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ACCUMULATION OF OLEIC ACID IN *HAEMATOCOCCUS PLUVIALIS* (CHLOROPHYCEAE) UNDER NITROGEN STARVATION OR HIGH LIGHT IS CORRELATED WITH THAT OF ASTAXANTHIN ESTERS[1]

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The chlorophyte *Haematococcus pluvialis* accumulates large quantities of astaxanthin under stress conditions. Under either nitrogen starvation or high light, the production of each picogram of astaxanthin was accompanied by that of 5 or 3–4 pg of fatty acids, respectively. In both cases, the newly formed fatty acids, consisting mostly of oleic (up to 34% of fatty acids in comparison with 13% in the control), palmitic, and linoleic acids, were deposited mostly in triacylglycerols. Furthermore, the enhanced accumulation of oleic acid was linearily correlated with that of astaxanthin. Astaxanthin, which is mostly monoesterified, is deposited in globules made of triacylglycerols. We suggest that the production of oleic acid-rich triacylglycerols on the one hand and the esterification of astaxanthin on the other hand enable the oil globules to maintain the high content of astaxanthin esters.

Key index words: **astaxanthin; *Haematococcus pluvialis*; high light; nitrogen starvation; oleic acid; triacylglycerols**

Haematococcus pluvialis Flotow (Chlorophyceae, order Volvocales) is a unicellular green alga common in small, transient, freshwater bodies. When green cells encounter stress conditions such as nitrogen deficiency, high light intensity, phosphate starvation, or salt stress, the alga rapidly differentiates from a vegetative stage into a resting stage, forming aplanospores (Boussiba et al. 1999, Boussiba 2000). Within a few days, cells increase in volume, produce a very tough cell wall, and accumulate large amounts of a red ketocarotenoid, astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione), which is deposited in extraplastidial oil bodies (Grünewald et al. 2001).

Formation of chloroplastic and extraplastidial lipid bodies containing both triacylglycerols (TAG) and carotenoids under stress conditions such as high irradiance and nitrogen starvation was described in various green microalgae (e.g. *Dunaliella bardawil, Chlorella zofingiensis, Scenedesmus* [Thompson 1996], and *Haematococcus pluvialis* [Boussiba 2000]). A close interrelationship between TAG synthesis, β-carotene accumulation, and chloroplast lipid globule formation was demonstrated in the unicellular alga *D. bardawil* (Rabbani et al. 1998). The induced β-carotene synthesis in this alga is driven by TAG deposition that was ascribed as a plastid-localized sink for the end product of carotenoid biosynthetic pathway. A positive correlation between the oleic acid (18:1) and carotene cellular contents with irradiance was found in *Dunaliella salina* (Mendoza et al. 1999). The authors related these findings to changes in the balance between storage and photosynthetic related fatty acids during the adaptation to high light. The accumulation of neutral lipids under conditions of nutrient deficiency in *H. pluvialis* was suggested to serve as a matrix for solubilizing the esterified astaxanthin in the lipid globules (Sprey 1970, Boussiba 2000). To further substantiate this assumption, we compared the effects of nitrogen starvation and high light, respectively, on astaxanthin accumulation, lipid content, and fatty acids profiles in this alga. Our data indicate that production of oleate-rich TAGs is essential for astaxanthin accumulation. We hypothesize that this composition enables the oil globules to maintain a higher content of astaxanthin esters.

MATERIALS AND METHODS

Haematococcus pluvialis Flotow was obtained from the Culture Collection of the University of Göttingen, Germany.

Growth conditions. Algal cultures were cultivated in a 4-cm wide 600-mL glass column containing 500 mL of modified BG-11 medium (Boussiba and Vonshak 1991) that was placed in a temperature-regulated water bath at 25° C. Cultures were stirred by bubbling with a mixture of 1.5% CO_2 in air. 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 green cells (nonflagellated) were cultivated on modified BG-11 medium for 5 days at a light intensity of 75 μmol photons$\cdot$m$^{-2}\cdot$s^{-1} normal light (NL), resuspended in full or nitrogen-free medium to a cell number of 2×10^5 cells$\cdot$mL^{-1}, and grown under the same conditions. Red cells were obtained by exposing green cells to astaxanthin-inductive conditions, that is, a light intensity of 350 μmol photons$\cdot$m$^{-2}\cdot$s^{-1} high light (HL) or nitrogen-free medium under NL conditions. Each experiment was repeated at least three times.

