The Carotenoid Hydrocarbons of *Euglena gracilis* and Derived Mutants

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Received for publication August 5, 1974 and in revised form October 11, 1974

ABSTRACT

An examination of the carotene fractions extracted from *Euglena gracilis* Z and pressure-bleached *Euglena* mutants PR-1, PR-2, PR-3, and PR-4 revealed phytoene in mutants PR-1, PR-2, and PR-3. Photosynthetic *E. gracilis* Z cultured at different light intensities showed no detectable phytoene, nor was phytoene found in mutant PR-4. However, dark-cultured *E. gracilis* Z yielded readily assayable amounts of phytoene. With the exception of PR-4, in which no C_w carotenoids were detected, the following carotenes were identified in all from their mass spectra: phytoene, phytofluene, α-carotene, β-zeaxarotene, and β-carotene. Of these, phytoene and β-zeaxarotene had not previously been unequivocally identified in *Euglena*.

In early studies of the carotenoid pigments of *Euglena*, phytoene, the presumed precursor of the C_w carotenoids, was not reported from any wild type or mutant species (6, 7, 12). These results led to the suspicion that in *Euglena*, phytoene might have been bypassed until it was recently reported that there is evidence for the presence of phytoene in mutant strains SM-L1, PBZ-G3, and HB-G of *E. gracilis* var. *bacillaris* (10) which are known to synthesize an altered pattern of unsaturated carotenoids (6). However, the same study demonstrated that phytoene is absent from mutant PBZ-G4 and from *Astasia longa*, in neither of which could any C_w compounds be identified (10). Kivic and Vesek (11) have shown that those mutants which synthesized carotenoids contained plastids, albeit immature ones, whereas those which failed to synthesize carotenoids and *Astasia* are aplastic. The implication is that carotenoid synthesis in the flagellate is solely intraplastidic or at least depends upon the presence of a plastid system.

The similarity of the carotenoid patterns of the *bacillaris* and Z strains of *E. gracilis* (12) suggested that a re-examination of the pigments of the Z strain and some of its mutants might prove fruitful in determining the ubiquity of phytoene as well as the relationship between carotenogenesis and plastids.

Since several of these mutants have also been studied for the presence of plastids by Kivic and Vesek (11). Therefore, we selected for experimentation the photosynthetic wild type *E. gracilis* Z and the nonphotosynthetic mutants PR-1, -2, and -3 (8) and PR-4, all of which were derived from the wild type after being subjected to high hydrostatic pressure. The carotenoids of these organisms were isolated chromatographically and identified from R_p values (2), co-chromatography with authentic samples, absorption spectra (3), and mass spectra (13, 14).

MATERIALS AND METHODS

Cell Culture Conditions. Cultures of *Euglena gracilis* Klebs strain Z and mutants were grown in 7-liter batches in aerated 3-gallon carboys. In all cases, growth temperature was 27 ± 2°C under continuous illumination from fluorescent lights.

Illumination energy was measured with a YSI Model 65 radiometer and a Model 6551 probe. For low light experiments, illumination was from two 40-w cool white and two 40-w day-lite lamps 51 cm above the culture surface yielding 9 × 10^8 ergs cm^-2 sec^-1 at surface level with the probe facing the light source. For high light experiments, four additional 40-w cool white lamps, a pair on each side, at 18 cm from the culture vessels were used. The impinging energy taken as the sum of the values from the left, right, and above was 2.4 × 10^9 ergs cm^-2 sec^-1. For dark experiments, cultures were protected from the light with several layers of opaque, black plastic; they were otherwise subjected to conditions identical to those of illuminated cultures.

Pigment Extraction. Cells were harvested by centrifugation with the aid of a Sorvall KSB continuous flow apparatus at 17000g and a flow rate of 500 to 600 ml/min. Pellets were washed once in cold-distilled H_2O and extracted with acetone according to Gross and Stroz (10). Each extract was transferred to diethyl ether, evaporated, and the residue was saponified by the standard procedure (2). The unsaponifiable material from each sample was chromatographed on a column of neutral alumina (Brockman grade III), and the hydrocarbons were eluted with 0.5% (v/v) ether in petroleum ether (b.p. 30–60°C). Phytoene and β-carotene concentrations were estimated from this fraction using the E_2_at values of 1250 for phytoene at 285 nm and 2505 for β-carotene at 450 nm (3). A Unicam SP 800 or Cary-14 recording spectrophotometer was used for determination of all spectra.

