The Methylerythritol Phosphate Pathway Contributes to Carotenoid But Not Phytol Biosynthesis in *Euglena gracilis*

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The biosynthesis of diadinoxanthin and β-carotene in *Euglena gracilis* was examined using [1-^{13}C]-D-glucose and [5,5-$^{2}H_2$]-1-deoxy-D-xylulose. In contrast to previous studies on isoprenoid biosynthesis in *E. gracilis*, the results demonstrate a role for the methylerythritol phosphate (MEP) pathway, along with the mevalonate pathway, in carotenoid biosynthesis. Interestingly, the MEP pathway is not involved in the biosynthesis of phytol, a result not previously observed for other chloroplast-containing organisms.

Since the discovery of the methylerythritol phosphate (MEP) pathway to isoprenoids,[1−3] numerous investigations have demonstrated that carotenoids and the phytyl side chain of chlorophyll are synthesized either predominantly or exclusively from 1-deoxy-D-xylulose 5-phosphate (DXP) in higher plants,[4,5] algae,[6] and cyanobacteria.[7] In liverworts and hornworts, differential labeling of these isoprenoids occurs, with the mevalonate pathway labeling the farnesyl diphosphate (FPP)-derived portion of the molecules and the MEP pathway labeling the final isopentenyl diphosphate (IPP)-derived units.[8,9] A common theme that arises is that carotenoids and phytol are biosynthesized from the same pool of geranylgeranyl diphosphate (GGPP) and that chloroplast-containing organisms all harbor the methylerythritol phosphate pathway.[5] The only organism identified to date that does not follow this pattern is the phytoflagellate *Euglena gracilis*, which apparently synthesizes sterols and phytol exclusively via the mevalonate pathway.[5] This is a curious finding considering that *Euglena* is thought to have obtained its chloroplasts from a secondary endosymbiotic event in which a green alga was putatively engulfed.[10]

Studies on isoprenoid biosynthesis in *E. gracilis* have a long history. In 1960, experiments conducted with radiolabeled acetate and mevalonate suggested a mevalonate pathway to β-carotene, although incorporation levels of precursors were quite low.[11] Biosynthetic experiments in the 1970s using 1-^{13}C-acetate suggested phytol biosynthesis via the mevalonate pathway.[12] More recent experiments have supported this result and also noted the lack of direct incorporation of deoxyxylulose into phytol.[5] Further, ergosterol biosynthesis was found to occur via the mevalonate pathway, consistent with results observed for plants and several algae.[13,14] Carotenoid biosynthesis in *E. gracilis*, however, has not been examined thoroughly using stable-isotope precursors. An initial experiment on isoprenoid biosynthesis in *E. gracilis* that we conducted using 6,6-$^{2}H_2$-D-glucose, however, suggested that the MEP pathway might actually have a small contribution to carotenoid biosynthesis. This result, coupled with the report that indicated that *E. gracilis* did not utilize the MEP pathway, led to a more detailed reexamination of carotenoid biosynthesis in this organism.

Glucose labeled at position 1 (or 6) has been utilized by many groups to distinguish between the two pathways because of the divergent fate of the label (Figure 1).[14] Distinctive labeling at positions 2 and 4 of IPP is expected via the mevalonate pathway, while labeling at position 1 of IPP is indicative of the MEP pathway. The methyl group of IPP (C5) is labeled via both pathways. For the incubation experiments with *E. gracilis*, diadinoxanthin and β-carotene were the main carotenoids that were isolated and analyzed for incorporation of isotopes. An initial experiment with 6,6-$^{2}H_2$-D-glucose provided weak labeling of diadinoxanthin at H3 and H28, two positions derived from C1 of IPP, along with strong labeling of the carotenoid methyl groups (data not shown). While not diagnostic for the MEP pathway because indirect labeling from a deuterated NADPH pool could explain the C1 labeling via the mevalonate pathway, participation of the MEP pathway was clearly a possibility. An experiment with 1-^{13}C-glucose was conducted to provide further information about the use of the two pathways. When both carotenoids were examined after labeling with 1-^{13}C-glucose, it was apparent that more than one pathway were operating (Figure 2). Variable labeling of the different isoprene units was observed, with only position 3 of the isoprene unit not being labeled, a site not labeled by either pathway. The observation of labels at positions 1, 2, and 4, at varying levels, suggests that both pathways contribute to carotenoid biosynthesis. Interestingly, the phytol isolated in this experiment qualitatively displayed the same labeling at carbons derived from positions 2, 4, and 5 of IPP as observed by Disch et al.[14] This carotenoid result indicates a clear difference from the phytol labeling, and it strongly implies that the MEP pathway does indeed operate in *E. gracilis*.