Measurements of growth parameters and pigment content. Samples were taken at indicated times, and the growth parameters were measured immediately. Cell number was determined by using a hemacytometer. Dry weight was measured by filtering a 5-mL sample through preweighed Whatman GF/C filters (Whatman, Maidstone, UK) and drying the cell mass at 70° C overnight. For chl determination, cells were harvested by centrifugation (2300*g*, 5 min), the pellet was resuspended in DMSO, and the mixture was heated for 10 min at 70° C. The procedure was repeated until a white pellet was obtained. The absorbance of the combined DMSO extracts was determined at 666 nm by an HP8452A spectrophotometer, and the chl content was calculated accord-

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ing to Seely et al. (1972). For astaxanthin determination, harvested cells were treated with a solution of 5% KOH in 30% (v/v) methanol to destroy the chl. The supernatant was discarded, five drops of acetic acid were added to reduce the pH, and the remaining pellet was extracted twice with DMSO to recover the astaxanthin. The absorbance of the combined extracts was determined at 490 nm. The amount of pigment was calculated using pure astaxanthin (Sigma Chemical Co., St. Louis, MO, USA) as a standard ($E^{1\%}{}_{cm}$ 1795 in DMSO). Under both nitrogen starvation (Boussiba et al. 1999) and high light (unpublished data) conditions, RP-HPLC analysis has shown that astaxanthin esters accounted for over 90% of total carotenoids.

Lipid extraction. Biomass was harvested, centrifuged, and lyophilized. Freeze-dried samples of *H. pluvialis* biomass (50 mg) were treated with 200 μL DMSO for 5 min at 70° C and further extracted with 5 mL of methanol at 4° C for 1 h. The mixture was centrifuged, the supernatant collected, and the pellet reextracted with methanol. Peroxide-free diethyl ether, containing 0.01% BHT, hexane, and water, was added to the methanol extract to form a final ratio of 1:1:1:1 (v/v/v/v). The mixture was shaken, centrifuged for 5 min at 2000*g*, and the upper phase collected. The lower phase was acidified with acetic acid to pH 3–4 and reextracted with a mixture of diethyl ether:hexane (1:1, v/v). The combined upper phases were evaporated to dryness under vacuum and kept at −20° C, under argon, in a small volume of chloroform.

Lipid fractionation. Total lipid extracts were fractionated into classes (neutral, glycolipids, phospholipids) on SEP-PAK cartridges (Waters, Milford, MA, USA) by sequential elution with chloroform, acetone, and methanol as previously described (Cohen et al. 1992). Neutral lipids were further resolved by TLC (silica gel 60, 20 × 20-cm plates with a concentrating zone, 0.25 mm thickness, Macherey-Nagel, Düren, France) using a solvent system of petroleum ether:diethyl ether:acetic acid (70:30:1, v/v/v). Lipids were localized by brief exposure to I_2 vapors and by comparison with the Rf of standards.

Fatty acid analysis. Samples of freeze-dried biomass, lipid extracts, or individual lipids were transmethylated with 2% H_2SO_4 in methanol:toluene (9:1, v/v) under argon atmosphere at 80° C for 1.5 h. Neutral lipids and astaxanthin esters were hydrolyzed by 5% KOH in 95% ethanol before methylation (Christie 1989). Heptadecanoic acid (Sigma Chemical Co.) was added as an internal standard. Gas chromatographic analysis of fatty acids methyl esters was performed on a Supecowax 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 shown represent mean values with a range of less then 5% for major peaks (over 10% of fatty acids) and 10% for minor peaks, of at least two independent samples, each analyzed in duplicate.

