Microbiol.* in press.
7. Britton, G., Powls, R. and Schultze, R. M. (1977) *Arch. Microbiol.* in press.
8. Davies, B. H. and Taylor, R. F. (1976) *Pure Appl. Chem.* **47**, 211.
9. Aung Than, Bramley, P. M., Davies, B. H. and Rees, A. F. (1972) *Phytochemistry* **11**, 3187.
10. Porter, J. W. (1969) *Pure Appl. Chem.* **20**, 449.
11. Qureshi, A. A., Andrewes, A. G., Qureshi, N. and Porter, J. W. (1974) *Arch. Biochem. Biophys.* **162**, 93.
12. Davis, J. B., Jackman, L. M., Siddons, P. T. and Weedon, B. C. L. (1966) *J. Chem. Soc. C*, 2154.
13. Davies, B. H., Holmes, E. A., Loeber, D. E., Toube, T. P. and Weedon, B. C. L. (1969) *J. Chem. Soc. C*, 1266.
14. Davies, B. H. and Rees, A. F. (1973) *Phytochemistry* **12**, 2745.
15. Goodwin, T. W. (1952) *Biochem. J.* **51**, 458.
16. Taylor, R. F. and Davies, B. H. (1975) *J. Chromatog.* **103**, 327.
17. Raymundo, L. C. and Simpson, K. L. (1972) *Phytochemistry* **11**, 397.
18. Qureshi, A. A., Qureshi, N., Kim, M. and Porter, J. W. (1974) *Arch. Biochem. Biophys.* **162**, 117.
19. Weeks, O. B. (1971) in *Aspects of Terpenoid Chemistry and Biochemistry* (Goodwin T. W. ed.) p. 301. Academic Press, New York.
20. Gregonis, D. E. and Rilling, H. C. (1974) *Biochemistry* **13**, 1538.
21. Qureshi, A. A., Kim, M., Qureshi, N. and Porter, J. W. (1974) *Arch. Biochem. Biophys.* **162**, 108.
22. Kushwaha, S. C., Kates, M. and Porter, J. W. (1975) Abstr. Contributed Papers Fourth Internat. Symp. on Carotenoids, Berne, p. 29.
23. Davies, B. H. (1973) *Pure Appl. Chem.* **35**, 1.
24. Brown, D. J., Britton, G. and Goodwin, T. W. (1975) *Trans. Biochem. Soc.* **3**, 741.
25. Krinsky, N. I. (1971) in *Carotenoids* (Isler, O. ed.) p. 667. Birkhäuser, Basel.
26. Powls, R. (1972) Proc. 2nd Internat. Congr. Photosynthesis Research **3**, 2611.
27. French, C. S. and Prager, L. (1969) in *Progress in Photosynthesis Research* (Metzner, H. ed.). Vol. 2, p. 555.
28. Faludi-Dániel, A., Nagy, A. H. and Nagy, A. (1969) in *Progress in Photosynthesis Research* (Metzner, H. ed.). Vol. 2, p. 592.
29. Kessler, A., Arthur, W. and Brugger, J. E. (1957) *Arch. Biochem. Biophys.* **71**, 326.
30. Davies, B. H. (1965) in *Chemistry and Biochemistry of Plant Pigments* (Goodwin, T. W. ed.) p. 489. Academic Press, New York.
31. Zeichmeister, L. (1962) Cis–Trans *Isomeric Carotenoids, Vitamins A and Arylpolyenes*. Springer Verlag, Vienna.