Pigment Identification. The standard methods of Britton and Goodwin (2) were used to isolate and identify the carotenoids. The individual carotenes were separated from alumina column chromatographic fractions by TLC on MgO: Kiesel-
guhr G (1:1) with petroleum ether as the developing solvent. This procedure yielded three bands; a UV-absorbing band (Rr 0.95), a greenish white fluorescing band under UV (Rr 0.6-0.7) and a yellow colored band (Rr 0-0.3). TLC of the first two bands on Silica Gel GF254 with petroleum ether as developing solvent yielded phytoene and phytofluene, respectively; the third band gave three fractions identified as $\beta$-carotene (Rr 0.5), $\beta$-zeacarotene (Rr 0.4), and $\zeta$-carotene (Rr 0.35).

The bands eluted from TLC were assayed in the Unicam SP-800 spectrophotometer and were subsequently analyzed in an AEL, MS-12, mass spectrometer at 70 and 12 eV, respectively, using a direct insertion probe and a source temperature of 200 C. The mass spectrometer is capable of identifying amounts of carotenoids of the order of less than $1 \times 10^{-6}$ g.

## RESULTS

The absorption spectra of the initial acetone extracts from all the mutants studied had characteristic peaks at 272, 282, and 293 nm, closely resembling but not identical to the absorption maxima of phytoene. It was confirmed by elution of the band remaining at the origin after silica gel TLC and petroleum ether as the developing solvent, and by co-chromatography with authentic ergosterol (Calbiochem), that the observed peaks were caused by large amounts of ergosterol which tended to mask the phytoene spectrum in agreement with previous results (10).

From the absorption spectra of the hydrocarbon fractions, however, it was clear that three of the mutants, PR-1, -2, and -3 each contained phytoene ($\lambda_{\text{max}}$ at 275, 285, 296 nm) as did the dark-grown Z strain. No phytoene was detected in PR-4 or in the light-grown, photosynthetically active Z strain, although the latter contained large amounts of $\beta$-carotene. Further chromatography of the hydrocarbon fractions of each mutant and the dark-grown extract allowed phytoene, phytofluene, $\zeta$-carotene, $\beta$-carotene, and $\beta$-zeacarotene to be isolated. Each compound was identified by comparison of its absorption and mass spectra and chromatographic behavior with those of authentic samples.

Thus, phytoene had $\lambda_{\text{max}}$ (petroleum ether) at 275, 285, and 296 nm (3), and the mass spectra (13, 14) had the parent ion, $M^+$, at 544 (50%, C$_{40}$H$_{50}$) with fragment ions at $m/e$ 450 (1%, M-94; metastable at $m/e$ 372, 450/544 = 372.2), and at $m/e$ 339 (36%, M-205; metastable at $m/e$ 211, 339/544 = 211.3). The major fragmentation (M-205) was due to cleavage of the C-11, 12 bonds allyclic to the triene chromophore.

Phytofluene, with $\lambda_{\text{max}}$ at 331, 348, and 367 nm (3), had its parent ion, $M^+$, at $m/e$ 542 (39%, C$_{40}$H$_{50}$) and major fragment ions at $m/e$ 405 (3%, M-137; metastable at $m/e$ 303, 405/542 = 302.6) and at $m/e$ 337 (10%, M-205; metastable at $m/e$ 210, 337/542 = 209.5) due to cleavage of the bisallylic bonds at C-7,8 and C-11',12', respectively (13, 14).

The $\lambda_{\text{max}}$ for $\beta$-carotene were at 425, 450, and 476 nm (3) and the parent ion, $M^+$, was at $m/e$ 536 (89%, C$_{40}$H$_{50}$) with fragments at $m/e$ 444 (8%, M-92; metastable at $m/e$ 368, 444/536 = 367.8 and at $m/e$ 430 (2%, M-106) (13).