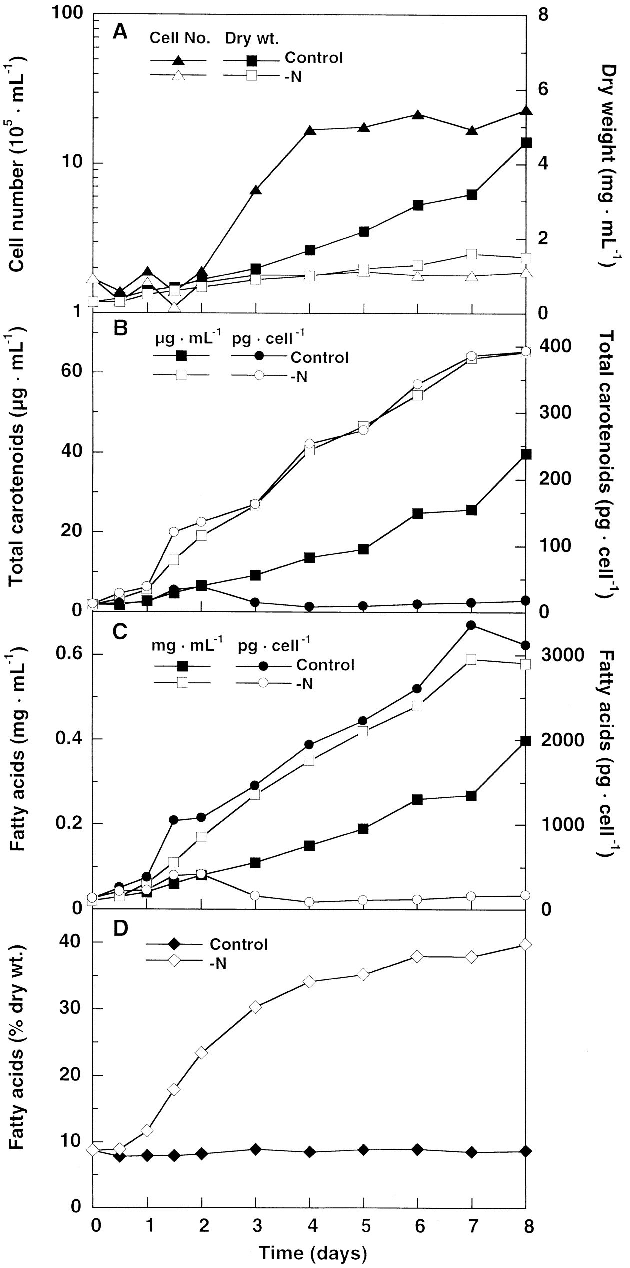

FIG. 1. Effect of nitrogen starvation on cell number and dry weight (A), total carotenoid content (B), and total fatty acid content (C and D) in *Haematococcus pluvialis.* In the control culture, the total carotenoids included mostly primary carotenoids, whereas in the nitrogen-starved culture, astaxanthin, mostly in its monoester form, comprised over 99% of total carotenoids. Cultures of green cells (initial cell number 2 × 10^5 cells·mL^{-1}), cultivated in modified BG-11 medium for 5 days at a light intensity of 75 μmol photons·m^{-2}·s^{-1}, were resuspended with nitrogen-free or full medium to a cell number of 2 × 10^5 cells·mL^{-1}. Each datum point in this and the following figures represents the mean of at least three independent experiments, varying by less than 5%.

RESULTS

Nitrogen starvation. After nitrogen starvation, green vegetative cells of *H. pluvialis* ceased to divide and gradually turned into red cysts, whereas the cell number of the control cultures increased exponentially after a 2-day lag for another 2 days (Fig. 1A). Sharp increases were noted in the volumetric and cellular contents of astaxanthin (up to 350 pg·cell^{-1}, Fig. 1B) and of fatty acids (up to 3400 pg·cell^{-1}, Fig. 1C; 39.8% of dry weight, Fig. 1D) in the nitrogen-starved cultures. A linear correlation was found between the increases in the cellular contents of astaxanthin and fatty acids (Fig. 2, $R^2 = 0.9904$). In the control cultures, there was no increase in the cellular content of either total carotenoids (mostly β-carotene) or fatty acids; however, due to the increase in cell number, the content per culture increased.

