

## INHIBITION OF ASTAXANTHIN SYNTHESIS UNDER HIGH IRRADIANCE DOES NOT ABOLISH TRIACYLGLYCEROL ACCUMULATION IN THE GREEN ALGA *HAEMATOCOCCUS PLUVIALIS* (CHLOROPHYCEAE)<sup>1</sup>

Mirash Zhekisheva, Aliza Zarka, Inna Khozin-Goldberg, Zvi Cohen, and Sammy Boussiba<sup>2</sup>

Microalgal Biotechnology Laboratory, The Jacob Blaustein Institute for Desert Research, Ben-Gurion University of the Negev, Sede Boqer Campus, Sede Boqer 84990, Israel

Under stress conditions, *Haematococcus pluvialis* Flotow accumulates fatty acid–esterified astaxanthin, in extraplastidial lipid globules. The enhanced accumulation of fatty acids, mainly in triacylglycerols (TAG), among which oleic acid predominates, is linearly correlated with that of astaxanthin. We used inhibitors of either carotenoid or lipid biosynthesis to assess the interrelationship between carotenogenesis and TAG accumulation under high light irradiance as the stress factor. The two carotenogenesis inhibitors used—norflurazon, an inhibitor of phytoene desaturase, and diphenylamine (DPA), an inhibitor of  $\beta$ -carotene C-4 oxygenase—suppressed the accumulation of astaxanthin in a concentration-dependent manner. Concurrently, the accumulation of neutral lipids was significantly less affected. The lipid biosynthesis inhibitor sethoxydim, which inhibits acetyl-CoA carboxylase, significantly decreased *de novo* fatty acid synthesis and, in concert, drastically inhibited astaxanthin formation. In the presence of various concentrations of the three inhibitors, the inhibition of astaxanthin was not accompanied by a proportional decrease in oleic acid, which was used as a marker for TAG fatty acids. When astaxanthin synthesis was completely inhibited, the volumetric content of oleic acid was about 60% of the control value when the two carotenogenesis inhibitors (0.05  $\mu$ M norflurazon or 20  $\mu$ M DPA) were used and 27% of the control when the lipid-synthesis inhibitor (50  $\mu$ M) was used. We suggest therefore that TAG accumulation under high irradiance is not tightly coupled with astaxanthin accumulation, although the correlation between these two processes was demonstrated earlier. Furthermore, we propose that the accumulation of a certain amount of TAG is a prerequisite for the initiation of fatty acid–esterified astaxanthin accumulation in lipid globules.

**Key index words:** astaxanthin; diphenylamine; *Haematococcus pluvialis*; high light irradiance; norflurazon; oleic acid; sethoxydim; triacylglycerols

**Abbreviations:** ACCase, acetyl-CoA carboxylase; DPA, diphenylamine; GL, glycolipid; HL, high

light; NL, neutral lipid; PL, phospholipid; PUFA, polyunsaturated fatty acid; TAG, triacylglycerol

The ketocarotenoid astaxanthin, which is a secondary carotenoid, is widely used as a red colorant in marine fish aquaculture (Johnson and An 1991, Benemann 1992). Because of its high antioxidative activity, astaxanthin also has several clinical applications (Palozza and Krinsky 1992, Lorenz and Cysewski 2000, Osterlie et al. 2000). The richest natural source of this carotenoid is the green alga *Haematococcus pluvialis* Flotow (Chlorophyceae, order Volvocales), which accumulates astaxanthin up to 4% of its dry biomass, in extraplastidial lipid globules under various environmental stress conditions (Kobayashi et al. 1992, Tjahjono et al. 1994, Harker et al. 1996, Boussiba 2000, Sarada et al. 2002).

Carotenoid biosynthesis in higher plants and green algae occurs via the following pathway: synthesis of the five-carbon building units isopentenyl diphosphate and its isomer dimethylallyldiphosphate, condensation of the C5 units, formation of phytoene, desaturation, cyclization, and modification (Lichtenthaler 1999). Several inhibitors of carotenogenesis were used for elucidating the biosynthetic pathway of carotenoids in microalgae. Fosmidomycin, an inhibitor of 1-deoxy-D-xylulose-5-phosphate reductoisomerase, a plastid enzyme of the nonmevalonate pathway leading to IPP formation, suppressed astaxanthin synthesis in flagellates of *H. pluvialis* (Hagen and Grünwald 2000). Norflurazon is an inhibitor of phytoene desaturase, which is the first enzyme responsible for the introduction of the conjugated double bonds (Böger and Sandmann 1983). In *H. pluvialis*, norflurazon at 100  $\mu$ M inhibited the accumulation of secondary carotenoids, resulting in the accumulation of high levels of phytoene, accounting for 60% of total carotenoids (Harker and Young 1995). In *Dunaliella bardawil*, the desaturation of phytoene to phytofluene was inhibited by 0.3  $\mu$ M norflurazon, resulting in the accumulation of phytoene, whereas at somewhat lower concentration (0.1  $\mu$ M) the accumulation of phytofluene was also detected (Shaish et al. 1990). Diphenylamine (DPA), another carotenogenesis inhibitor that inhibits  $\beta$ -carotene C-4 oxygenase, when applied at a low concentration (30  $\mu$ M) specifically inhibited the conversion of

<sup>1</sup>Received 20 January 2005. Accepted 30 April 2005.

<sup>2</sup>Author for correspondence: e-mail sammy@bgu.ac.il.

$\beta$ -carotene to ketocarotenoid in *H. pluvialis* and consequently also the synthesis of astaxanthin, resulting in the accumulation of  $\beta$ -carotene (Fan et al. 1995). At higher concentrations (60 and 90  $\mu\text{M}$ ), phytoene, rather than  $\beta$ -carotene, was found by the same authors to accumulate, indicating the inhibition of an earlier step (e.g. desaturation). However, Harker and Young (1995) demonstrated the accumulation of  $\beta$ -carotene in cells treated with 100  $\mu\text{M}$  DPA. In *D. bardawil*, DPA (40.6  $\mu\text{M}$ ) inhibited the accumulation of  $\beta$ -carotene with the accompanied accumulation of phytoene (Vorst et al. 1994).