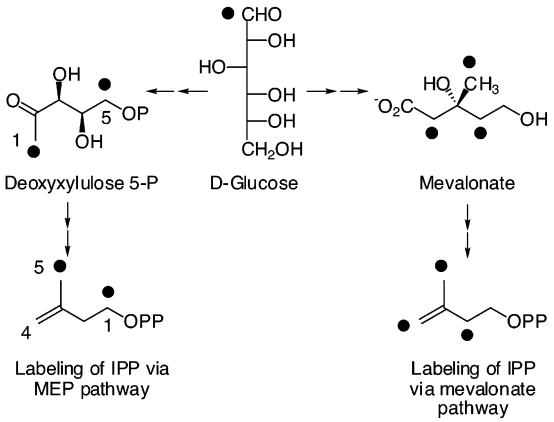

Figure 1. Fate of 1-^{13}C-glucose via the MEP and mevalonate pathways.

The culture medium for an additional experiment was supplemented with [5,5-$^{2}H_2$]-deoxyxylulose (DX) in order

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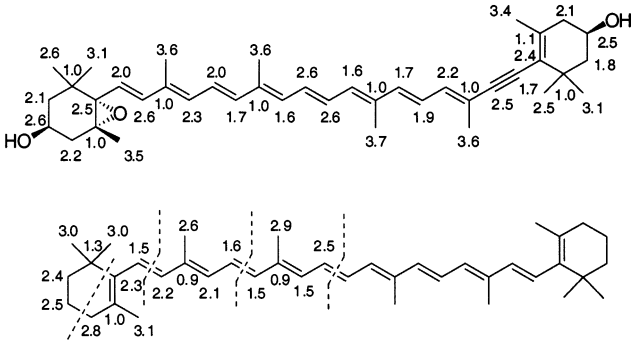

Figure 2. ^{13}C incorporation levels for diadinoxanthin (top) and β-carotene (bottom) labeled by 1-^{13}C-glucose. Dashed lines indicate the individual isoprene units.

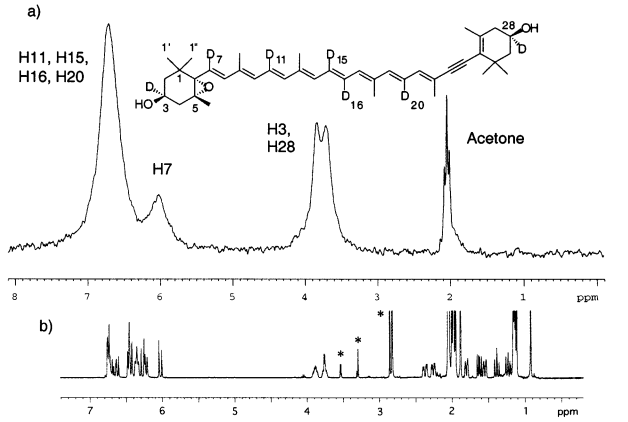

Figure 3. (a) ^2H NMR spectrum of diadinoxanthin labeled by [5,5-$^2H_2$]-deoxyxylulose; (b) ^1H NMR spectrum of diadinoxanthin in acetone-d_6. Peaks marked with an asterisk are not associated with diadinoxanthin. The methyl peaks have been truncated to fit the overall figure.

to confirm this result. If DX is incorporated intact, only labels at carbons derived from position 1 of IPP should be observed. If DX is metabolized prior to incorporation, less specific labeling might be encountered. Both diadinoxanthin and β-carotene were extensively labeled by DX, with deuterium only appearing at position 1 derived carbons (Figure 3). On the basis of integration of both ^1H and ^2H NMR spectra, the incorporation of deuterium at each position in diadinoxanthin was >70%, while the incorporation of β-carotene was ~40%. These high levels were confirmed by the mass spectrometric data. Strong signals were observed at $m/z = 589$ for diadinoxanthin and $m/z = 546$ for β-carotene. These ions correspond to M + 7 (diadinoxanthin MW = 582) and M + 10 (β-carotene MW = 536). These ions can arise only if each individual isoprene unit within the carotenoid has been labeled by [5,5-$^2H_2$]-DX. Apparently under the conditions of the incubation experiment, once *E. gracilis* has assimilated DX, there is very little dilution with endogenous DXP and the labeled DX is efficiently used for carotenoid synthesis. The high level of labeling and the specificity of incorporation remove any doubts that the MEP pathway contributes to carotenoid biosynthesis.