The fatty acid composition of control cultures of *H. pluvialis* was characterized by the presence of various

C_{16}, C_{18} and C_{20} polyunsaturated fatty acids (PUFAs), which amounted to 69.1% of total fatty acids. After 1 day of nitrogen starvation, the proportion of 18:1 increased sharply to 24.1%, compared with 5.0% in the control, whereas that of PUFAs decreased to 52.8% (data not shown). In the following days, there was a further decrease in the level of desaturation that was expressed by the increase in the proportion of 18:2 at the expense of 16:4 and 18:3. The accumulation of oleic acid that was linearly correlated with that of astaxanthin (Fig. 2, inset, $R^2 = 0.9967$) led us to suggest that the oleic acid is mainly accumulated in TAG and that the lipid globules may have a role as a depository for the pigment.

High light. Exponentially growing (green) cells of *H. pluvialis* cultivated for 5 days under a light intensity of 75 μmol photons$\cdot$m$^{-2}\cdot$s^{-1} were diluted and exposed to a light intensity of 350 μmol photons$\cdot$m$^{-2}\cdot$s^{-1}. After a 2-day lag, cells of both control and high light cultures started to divide. The cell number (Fig. 3A) and the chl per culture (Fig. 3B) of both cultures attained similar values. The cellular chl content more than tripled in the control culture compared with a rather small increase under high light (Fig. 3B). After 4 days, it decreased to a similar level in both cultures and increased slightly thereafter. Changes in cell dry weight could be differentiated into three stages. In the first stage, cell dry weight increased sharply under high light within the first 12 h, reaching a maximum of 8 ng$\cdot$cell^{-1} after 2 days (Fig. 4A). Cell dry weight decreased during the next 2 days and increased slightly again in the last 2 days. A similar pattern of lower magnitude was observed in the control. The culture cell dry weight, however, increased continuously in both cultures but especially under high light, reaching 7.1 mg$\cdot$mL^{-1} compared with 2.9 mg$\cdot$mL^{-1} in the control (Fig. 4A).

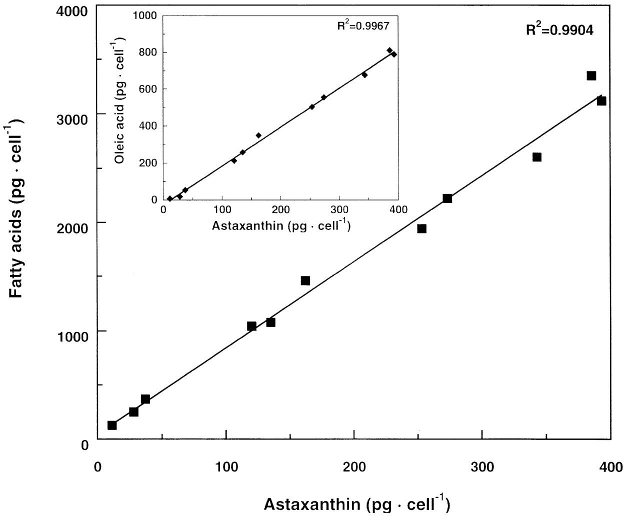

FIG. 2. Correlation between cellular content of fatty acids (oleic acid in inset) and astaxanthin after nitrogen starvation.