The capability of *H. pluvialis* to accumulate high amounts of astaxanthin was suggested to be due to its ability to deposit the fatty acid-esterified pigment in cytoplasmic lipid globules (Sprey 1970, Boussiba 2000). Zhekisheva et al. (2002) reported that intensive *de novo* fatty acid synthesis occurs when cells accumulate astaxanthin. Under nitrogen starvation or high irradiance, the accumulation of oleic acid in lipids, mainly in triacylglycerols (TAG), was linearly correlated with the accumulation of astaxanthin monoesters (Zhekisheva et al. 2002). Furthermore, the inhibition of fatty acid synthesis by cerulenin, an inhibitor of 3-ketoacyl-acyl carrier protein synthase, resulted in the inhibition of astaxanthin accumulation in *H. pluvialis* under high light (Schoefs et al. 2001).

The first step of the *de novo* fatty acid synthesis in chloroplasts, catalyzed by acetyl-CoA carboxylase (ACC-Case), is the formation of malonyl CoA. In most higher plants, two forms of ACCase were identified: a prokaryotic multi-subunit form, localized in the chloroplast, and an eukaryotic multidomain form, found in the cytosol. The herbicide sethoxydim inhibits ACCase activity and disrupts acyl lipid biosynthesis specifically in grasses possessing the multidomain form in both plastids and cytosol (Konishi and Sasaki 1994, Harwood 1999). When this herbicide was administrated to *D. bardawil*, it inhibited both lipid and  $\beta$ -carotene accumulation (Rabbani et al. 1998). The multidomain form of ACCase was demonstrated in *Isochrysis galbana* (Prymnesiophyceae) (Livne and Sukenik 1992) and in the diatom *Cyclotella cryptica* (Roessler 1990).

Although astaxanthin accumulation was previously shown to be correlated with fatty acid biosynthesis in *H. pluvialis*, the detailed interdependence between both processes has not been investigated. This study aimed to clarify this issue by the use of the carotenogenesis inhibitors, norflurazon and DPA, and the fatty acid synthesis inhibitor sethoxydim.

#### MATERIALS AND METHODS

**Materials.** *Haematococcus pluvialis* Flotow 1844 em. Wille K-0084 was obtained from the Scandinavia Culture Center for Algae and Protozoa at the University of Copenhagen, Denmark.

**Growth conditions.** Algal cultures were cultivated in 4-cm-wide 600-mL glass columns containing 500 mL of modified BG-11 medium (Boussiba and Vonshak 1991) that were placed in a temperature-regulated water bath at 25° C. The

culture medium was aerated with air stream enriched with 1.5% CO<sub>2</sub>. Illumination was provided by cool-white fluorescent lamps (20 W) external to the water bath. Irradiance was measured at the center of the column with a quantum meter (Lambda L1-185, Lambda Probes and Diagnostics, Graz, Austria). Cultures of nonflagellated green cells were cultivated for 5 days at a light intensity of 75  $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ , resuspended in modified BG-11 medium to a cell number of  $2 \times 10^5$  cells  $\cdot \text{mL}^{-1}$ , and grown under astaxanthin-inductive conditions, that is, photon flux density of 350  $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  (high light, HL). As a result, the cells became red. On the basis of our previous finding that cells growing at a light intensity of 75  $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  for 24 h contain the same amount of secondary carotenoids and lipids as in zero time cells (Zhekisheva et al. 2002), the latter were used as the noninduced control. Likewise, based on another finding that during the 24 h of HL intense accumulation of carotenoids and lipids took place without an accompanying change in cell number, the volumetric content of the pigments and lipids reported here relates to their cellular content.

**Measurements of growth parameters and pigment content.** Growth parameters were measured in culture samples immediately after the end of 24-h exposure to HL. Chlorophyll and total carotenoids were determined by spectrophotometer, as described previously (Zhekisheva et al. 2002).

**Pigment analysis.** The pigments were resolved by HPLC with a Varian chromatography system equipped with a Prostar 240 solvent-delivery module and Prostar 330 photodiode array detector (Varian Analytical Instruments, Walnut Creek, CA, USA). Cells were ground with mortar and pestle under liquid nitrogen and extracted with methanol. The pigment extract was filtered through 0.45- $\mu\text{m}$  filters and separated according to Yuan et al. (1997) by using a C18 reverse-phase column (5  $\mu\text{m}$ , 250 mm Lichrosphere 100, Merck, Darmstadt, Germany). Esterified astaxanthin and  $\beta$ -carotene were quantified by integrating the peak area and comparing it with commercial standards (Sigma Chemical Co., St. Louis, MO, USA). Phytoene, which was separated from the total extract on SEP-PAK cartridges (Waters, Milford, MA, USA) by elution in 5% diethyl ether in hexane, was quantified by spectrophotometer using an absorbance coefficient in hexane of  $E_{1\%}^{1\text{cm}} = 915$ .

**Lipid extraction.** Biomass was harvested, centrifuged, and lyophilized. Samples (50 mg) were extracted with 200  $\mu\text{L}$  DMSO for 5 min at 70° C and further extracted with 5 mL of methanol at 4° C for 1 h. For phase separation, peroxide-free diethyl ether containing 0.01% butylated hydroxytoluene, hexane, and water was added to the methanol extract at a volumetric ratio of 1:1:1:1 (Zhekisheva et al. 2002). The lipid-containing upper phase was evaporated to dryness under vacuum and kept at -20° C under argon atmosphere in a small volume of chloroform.

**Lipid fractionation.** Total lipid extracts were fractionated into three classes—neutral lipids (NL), glycolipids (GL), and phospholipids (PL)—on silica cartridges (Bond-Elute JR-SI, Varian) by sequential elution with chloroform, acetone, and methanol, as previously described (Cohen et al. 1992). Neutral lipids were further resolved to TAG, diacylglycerols, unesterified fatty acids, and astaxanthin esters by TLC (silica gel 60, with a concentrating zone, 20  $\times$  20-cm plates, 0.25 mm thickness, Macherey-Nagel, Düren, France) using a solvent system of petroleum ether:diethyl ether:acetic acid (70:30:1, v/v). Lipids were visualized by brief exposure to I<sub>2</sub> vapors and were identified by comparison with the R<sub>f</sub> of standards.