An additional finding from this labeled DX experiment involved phytoene, the universal precursor to carotenoids. During the purification of β-carotene, a related compound was found to coelute under the usual purification conditions. By using modified conditions, pure β-carotene was obtained, as well as the related compound, which was determined to be phytoene by NMR and mass spectral data. The phytoene was shown by deuterium NMR spectroscopy and MS to have extensive specific labeling from DX. This result was unexpected because under normal growth conditions phytoene does not accumulate to a significant extent to be isolated from the standard culture. It is clear that with the amount of deuterated DX used normal metabolism is altered. The accumulation of phytoene likely represents an isotope effect on the desaturation and/or cyclization steps that are involved in carotenoid biosynthesis. Phytol was also labeled with deuterium from [5,5-$^2H_2$]-DX, although to a much smaller extent than the carotenoids (<1%), and the labeling pattern observed for phytol was essentially the same as the pattern obtained with 6,6-$^2H_2$-glucose. This suggests that the deoxyxylulose is catabolized, presumably to acetate, prior to incorporation into the phytyl side chain.

As has been concluded by Lichtenthaler, the synthesis of isoprenoids in *E. gracilis* is unique.[5] The absence of a contribution of the MEP pathway to phytol biosynthesis is a distinct difference from other chloroplast-containing organisms. Although our results indicate that the MEP pathway is present, they do not explain the divergence of carotenoid and phytol biosynthesis. Further studies on phytol biosynthesis in *E. gracilis* will be reported separately.

Experimental Section

General Experimental Procedures. The labeled precursors 1-^{13}C-D-glucose and 6,6-$^2H_2$-D-glucose were purchased from Cambridge Isotope Laboratories, Inc. 1-Deoxy-5,5-$^2H_2$-D-xylulose was synthesized from diisopropyl-D-tartrate using a modification of a literature procedure.[15] Lithium aluminum deuteride was used in place of lithium aluminum hydride to introduce the deuterium labels. NMR spectra were recorded on a Bruker AM400 spectrometer operating at 400.13 MHz for ^1H, 100.13 MHz for ^{13}C, and 61.42 MHz for ^2H. NMR spectra were referenced to $CHCl_3$ or $CDCl_3$ at 7.26 ppm for ^1H and ^2H spectra, respectively, and to the centerline of the $CDCl_3$ triplet at 77.00 ppm for ^{13}C spectra. NMR spectra recorded in acetone or acetone-d_6 were referenced to 2.05 ppm for ^1H and ^2H NMR and 30.7 ppm for the centerline of the septet for ^{13}C NMR. The inverse-gated decoupling pulse sequence[16] was used for recording ^{13}C NMR spectra for determining ^{13}C isotopic enrichments. Incorporation levels for ^{13}C signals of isoprenoids were calculated on the basis of the intensities of the target peak relative to the intensity of an unenriched peak. This ratio was compared with the ratio for the corresponding peaks from the natural abundance ^{13}C NMR spectrum to determine the relative incorporation level. Levels of incorporation from the deuterium NMR spectrum were based on the integration of the natural abundance deuterium signal of $CHCl_3$ or acetone (0.016%).

Biological Material. An axenic culture of *Euglena gracilis* UTEX 753 was obtained from the University of Texas Culture Collection and was grown at 30 °C with continuous low-level lighting on a basal medium[17] supplemented with 1 g of D-glucose/L. This strain is equivalent to the *E. gracilis* Z strain that was used by Ahrens et al. (CCAP 1224/5Z).[12]

Biosynthetic Experiments. Seed cultures (200 mL) were inoculated with stock cultures and were grown in basal medium with 1 g/L of D-glucose (0.9 g/L unlabeled D-glucose + 0.1 g/L labeled glucose precursors). For the labeled DX experiment, 1 g of [5,5-$^2H_2$]-deoxyxylulose was added to the culture medium prior to inoculation. Production cultures of 5 L (^{13}C labeling) or 6 L (deuterium labeling) were aerated and grown at 30 °C for 7 days under constant illumination with 40 W cool-white fluorescent lights (200−210 ftc). Cultures were harvested by centrifugation at 7000 rpm (8670*g*), frozen at −80 °C, and lyophilized (dry wt 0.2−0.4 g/L). Due to an initial decrease in growth rate in the medium supplemented with labeled DX, the *E. gracilis* culture for this experiment was grown for 14 days prior to harvest.