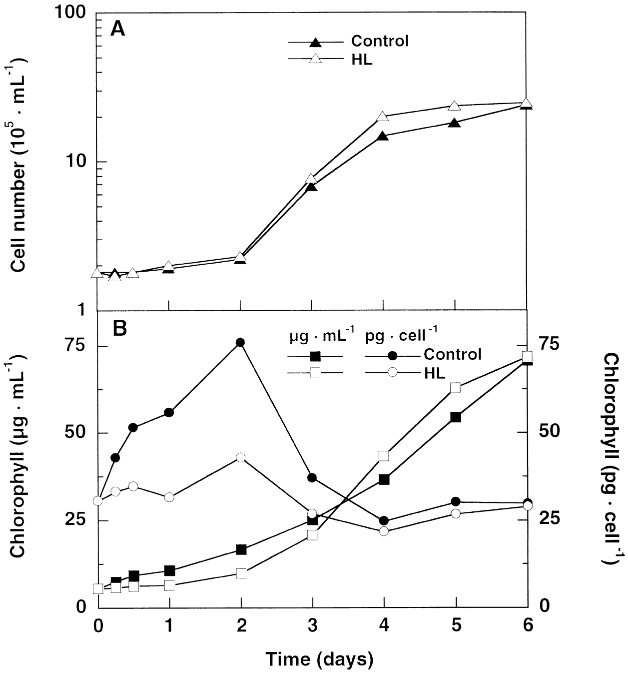

FIG. 3. Effect of high light exposure (350 μmol photons$\cdot$m$^{-2}\cdot$s^{-1}) on cell number (A) and chl content (B). Cultures of green cells, cultivated in modified BG-11 medium for 5 days at a light intensity of 75 μmol photons$\cdot$m$^{-2}\cdot$s^{-1}, were resuspended in full medium to a cell number of 2 × 10^5 cells$\cdot$mL^{-1} and exposed to high light (HL, 350 μmol photons$\cdot$m$^{-2}\cdot$s^{-1}) or normal light (Control, 75 μmol photons$\cdot$m$^{-2}\cdot$s^{-1}).

The changes in the cellular content of total carotenoids (Fig. 4B) and fatty acids (Fig. 4C) mimicked that of the cell dry weight, rising sharply in the first 2 days and decreasing in the following 2 days. The control did not change appreciably. The maximal fatty acid content was much lower than that achieved under nitrogen starvation (12.4% ± 2.8 vs. 39.8% ± 1.1, Figs. 1D and 4D). The volumetric contents (mg fatty acid$\cdot$mL^{-1}), however, increased continuously, especially in the last 2 days, whereas that of the control did not change appreciably (Fig. 4C). When cells started to divide, the cellular fatty acid content decreased, whereas culture levels slightly increased, indicating that the accumulated fatty acids were mostly diluted rather than consumed.

The response of the fatty acid composition to high light was similar to that observed under nitrogen starvation. The most outstanding change was noted in the proportion of 18:1 that increased, from 5.2% (of total fatty acids) in the control to 12.5% after 6 h and to 19.8% after 1.5 days, and decreased thereafter (data not shown).

To elucidate the effect on fatty acids in different lipid classes, we separated the lipid extract into three fractions: glycolipids, phospholipids, and neutral lipids. TAGs were separated from the latter. In the first day, the cell content of neutral lipids increased dramatically from 3.6 to 278 pg$\cdot$cell^{-1}, whereas that of the