**Fatty acid analysis.** Samples of freeze-dried biomass, lipid extracts, or individual lipids were transmethylated with 2% H<sub>2</sub>SO<sub>4</sub> in dry methanol under argon atmosphere at 80° C for 1.5 h. Toluene was added (10%, v/v) to facilitate solubilization

of the neutral lipids. Neutral lipids and astaxanthin esters were hydrolyzed by 5% KOH in 95% ethanol followed by transmethylation (Christie 1989). Gas chromatographic analysis of fatty acid methyl esters was performed on a Supelco-wax 10 (Supleco Inc., Bellefonte, PA, USA) fused silica capillary column (30 m × 0.32 mm) using a temperature gradient of 185° C to 210° C. Fatty acid methyl esters were identified by co-chromatography with authentic standards (Sigma Chemical Co.) and by comparison of their equivalent chain length (Ackman 1969). The data presented in the tables and figures represent mean values of at least three independent experiments with a range of less than 5% for major peaks (over 10% of fatty acids) and 10% for minor peaks. Each sample was analyzed in duplicate.

**Inhibition of carotenoid and fatty acid biosynthesis.** Two carotenogenesis inhibitors were used: norflurazon (4-chloro,5-methylamino-2- $\alpha$ - $\alpha$ , trifluoro-methyl-phenyl-3(2H) pyridazine, Sandoz Agro, Inc., Des Plaines, IL, USA) and DPA (BDH Chemicals Ltd., Poole, UK). The fatty acid inhibitor used was sethoxydim (Riedel-de Haën, Seelze, Germany). Aliquots were taken from the stock solutions (0.1 mM of norflurazon, 10 mM of DPA, and 10 mM of sethoxydim in DMSO) and added to the cultures at the onset of induction to make a range of concentrations of 0.01–0.05  $\mu$ M norflurazon, 5–30  $\mu$ M DPA, and 10–50  $\mu$ M sethoxydim. The DMSO was added to the cells of the control at concentrations lower than 1%.

**Isolation of lipid globules.** Red cells were frozen in liquid nitrogen and ground with mortar and pestle. Grinding medium contained 10 mM 4-morpholinepropanesulfonic acid (pH 7.0), 10 mM KCl, 5 mM EDTA, 1% BSA (fatty acid free), 0.005% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 0.5 mg · mL<sup>-1</sup> leupeptine, 1 mM dithiothreitol, and 0.6 M sucrose. The homogenate was centrifuged at 1500 g for 10 min to remove cell debris and unbroken cells. The supernatant and the floating layer were gently mixed and subjected to flotation gradient centrifugation at 25,000 g. Discontinuous gradient contained 0.4 M sucrose and 0.2 M sucrose in 10 mM 4-morpholinepropanesulfonic acid, 5 mM EDTA, 10 mM KCl, and 1 mM dithiothreitol. The floating oil layer above the 0.2 M sucrose buffer was collected, mixed with an equal volume of 0.6 M sucrose buffer, and overlaid with 0.2 M sucrose. The flotation centrifugation step was repeated to recover oil bodies.

**Statistics.** Each experiment was repeated at least three times. Each datum point in the tables and figures represents a mean of at least three independent experiments varying by less than 5%.

## RESULTS

**Effect of norflurazon and DPA on carotenoid and fatty acid biosynthesis.** Exponentially growing (green non-flagellated) cells of *H. pluvialis* were exposed to HL (350  $\mu$ mol photons · m<sup>-2</sup> · s<sup>-1</sup>) to induce carotenoid accumulation. Two carotenogenesis inhibitors, norflurazon and DPA, were added to the cultures at various concentrations at the time of inoculation, and their effect on carotenoid and fatty acid accumulation was determined after 24 h on per volume basis, which is, as explained in Materials and Methods, related to their cellular content. It is worthy to mention that *H. pluvialis* cells survived all inhibitors treatments applied in this work and were able to divide after a lag period of 24–48 h.

As compared with zero-time control, cells subjected to HL and not treated with any inhibitor (HL control) accumulated considerable amount of carotenoids (Table 1). Of the 12.3  $\mu$ g · mL<sup>-1</sup> of total carotenoids, esterified astaxanthin constituted 9.2  $\mu$ g · mL<sup>-1</sup>; the rest was xanthophylls—zeaxanthin, violaxanthin, antheraxanthin, neoxanthin, and  $\beta$ -carotene (Fig. 1). Chlorophyll content also increased, but to a lesser extent, during 24 h of exposure to HL. At a concentration of 0.01  $\mu$ M, norflurazon had no effect on total carotenoid accumulation. However, at 0.02  $\mu$ M, the content of carotenoids and astaxanthin was about half that of the control (6.7 and 4.2  $\mu$ g · mL<sup>-1</sup>, respectively). At 0.05  $\mu$ M, only 26% of the total carotenoid content of control (3.2  $\mu$ g · mL<sup>-1</sup>) was accumulated with trace amounts of astaxanthin (Table 1). Concomitantly, phytoene content increased by about 7-fold from 0.3 to 2  $\mu$ g · mL<sup>-1</sup>. At the same time, chl synthesis was not affected significantly even in the presence of the highest norflurazon concentration (Table 1).

Addition of 30  $\mu$ M DPA to the HL-exposed cultures completely inhibited the accumulation of astaxanthin, but no  $\beta$ -carotene accumulation was observed (Fig. 1). The HPLC analysis of these cells revealed the accumulation of  $\xi$ -carotene (data not shown). This finding

TABLE 1. Effect of norflurazon, DPA, and sethoxydim on the content ( $\mu$ g · mL<sup>-1</sup>) of pigments and total fatty acids in *Haematococcus pluvialis* grown under high irradiance for 24 h.