Isolation of Carotenoids. The lyophilized cell material was extracted three times at room temperature with 2:1

$CHCl_3$−MeOH to provide a crude extract that was loaded onto a flash silica column. Stepwise elution with 20% EtOAc−hexanes, 40% EtOAc−hexanes, and 50% EtOAc−hexanes provided a β-carotene-containing fraction in the 20% eluent and a diadinoxanthin-containing fraction in 50% EtOAc−hexanes. After these fractions were concentrated, diadinoxanthin (2 mg/L av yield) was purified using a Sephadex LH-20 column with MeOH as eluent and β-carotene (1 mg/L av yield) was purified by preparative silica TLC with 4:4:1 cyclohexane−hexanes−toluene. When the [5,5-$^2H_2$]-DX incubation experiment was conducted, β-carotene was separated from phytoene by using preparative-RP-C18 TLC with a mixture of 20:35:45 petroleum ether−acetonitrile−MeOH. The purified carotenoids were analyzed by MS and NMR to confirm their identities. Phytol was obtained from the dark green 40% EtOAc−hexanes fraction after saponification overnight with KOH in MeOH (10% w/v). The crude phytol obtained from an ether extract of the saponification mixture was purified by flash silica chromatography using 15% EtOAc−hexanes. The isolated phytol was decolorized by passing the solution over a short column of charcoal and Florisil and washing with ether to provide 4 mg/L average yield of pure phytol.

The ^{1}H NMR assignments for diadinoxanthin in $CDCl_3$ and ^{1}H and ^{13}C spectra for β-carotene, phytol, and phytoene have been reported.[18−21] The ^{1}H NMR and ^{13}C NMR assignments for diadinoxanthin are based on HSQC and HMBC experiments. Diadinoxanthin: ^{1}H NMR (acetone-d_6): 6.75 (m, H15 + H16), 6.70 (m, H11), 6.64 (dd, J = 14.9, 11.5 Hz, H20), 6.47 (d, J = 11.5 Hz, H21), 6.44 (m, H12 + H19), 6.38 (br d, H14), 6.34 (br d, H17), 6.28 (d, J = 15.5 Hz, H8), 6.23 (d, J = 11.5 Hz, H10), 6.03 (d, J = 15.5 Hz, H7), 3.89 (m, H28), 3.76 (m, H3), 2.38 (dd, J = 17.5, 4.3 Hz, H27a), 2.26 (dd, J = 14.2, 4.6 Hz), 2.03 (m, H27b), 2.01 (s, H22′), 1.99 (s, H18′), 1.98 (s, H9′), 1.96 (s, H13′), 1.89 (s, H26′), 1.81 (br d, J = 10.6 Hz, H29a), 1.63 (dd, J = 14.2, 8.8 Hz, H4a), 1.57 (br d, J = 12.6 Hz, H2a), 1.39 (dd, J = 12.1, 12.0 Hz, H29b), 1.23 (m, H2b), 1.17 (s, H30″), 1.15 (s, H1″), 1.14 (s, H5′), 1.12 (s, H30′), 0.93 (s, H1′). ^{13}C NMR (acetone-d_6): 139.16 (C12), 138.97 (C19), 138.85 (C26), 137.87 (C8), 137.60 (C13), 137.15 (C18), 135.96 (C21), 135.49 (C9), 134.64 (C14), 133.82 (C17), 132.96 (C10), 131.67 (C15), 131.26 (C16), 126.04 (C7), 126.04 (C11), 125.20 (C20), 124.93 (C25), 119.92 (C22), 99.21 (C23), 90.25 (C24), 70.78 (C6), 67.47 (C5), 64.36 (C28), 63.83 (C3), 48.14 (C2), 47.66 (C29), 42.39 (C27), 41.97 (C4), 37.11 (C30), 35.88 (C1), 31.11 (C30″), 30.02 (C1″), 29.16 (C30′), 25.48 (C1′), 22.76 (C26′), 20.44 (C5′), 18.26 (C22′), 13.14, (C9′), 12.93 (C13′), 12.85 (C18′).

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Supporting Information Available: Deuterium NMR spectra of β-carotene, phytoene, and phytol and a listing of isotopomer intensities for the mass spectra of carotenoids. This material is available free of charge via the Internet at http://pubs.acs.org.

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