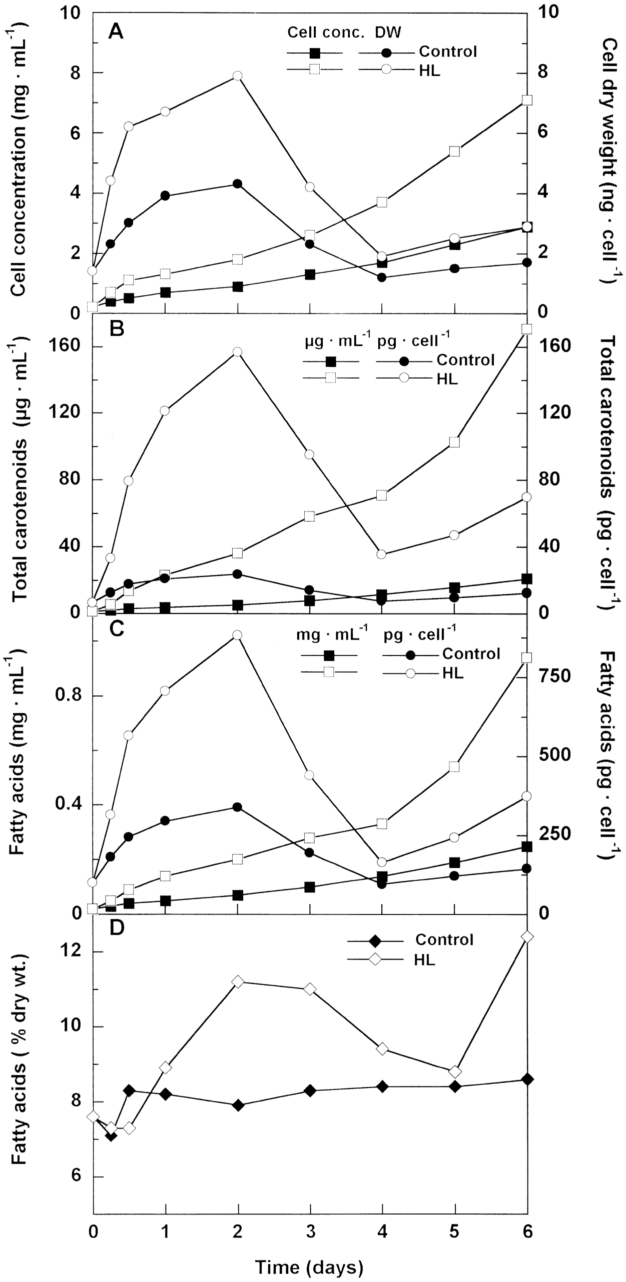

FIG. 4. Effect of high light (HL, 350 μmol photons·m^{-2}·s^{-1}) exposure on volumetric and cellular dry weight (A), total carotenoid content (B), and total fatty acid content (C and D) in *Haematococcus pluvialis*. In the control culture, carotenoids included mostly primary carotenoids, whereas in the high light culture, except for the first time point, astaxanthin in its ester form constituted 82%–92% of total carotenoids. For growth conditions see Figure 3.

polar lipids did not change (Fig. 5). In the next day, neutral lipids further increased to 455 and decreased thereafter to 45.9 pg·cell^{-1}, whereas polar lipids increased slightly. The increase in cellular fatty acid content during the early accumulation period was correlated with that of total carotenoids (Fig. 6), which were shown by TLC to contain 99% astaxanthin. Cell division, which occurred between day 2 and day 4, was accompanied by a sharp decrease in the cellular content of TAG. In the first day, the major change observed in the fatty acid composition of the glycolipids was the increase in desaturation of 18:2 to 18:3ω3, which was reflected in the change in their proportion, from 22.8% and 27.2% to 5.2% and 44.7%, respectively (Table 1). An increase in desaturation of 16:1 and 16:2 to 16:3 and 16:4 was also observed. A similar increase, although less pronounced, was observed in the phospholipids. In the following days the pattern reversed, and in the glycolipids 18:2 and 16:0 increased at the expense of 18:3ω3 and 16:4ω3, respectively, whereas in the phospholipids 18:1 increased at the expense of 18:3ω3. However, the most profound change was observed in TAG, where the proportion of 18:1 increased within the first 6 h from 13.2% to 34.4%, at the expense of the PUFAs. This increase, compounded with the increase in the content of TAG, affected the sharp increase observed in the proportion of 18:1 in total lipids. After the first day, the proportion of 18:1 gradually decreased. As in the case of nitrogen starvation, the accumulation of astaxanthin was well correlated with that of 18:1 (Fig. 6, inset).

DISCUSSION

The content of astaxanthin in *H. pluvialis*, which may exceed 4% of dry weight, is by far the highest value reported for any microorganism, including bacteria, fungi, and other microalgae (Boussiba 2000). This may have to do with the efficient deposition of the pigment in lipid globules in its esterified form (Bidigare et al. 1993). It was thus reasonable to assume that the fatty acid metabolism under conditions inductive for pigment accumulation would be one of the key factors controlling astaxanthin biosynthesis in this alga.