Treatment	Total carotenoids	Esterified astaxanthin	$\beta$ -Carotene	Chl	Total fatty acids	Oleic acid
0-time control	1.4	Trace	0.1	4.4	20.5	0.96
HL control	12.3 (100) <sup>a</sup>	9.5 (100)	0.3 (100)	6.7 (100)	133.2 (100)	27.1 (100)
Norflurazon ( $\mu$ M)						
0.01	14.5 (118)	10.9 (115)	0.3 (100)	7.7 (115)	139.2 (104)	28.6 (105)
0.02	6.7 (56)	4.2 (44)	0.2 (87)	6.6 (98)	111.1 (83)	22.0 (82)
0.05	3.2 (26)	0 (0)	0.2 (63)	6.1 (91)	98.5 (74)	16.1 (59)
DPA ( $\mu$ M)						
5	13.1 (106)	9.8 (103)	1.0 (333)	6.6 (98)	137.3 (103)	30.5 (112)
10	7.6 (62)	2.6 (27)	1.9 (633)	6.0 (89)	143.4 (108)	30.1 (111)
20	4.8 (39)	1.5 (16)	2.0 (666)	5.7 (85)	91.4 (68)	17.0 (63)
Sethoxydim ( $\mu$ M)						
10	13.0 (106)	6.9 (73)	n.d.	7.2 (107)	102.2 (77)	18.5 (68)
20	5.7 (46)	0.8 (8)	n.d.	6.6 (98)	72.1 (54)	10.7 (39)
50	3.3 (27)	0 (0)	n.d.	6.1 (91)	61.0 (46)	7.3 (27)

HL control, high light exposed; n.d., not determined.

<sup>a</sup>Values in the brackets represent percent of HL control.

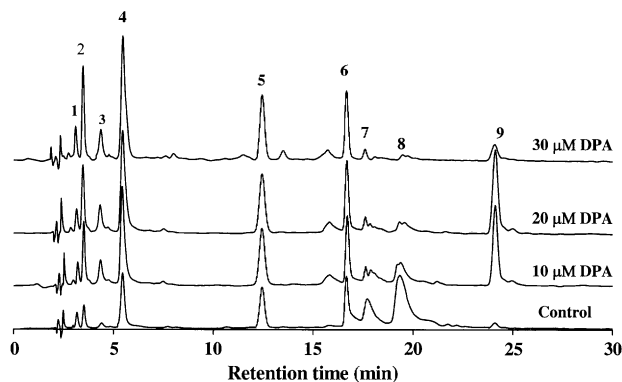


FIG. 1. Pigment profile (analyzed by HPLC) of *Haematococcus pluvialis* induced to accumulate astaxanthin by high irradiance for 24 h as affected by DPA at different concentrations. Peaks: 1, Neoxanthin; 2, violaxanthin; 3, antheraxanthin; 4, zeaxanthin; 5, chl *b*; 6, chl *a*; 7 and 8, esterified astaxanthin; 9,  $\beta$ -carotene.

indicated an inhibition of the  $\xi$ -carotene desaturase rather than the  $\beta$ -carotene C-4 oxygenase. However, at lower DPA concentrations (e.g. 5–20  $\mu\text{M}$ ),  $\beta$ -carotene content increased by 3.3- to 6.7-fold (Table 1) concurrently with the increase in the level of xanthophylls (zeaxanthin, antheraxanthin, and violaxanthin) (Fig. 1). In the presence of 10 and 20  $\mu\text{M}$  DPA, total carotenoid accumulation decreased to 62% and 28% that of the control, respectively (Table 1), whereas astaxanthin content decreased to 39% and 16%, respectively.

In HL control cultures, accumulation of astaxanthin was accompanied by an intense synthesis of lipids, as shown by an increase in the volumetric content of total fatty acids, from 20 in zero-time control to 133  $\mu\text{g}\cdot\text{mL}^{-1}$  (Table 1). Among total fatty acids, oleic acid was dominant in these cells, accounting for 29.3  $\mu\text{g}\cdot\text{mL}^{-1}$ . Administration of 0.05  $\mu\text{M}$  norflurazon or 20  $\mu\text{M}$  DPA, which almost entirely abolished the synthesis of astaxanthin, reduced the volumetric content of total fatty acids to 74% and 78% of control, respectively. At the same inhibitor concentrations, oleic acid was reduced to 51% or 58% of the control, respectively (Table 1). Hence, the impact of norflurazon and DPA on lipid synthesis was significantly lower than their effect on astaxanthin synthesis.

**Effect of sethoxydim on the accumulation of fatty acids and carotenoids.** The fatty acid synthesis inhibitor sethoxydim reduced the HL-induced accumulation of both fatty acids and carotenoids, especially that of astaxanthin (Table 1). Although the volumetric content of fatty acids was reduced to 54% and 46% by 20 and 50  $\mu\text{M}$  sethoxydim, respectively, that of total carotenoids was reduced to 46% and 27%, respectively. At the same inhibitor concentrations, oleic acid was reduced to 35% or 25% of the control, respectively (Table 1). The content of esterified astaxanthin was practically annulled at 50  $\mu\text{M}$  sethoxydim. The content of chl was not affected significantly by these concentrations of sethoxydim.

**Effect of inhibitors on fatty acid content and composition.** The three main lipid fractions—NL, GL, and

PL—as well TAG, isolated from the NL fraction, were determined in cells treated with the three inhibitors used above, which were applied at a concentration causing about 50% reduction in total carotenoid accumulation, that is, 0.02  $\mu\text{M}$  norflurazon, 20  $\mu\text{M}$  DPA, and 20  $\mu\text{M}$  sethoxydim (Fig. 2).

In green cells growing exponentially under noninductive conditions (75  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ), membranar lipids, GL and PL, were the major lipid classes. As a result of the exposure to high irradiance, all three lipid fractions increased, especially NL, which became the predominant lipid class (Fig. 2). A major part of NL was TAG. Although NL content increased sharply from 1.9 to 78.9  $\mu\text{g}\cdot\text{mL}^{-1}$ , TAG content increased from 0.4 to 53.2  $\mu\text{g}\cdot\text{mL}^{-1}$ . The GL and PL contents also increased significantly under HL, from 40.3 and 17.2  $\mu\text{g}\cdot\text{mL}^{-1}$  to 101.5 and 29.1  $\mu\text{g}\cdot\text{mL}^{-1}$ , respectively.

All three inhibitors used reduced the volumetric contents of NL, which nevertheless remained the dominant class (Fig. 2). The cells treated with norflurazon and DPA contained, among their NL, significant amounts of diacylglycerols and nonesterified fatty acids in addition to TAGs. Sethoxydim (20  $\mu\text{M}$ ) had the strongest effect on NL, reducing the volumetric content of both NL and TAG by 50%. The content of GL was also reduced by 36%, 27%, and 25% to that of the control by norflurazon, DPA, and sethoxydim, respectively. However, the inhibitors had a very little impact on the volumetric content of PL. Because of the apparent decrease in NL content, the proportion of membrane lipids (GL and PL) in total cell lipids was significantly higher in sethoxydim-treated cells than in the untreated control (Fig. 2).