$\beta$-Zeacarotene had $\lambda_{\text{max}}$ at 405, 427, and 452 nm (3) and $M^+$ at $m/e$ 538 (100%, C$_{40}$H$_{50}$) with major fragment ions at $m/e$ 446 (1%, M-92; metastable at $m/e$ 370, 446/538 = 369.7) and at $m/e$ 401 (6%, M-137; metastable at $m/e$ 299, 401/538 = 298.9) due to cleavage of the bisallylic C-7',8' bonds (13).

$\zeta$-Carotene had $\lambda_{\text{max}}$ at 378, 400 and 424 nm (3, 13) and $M^+$ at $m/e$ 540 (72%, C$_{40}$H$_{50}$) with a major fragment ion at $m/e$ 403 (12%, M-137; metastable at $m/e$ 301, 403/540 = 300.8) reflecting cleavage of the bisallylic bonds at C-7,8 and C-7',8' adjacent to the main chromophore (13, 14). This result and the absence of strong fragment ions at $m/e$ 471 (M-69) and $m/e$ 335 (M-205) identify this compound as the symmetrical $\zeta$-carotene (7,8,7',8'-tetrahydro-\(\psi\), \(\psi\)-carotene) rather than the unsymmetrical 7,8,11,12-tetrahydro-\(\psi\), \(\psi\)-carotene found in some bacteria (4, 13).

All the colored mutants studied, as well as the dark-grown wild type, gave like results. On the other hand, in the light-grown, actively photosynthetic stock, no phytoene was evident. Even though phytofluene is often a minor component in comparison with phytoene, phytofluene could be detected as could the other carotenoid hydrocarbons. Carotenoids were not detected in the white mutant, PR-4.

The effect of light intensity on carotenoids was estimated from the respective $E_{\text{ilm}}$ values of phytoene and $\beta$-carotene. The results shown in Table I indicate that more $\beta$-carotene is formed in high than low light intensity in the mutants as well as in the light-grown parent strain corroborating earlier reports of stimulated carotenoid synthesis in light- as opposed to dark-grown Euglena (5, 7, 9). It is also clear that phytoene synthesis is light-stimulated in the mutants, whereas in the wild type, it is detectable only in dark-grown cells (Table I).

## DISCUSSION AND CONCLUSIONS

That phytoene is present in Euglena has now been established unequivocally from its mass spectra. It had not been detected in several early studies of Euglena carotenoids (6, 7, 12), probably due to the less satisfactory methods of separation and identification then in use. Although phytoene has not been detected in the actively photosynthetic Euglena, it is assumed to be there, albeit in amounts that fail to accumulate to detectable levels; thus the rate-limiting step in carotenogenesis would appear to occur just prior to phytoene in the photosynthetic flagellate so that all synthesized phytoene would be rapidly converted to the more unsaturated phytofluene. The clear-cut identification of phytoene in nonphotosynthetic, dark-grown organisms attests to its presence as a natural component.

Some mutants, like PBZ-G4 (10) and PR-4 apparently synthesize E$_{\text{ilm}}$ possibly as a way to detoxify whatever. Unlike their colored
counterparts which synthesize carotenoids and have been shown to contain proplastids, these mutants appear white and have been verified as plastidless (11).

The carotene pattern of the wild type Euglena and those mutants which do form carotenoids reveals no surprises, the common intermediates having been identified, including β-carotene which had not been demonstrated previously. Only quantitative differences have been uncovered among the carotenes.

Photostimulation of carotenogenesis has been reported in both photosynthetic and nonphotosynthetic organisms including Euglena (1, 5, 7, 9). The results presented in Table I support these reports. In addition, the present data demonstrate that there is also an illumination-dependent increase in phytoene.

Acknowledgments—We wish to thank Mr. J. R. Ireland (University of Liverpool) for technical assistance in determination of mass spectra, and Mr. Andre L. Curtis (Indiana State University) for invaluable aid and patience in the laboratory. We are grateful to Prof. T. W. Goodwin for discussions, review of the manuscript, and continued interest and encouragement during the course of this study.

LITERATURE CITED