Nitrogen starvation induced a sharp increase in the content of both astaxanthin and TAG of *H. pluvialis*. The increase in TAG content was not surprising. In-

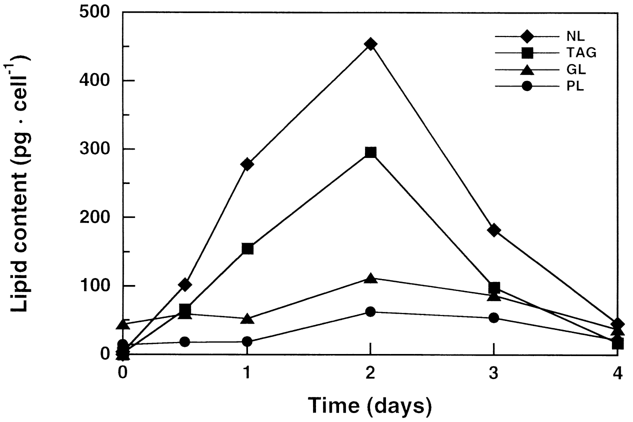

FIG. 5. Effect of high light on the content of different lipid groups. NL, neutral lipids; TAG, triacylglycerols; GL, glycolipids; PL, phospholipids.

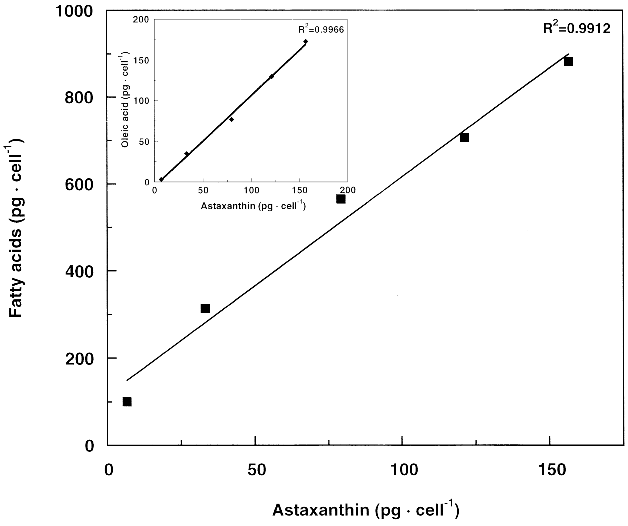

FIG. 6. Correlation between cellular content of fatty acids (oleic acid in inset) and astaxanthin under high light (350 μmol photons·m^{-2}·s^{-1}). Correlation was made for data obtained during the first 2 days of exposure to high light.

creases of even higher magnitudes were reported to occur in many microalgae under nitrogen starvation. Generally, the lipid contents increased up to 10%–25% of dry weight; however, contents as high as 72% and even 88% were reported (Cohen 1985). Imposing nitrogen limitation when light is in excess results in cessation of growth. Because photosynthetic fixation of carbon continues, the cellular C/N is thereby increased and energy is channeled into production of non-nitrogenous materials such as TAG, which serve as a sink for photosynthetically fixed carbon (Mayzaud et al. 1989). However, because massive lipid accumulation, under nitrogen starvation, could have occurred regardless of the pigment accumulation, to find out whether the enhancement of the TAG content in *H. pluvialis* is related or coincidental to the accumulation of astaxanthin, we chose to induce astaxanthin accumulation by an increase in light intensity.