The sharp increase in the content of NL under HL was accompanied by significant alteration in its fatty acid composition (Table 2). In green cells (0-time

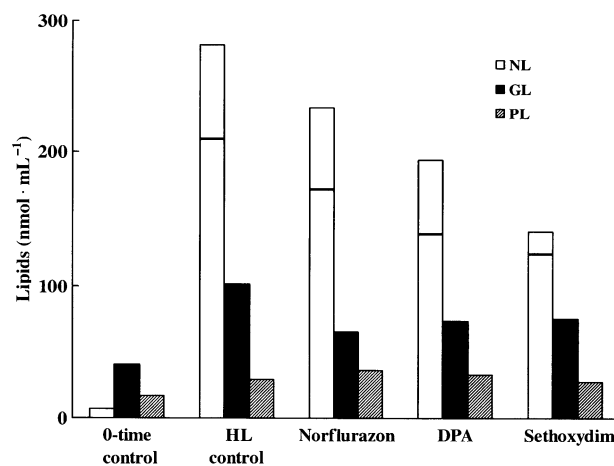


FIG. 2. Volumetric content of NL, GL, and PL in the cultures of *Haematococcus pluvialis* grown under high irradiance for 24 h as affected by norflurazon (0.02  $\mu\text{M}$ ), DPA (20  $\mu\text{M}$ ), and sethoxydim (20  $\mu\text{M}$ ) as compared with 0-time control and HL-exposed cells (HL control). The horizontal line inside the NL bar marks the portion of TAG in this fraction.

TABLE 2. Fatty acid composition of NL, TAG, GL, and PL of *Haematococcus pluvialis* grown under high irradiance for 24 h (HL control) as affected by norflurazon (0.02  $\mu$ M), DPA (20  $\mu$ M), and sethoxydim (20  $\mu$ M) as compared with 0-time control.

Lipid	Treatment	Fatty acid composition (% of total)														
		16:0	16:2 $\omega$ 6	16:3 $\omega$ 3	16:4 $\omega$ 3	18:0	18:1	18:2	18:3 $\omega$ 6	18:3 $\omega$ 3	18:4 $\omega$ 3	20:2 $\omega$ 6	20:3 $\omega$ 6	20:4 $\omega$ 6	20:5 $\omega$ 3	Others
NL	0-time control	23.6	5.6	1.0	6.2	2.4	11.2	26.0	1.6	12.1	1.5	1.4	0.7	3.8	1.2	1.7
	HL control	15.6	0.3	1.8	4.4	0.9	30.4	26.9	2.1	9.0	1.9	1.5	0.6	0.9	3.2	0.5
	Norflurazon	13.3	0.2	1.6	6.1	1.1	26.7	26.6	2.3	11.7	2.1	0.9	0.6	1.4	1.2	4.2
	DPA	13.9	0.5	1.4	4.0	0.9	29.3	24.7	3.2	12.0	2.2	1.2	1.0	1.0	3.4	1.3
	Sethoxydim	14.7	0.4	1.2	3.0	1.5	30.0	29.4	2.0	9.0	1.5	0.9	0.8	1.8	0.5	3.3
TAG	0-time control	18.3	3.5	0.8	6.7	2.7	16.2	21.5	4.1	10.1	3.4	1.7	1.0	6.9	2.5	0.6
	HL control	18.8	0.6	2.0	4.5	1.1	31.0	26.2	2.2	8.1	1.8	0.5	0.5	0.9	0.5	1.2
	Norflurazon	18.4	tr	1.7	4.2	1.1	30.1	27.1	2.3	8.6	2.1	0.6	0.5	1.1	0.4	1.7
	DPA	17.0	0.6	1.5	3.8	0.9	32.3	25.4	3.6	9.5	2.0	0.6	0.7	0.8	0.5	0.8
	Sethoxydim	18.0	0.9	1.3	2.8	1.3	29.7	30.1	2.3	7.1	1.5	0.7	0.6	1.7	0.6	1.4
GL	0-time control	13.4	13.2	2.1	12.0	0.3	4.1	30.3	1.0	18.3	1.0	0.4	0.5	1.6	0.5	1.3
	HL control	14.7	0.8	7.1	20.0	0.3	2.2	8.9	1.9	39.7	1.8	0.3	0.2	0.9	0.9	0.3
	Norflurazon	15.7	0.3	6.4	18.2	0.8	3.3	8.3	1.4	40.4	1.4	0.5	0.2	0.8	0.4	1.9
	DPA	13.5	0.2	5.6	21.5	0.8	3.1	8.3	1.1	41.6	1.0	0.4	0.3	0.6	0.4	1.6
	Sethoxydim	15.1	0.6	3.9	20.5	0.6	2.2	7.7	2.1	41.7	2.0	0.5	0.3	1.3	0.4	1.1
PL	0-time control	34.1	4.0	0.9	2.0	0.6	7.3	23.9	3.5	6.8	2.9	1.6	1.4	7.0	2.1	1.9
	HL control	34.8	0.7	2.6	2.2	0.5	6.3	18.3	4.9	16.1	4.3	1.4	0.4	4.5	1.6	1.4
	Norflurazon	31.8	0.7	3.2	2.8	0.9	5.5	17.2	4.0	21.3	3.6	1.2	0.4	3.3	1.2	2.9
	DPA	31.2	0.4	3.0	2.3	0.8	6.3	18.3	3.4	22.4	2.7	1.3	1.0	2.9	1.0	3.0
	Sethoxydim	32.2	0.8	1.9	2.5	0.7	5.5	16.3	5.8	16.8	5.5	1.7	0.7	4.9	1.4	3.3

control), the major fatty acids of NL and TAG were mostly linoleic (18:2) and palmitic (16:0) acids, followed by oleic (18:1), linolenic (18:3 $\omega$ 3), and hexatetraenoic (16:4 $\omega$ 3) acids (Table 2). Under HL, oleic acid content almost tripled in NLs and doubled in TAG at the expense of most polyunsaturated fatty acids (PUFA). However, 18:3 $\omega$ 3 decreased only slightly, whereas 18:2 even increased in TAG.

The fatty acid composition of GLs of red cells (HL control) was distinguishable from that of green cells by the presence of higher levels of C16 and C18 PUFA (Table 2) (Zhekisheva et al. 2002). The major fatty acids composing GL in green cells were 16:0, 16:2 $\omega$ 6, 16:4 $\omega$ 3, 18:2, and 18:3 $\omega$ 3 (Table 2). Under HL, the levels of the PUFA 16:3 $\omega$ 3, 16:4 $\omega$ 3, and 18:3 $\omega$ 3 significantly increased concomitantly with the drop in the proportion of their less unsaturated precursors. Oleic acid was only a minor component of the GL fraction. In both green and red cells, PL fatty acids were dominated by 16:0 and 18:2. Under HL the content of 18:3 $\omega$ 3 increased. The three inhibitors did not significantly affect the fatty acid composition of the major

lipid classes at the concentrations indicated above (Table 2).

In HL control lipid globules, which appeared intensely red due to the high concentration of astaxanthin esters (molar ratio of TAG to astaxanthin monoester was close to 3), TAG was the major lipid. The fatty acid composition of both TAG and esterified astaxanthin isolated from the lipid globules was similar to each other (Table 3) and was also similar to that of TAG extracted from whole cells (Table 2). However, the esterified astaxanthin was relatively rich in C20 unsaturated fatty acids in comparison with the TAG isolated from the lipid globules.

*Are astaxanthin accumulation and fatty acid synthesis interdependent?* In view of our previous results, which showed that the accumulation of oleic acid in total fatty acids was directly correlated with that of astaxanthin esters (Zhekisheva et al. 2002), we examined the interrelationship between the two processes by the use of the carotenogenesis and lipid synthesis inhibitors used above. As shown above (Table 2), under HL oleic acid increased the most among the fatty

TABLE 3. Fatty acid composition of TAG and astaxanthin monoesters (ASME) of isolated lipid globules of *Haematococcus pluvialis* grown under high irradiance for 24 h.

Lipid	Fatty acid composition (% of total)														
	16:0	16:2 $\omega$ 6	16:3 $\omega$ 3	16:4 $\omega$ 3	18:03	18:1	18:2	18:3 $\omega$ 6	18:3 $\omega$ 3	18:4 $\omega$ 3	20:2 $\omega$ 6	20:3 $\omega$ 6	20:4 $\omega$ 6	20:5 $\omega$ 3	Others
TAG	23.9	0.5	1.6	2.4	1.5	31.6	24.5	2.0	5.0	1.4	1.1	1.0	1.0	0.9	0.7
ASME	25.3	Trace	0.7	1.9	3.5	25.7	17.7	1.7	9.0	2.0	1.8	2.9	2.5	3.9	1.1

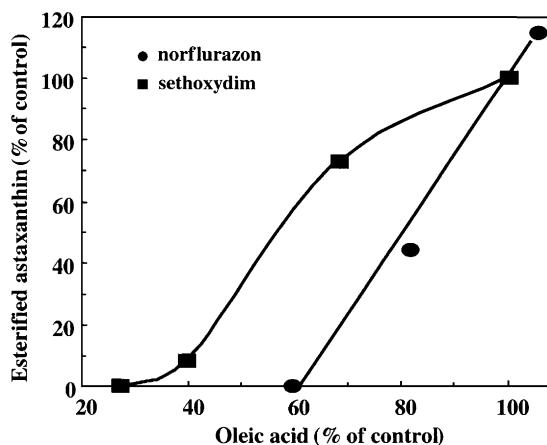


FIG. 3. Relationship between the content of esterified astaxanthin and oleic acid, expressed as percent of the HL control, in *Haematococcus pluvialis* as affected by various concentrations of norflurazon and (●) and sethoxydim (■). Values presented in Table 1 were used for plotting the curves.

acids of NL and TAG. We therefore used the increase in this fatty acid as an indicator of TAG accumulation under HL. The content of esterified astaxanthin and the content of oleic acid in the presence of various concentrations of norflurazon and sethoxydim (Table 1), expressed as percent of the HL control, were plotted each versus the other (Fig. 3).

As can be seen, the inhibition of astaxanthin synthesis by norflurazon was linearly correlated with that of oleic acid. However, this was true only for 40% of the oleic acid produced under these conditions. Indeed, oleic acid synthesis was not annulled, even when astaxanthin synthesis was completely inhibited. A 16-fold increase in the volumetric content of oleic acid, from  $0.96$  to  $16 \mu\text{g} \cdot \text{mL}^{-1}$  (Table 1), occurred during 24 h of HL in the presence of the highest concentration of norflurazon ( $0.05 \mu\text{M}$ ).

Sethoxydim inhibited *de novo* oleic acid synthesis. Its accompanying effect on astaxanthin accumulation was moderate if the content of oleic acid was higher than 60% of its HL control. Below this value, the synthesis of astaxanthin was sharply inhibited and abolished when the content of oleic acid decreased to about 30% of its corresponding control (Fig. 3).

#### DISCUSSION

In *H. pluvialis*, extraplastidial oil globules, containing both TAG and esterified astaxanthin, are formed during the exposure of cells to different stresses (Boussiba 2000). We previously showed that in cells subjected to HL or nitrogen starvation, both monoesterified astaxanthin and TAG were rich in oleic acid (Zhekisheva et al. 2002). These findings allowed us to suggest that an increase in the proportion of mono-unsaturated fatty acids is important for sequestering the high content of astaxanthin esters in oil globules. Moreover, a direct positive correlation between oleic

acid and esterified astaxanthin was established. Our results indicated that the accumulation of astaxanthin is accompanied, and perhaps preceded, by the accumulation of oleate-rich TAG. However, there was no indication which of these processes was the prerequisite for the other. The present experiments with the two carotenogenesis inhibitors, norflurazon and DPA, applied to HL-exposed *H. pluvialis* cells showed that whereas astaxanthin accumulation was severely inhibited, fatty acid synthesis was not proportionally decreased. Neutral lipids and particularly TAG were still produced, indicating that their synthesis was not solely dependent on the accumulation of carotenoids. Indeed, high irradiance induces TAG synthesis in many algae that do not necessarily accumulate carotenoids (Roessler 1990).

When cultures were treated with the ACCase inhibitor sethoxydim, a strong dependence of astaxanthin production on lipid synthesis was evident. The sharp increase in lipid synthesis within 24 h of high irradiance (Table 1) together with the 5-fold increase in biomass (Zhekisheva et al. 2002) indicates that under HL, *H. pluvialis* cells enhance the synthesis of fatty acids *de novo* and hence require a large content of malonyl-CoA. The inhibition of malonyl-CoA production, and consequentially TAG biosynthesis, by sethoxydim had a strong impact on astaxanthin formation. We propose therefore that in *H. pluvialis*, fatty acid synthesis plays a key role in the regulation of astaxanthin biosynthesis. We also suggest that under HL, the synthesis of astaxanthin is not the only driving force for fatty acid synthesis. This can be deduced from the results showing a high content of fatty acids in cells devoid of astaxanthin. By the use of another fatty acid synthesis inhibitor cerulenin, Schoefs et al. (2001) also indicated the importance of active fatty acid synthesis for astaxanthin accumulation in flagellated cells of *H. pluvialis* exposed to high irradiance. Fatty acid synthesis as a driving force for carotenoid accumulation was also suggested for *D. bardawil* (Rabbani et al. 1998). As in *H. pluvialis*, the inhibition of fatty acid synthesis in *D. bardawil* by sethoxydim completely abolished  $\beta$ -carotene accumulation in TAG droplets in the plastids. It is thus clear that enhanced TAG production is essential for carotenoid overproduction in these microalgae, and it is not dependent on the intracellular site of deposition (plastids or extraplastidial compartment). Moreover, the fatty acid esterification of astaxanthin in *H. pluvialis* represents another essential constraint for its enhanced accumulation. The similarity in the major fatty acid composition of TAG and esterified astaxanthin indicate that both acylations utilize the same pool of fatty acids.

The proportion of oleic acid (18:1) among fatty acids of TAG sharply increased in *H. pluvialis* under HL; at the same time, the proportion of other major fatty acids, 16:0 and 18:2, did not change. Three major molecular species of TAG (18:1/18:1/18:1, 18:1/18:1/18:2, and 16:0/18:1/18:2) were produced under HL, indicating that the major flux of *de novo*

synthesized fatty acids are channeled into TAG predominantly via the Kennedy pathway of TAG biosynthesis (Kennedy 1961).

The percentage of the PUFA 18:3 $\omega$ 3 and 16:4 $\omega$ 3 in NLs and in TAG decreased under HL. However, because of the sharp increase in TAG volumetric content under HL, the content of these fatty acids also significantly increased. Because the levels of these fatty acids also increased in GL, the major lipid class of chloroplast membranes, their increase in TAG indicates a turnover of chloroplastic fatty acids for extra-plastidial NL synthesis. Utilization of chloroplastic fatty acids for TAG synthesis was shown in ozone-fumigated (Sakaki et al. 1994) and senescent (Kaup et al. 2002) leaves of higher plants. The typical feature of this pathway is the appearance of the predominant thylakoid fatty acids 18:3 $\omega$ 3 and 16:3 $\omega$ 3 in TAG. The pathway involves lipolysis of GL and assembly of TAG, catalyzed by diacylglycerol acyltransferase (Kaup et al. 2002). The turnover of chloroplastic membrane lipids supports the suggestion that accumulation of astaxanthin in cytoplasmic lipid globules involves transport of its intermediates from the site of the early biosynthetic steps in the chloroplast (Grünewald et al. 2001).

We conclude that fatty acid synthesis in *H. pluvialis* exerts a strong control over astaxanthin esterification and deposition in oil globules. However, the lipid biosynthesis in this alga does not depend on astaxanthin accumulation. This may indicate that both processes differ on their signaling demands. Furthermore, we suggest that accumulation of a certain amount of TAG is prerequisite for the initiation of astaxanthin overproduction. Apparently, high irradiance induces several substantial alterations in lipid metabolism of *H. pluvialis* in concert with enhanced *de novo* biosynthesis of TAG, namely, the increase in the desaturation of chloroplastic lipids and the rapid flux of unsaturated fatty acids to the newly formed TAG. It is noteworthy that the inhibitors, either of carotenoid or lipid synthesis, did not affect notably the fatty acid pattern of the major lipid classes even when astaxanthin production was repressed, indicating that the mechanism of oil globules formation under HL is strictly regulated.

Finally, it is also worth mentioning that complete inhibition of astaxanthin accumulation by either carotenoid or lipid biosynthesis inhibitors did not result in cell death despite the exposure to high irradiance. In all these treatments, significant amounts of other secondary carotenoids were accumulated. Astaxanthin plays the main role in light protection (Wang et al. 2003); however, under the specific conditions used in the present work, the increase in other secondary carotenoids (probably xanthophylls) represents an alternative mechanism for the survival of *H. pluvialis* under HL. This observation is currently under investigation.

We thank Ms. Dorot Imber for editing this manuscript and useful suggestions.

- Ackman, R. G. 1969. Gas-liquid chromatography of fatty acids and esters. *Methods Enzymol.* 14:329–81.
- Benemann, J. 1992. Microalgae aquaculture feeds. *J. Appl. Phycol.* 4:233–45.
- Böger, P. & Sandmann, G. 1983. Pigment biosynthesis and herbicides interaction. *Photosynthetica* 28:481–93.
- Boussiba, S. 2000. Carotenogenesis in the green alga *Haematococcus pluvialis*: cellular physiology and stress response. *Physiol. Plant.* 108:111–7.
- Boussiba, S. & Vonshak, A. 1991. Astaxanthin accumulation in the green alga *Haematococcus pluvialis*. *Plant Physiol.* 32: 1077–82.
- Christie, W. 1989. *The Preparation of Derivatives of Fatty Acids. Gas Chromatography and Lipids.* The Oily Press Ltd., Ayr, Scotland, pp. 65–6.
- Cohen, Z., Didi, S. & Heimer, Y. M., 1992. Over-production of  $\gamma$ -linolenic and eicosapentaenoic acids by algae. *Plant Physiol.* 98:569–72.
- Fan, L., Vonshak, A., Gabbay, R., Hirshberg, J., Cohen, Z. & Boussiba, S. 1995. The biosynthetic pathway of astaxanthin in a green alga *Haematococcus pluvialis* as indicated by inhibition with diphenylamine. *Plant Cell Physiol.* 36:1519–24.
- Grünewald, K., Hirschberg, J. & Hagen, C. 2001. Ketocarotenoid biosynthesis outside of plastids in the unicellular green alga *Haematococcus pluvialis*. *J. Biol. Chem.* 276:6023–9.
- Hagen, C. & Grünewald, K. 2000. Fosmidomycin as an inhibitor of the non-mevalonate terpenoid pathway depresses synthesis of secondary carotenoids in flagellates of the green alga *Haematococcus pluvialis*. *J. Appl. Bot.* 74:137–40.
- Harker, M., Tsavalos, A. & Young, A. 1996. Factors responsible for astaxanthin formation in the chlorophyte *Haematococcus pluvialis*. *Biores. Technol.* 55:207–14.
- Harker, M. & Young, A. 1995. Inhibition of astaxanthin synthesis in the green alga, *Haematococcus pluvialis*. *Eur. J. Phycol.* 30: 179–87.
- Harwood, J. 1999. Graminicides which inhibit lipid synthesis. *Pestic. Outlook* 10:154–8.
- Johnson, E. A. & An, G. H. 1991. Astaxanthin from microbial sources. *Crit. Rev. Biotechnol.* 11:297–326.
- Kaup, M., Frosese, C. & Thompson, J. 2002. A role for diacylglycerol acyltransferase during leaf senescence. *Plant Physiol.* 129:1616–26.
- Kennedy, E. P. 1961. Biosynthesis of complex lipids. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 20:934–40.
- Kobayashi, M., Kakizono, T., Yamaguchi, K., Nishio, N. & Nagai, S. 1992. Growth and astaxanthin formation of *Haematococcus pluvialis* in heterotrophic and mixotrophic conditions. *J. Ferment. Bioeng.* 74:17–20.
- Konishi, T. & Sasaki, Y. 1994. Compartmentalization of two forms of acetyl-CoA carboxylase in plants and the origin of their tolerance toward herbicides. *Proc. Natl. Acad. Sci. USA* 91: 3596–601.
- Lichtenthaler, H. K. 1999. The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50:47–65.
- Livne, A. & Sukenik, A. 1992. Lipid synthesis and abundance of acetyl CoA carboxylase in *Isochrysis galbana* (Prymnesiophyceae) following nitrogen starvation. *Plant Cell Physiol.* 33:1175–81.
- Lorenz, R. & Cysewski, G. 2000. Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. *Trends Biotechnol.* 18:160–70.
- Osterlie, M., Bjerkeng, B. & Liaaen-Jensen, S. 2000. Plasma appearance and distribution of astaxanthin E/Z and R/S isomers in plasma lipoproteins of men after single dose administration of astaxanthin. *J. Nutr. Biochem.* 11:482–90.
- Palozza, P. & Krinsky, N. 1992. Astaxanthin and canthaxanthin are potent antioxidant in a membrane model. *Arch. Biochem. Biophys.* 297:291–5.
- Rabbani, S., Beyer, P., Lonting, J.V., Huguency, P. & Kleining, H. 1998. Induced  $\beta$ -carotene synthesis driven by triacylglycerol deposition in the unicellular alga *Dunaliella bardawil*. *Plant Physiol.* 116:1239–48.

- Roessler, P. G. 1990. Environmental control of glycerolipid metabolism in microalgae: commercial implications and future research directions. *J. Phycol.* 26:393–9.
- Sakaki, T., Tanaka, T. & Yamada, M. 1994. General metabolic changes in leaf lipids in response to ozone. *Plant Cell Physiol.* 35:53–62.
- Sarada, R., Bhattacharya, S. & Ravishankar, G. 2002. Optimization of culture conditions for growth of the green alga *Haematococcus pluvialis*. *World J. Microbial. Biotech.* 18:517–21.
- Schoefs, B., Rmiki, N. E., Rachadi, J. & Lemoine, Y. 2001. Astaxanthin accumulation in *Haematococcus* requires a cytochrome P450 hydroxylase and an active synthesis of fatty acids. *FEBS Lett.* 500:125–8.
- Shaish, A., Avron, M. & Ben-Amotz, A. 1990. Effect of inhibitors on the formation of stereoisomers in the biosynthesis of  $\beta$ -carotene in *Dunaliella bardawil*. *Plant Cell Physiol.* 31: 689–96.
- Sprey, B. 1970. Die lokalisierung von sekundärcarotinoiden von *Haematococcus pluvialis* Flotow em. Wille. *Protoplasma* 71: 235–50.
- Tjahjono, A., Hayama, Y., Kakizono, T., Terada, Y., Nishio, N. & Nagai, S. 1994. Hyper-accumulation of astaxanthin in a green-alga *Haematococcus pluvialis* at elevated temperature. *Biotechnol. Lett.* 16:133–8.
- Vorst, P., Baard, R., Mur, L., Korthals, H. & Vandenende, H. 1994. Effect of growth arrest on carotene accumulation and photosynthesis in *Dunaliella*. *Microbiology UK* 140:1411–7.
- Wang, B., Zarka, A., Trebst, A. & Boussiba, S. 2003. Astaxanthin accumulation in *Haematococcus pluvialis* (Chlorophyceae) as an active photoprotective process under high irradiance. *J. Phycol.* 39:1116–24.
- Yuan, J., Gong, X. & Chen, F. 1997. Separation and analysis of carotenoids and chlorophylls in *Haematococcus lacustris* by high-performance liquid chromatography photodiode array detection. *J. Agric. Food Chem.* 45:1952–6.
- Zhekisheva, M., Boussiba, S., Khozin-Goldberg, I., Zarka, A. & Cohen, Z. 2002. Accumulation of oleic acid in *Haematococcus pluvialis* (Chlorophyceae) under nitrogen starvation or high light is correlated with that of astaxanthin esters. *J. Phycol.* 38:325–31.