Astaxanthin is accumulated also when cultures of *H. pluvialis* are exposed to high light intensity (Boussiba and Vonshak 1991, Sun et al. 1998, Steinbrenner and Linden 2001). Although the accumulation of fatty acids under nitrogen starvation is a widely known phenomenon (Roessler 1990, Thompson 1996), sharp increases in the fatty acid content on transfer to high light have been less studied. We were thus interested to examine the correlation between astaxanthin and fatty acid accumulation under high light. The effect of high light intensity on lipid composition and fatty acid desaturation is not at all clear. Sukenik et al. (1989) showed that in *Nannochloropsis*, the share of TAG increases under high light irradiation, whereas that of monogalactosyldiacylglycerol (MGDG) decreases. In the latter, the proportion of the major PUFA, 20:5ω3, decreased. However, Adlerstein et al. (1997) showed an increase in the desaturation of 20:4ω6 to 20:5ω3 in the galactolipids of *Porphyridium cruentum* under high light. Klyachko-Gurvich et al. (1999) similarly showed that in both *Dunaliella* and *Chlamydomonas*, the proportions of the PUFAs 16:3 and 16:4 increase in the galactolipids under high light. The increase in desaturation of the polar lipids of *H. pluvialis*, predominantly the glycolipids, observed during the first day is in keeping with the latter findings.

TABLE 1. Changes in the fatty acid composition of major lipid groups of *Haematococcus pluvialis* after transfer to high light intensity.

		Fatty acids composition[a] (% of total)																	
Lipid	Time (h)	16:0	16:1 ω11	16:1 ω5	16:2 ω6	16:3 ω3	16:4 ω3	18:0	18:1	18:2	18:3 ω6	18:3 ω3	18:4 ω3	20:1	20:2 ω6	20:3 ω6	20:4 ω6	20:5 ω3	22:0
TAG	0	20.6	2.1	1.6	0.5	0.4	9.1	1.2	13.2	19.0	3.9	10.7	3.2	1.8	1.1	1.0	6.2	2.4	1.6
	6	21.1	1.9	1.1	0.3	2.3	2.8	0.9	34.4	20.4	2.9	5.3	1.6	0.6	0.3	0.2	1.3	0.5	1.2
	12	24.2	0.5	0.7	0.3	2.1	2.7	0.6	33.5	20.9	1.6	6.0	1.3	0.6	0.5	0.3	1.0	0.3	1.6
	24	27.5	—	0.5	0.2	1.8	3.1	0.6	31.7	18.8	1.5	5.8	1.3	—	0.7	0.5	1.0	1.0	2.4
	48	22.1	—	0.6	0.3	3.1	4.3	0.4	27.6	23.8	2.3	9.2	2.2	0.2	0.6	0.4	1.2	0.7	0.4
	72	18.5	—	0.6	0.2	3.0	3.8	0.4	26.3	25.9	2.0	9.9	1.7	0.4	1.3	0.6	2.0	1.2	1.8
	96	12.9	1.5	0.5	0.2	3.2	4.5	0.4	24.0	25.7	1.4	12.7	1.6	1.8	1.7	0.7	3.5	2.1	1.5
GL	0	5.0	1.8	8.5	3.5	2.9	20.9	1.4	1.9	22.8	0.7	27.2	1.3	—	tr	0.2	1.4	0.4	—
	6	6.1	0.8	2.4	2.4	8.0	25.1	0.5	3.0	10.0	1.3	37.9	1.2	tr	tr	—	0.9	0.2	—
	12	8.1	—	1.1	1.7	6.1	30.2	0.5	2.0	4.7	0.9	42.9	1.2	—	tr	—	0.5	tr	—
	24	7.1	—	0.8	1.0	5.8	28.9	0.5	2.6	5.2	0.8	44.7	1.1	0.2	—	—	0.6	tr	—
	48	12.5	—	1.4	0.7	6.6	23.5	0.2	2.7	8.7	1.6	37.5	1.9	0.2	0.2	tr	0.8	0.2	—
	72	14.2	—	1.2	0.7	5.4	24.0	tr	1.1	8.5	1.5	38.8	2.3	0.2	0.2	tr	0.8	0.3	—
	96	9.1	1.1	2.8	0.9	3.6	25.3	0.2	1.2	12.4	1.2	37.7	2.0	0.2	0.3	0.2	0.9	0.5	—
PL	0	33.7	—	4.3	0.6	2.7	3.5	0.3	6.0	20.2	3.9	10.3	3.6	0.3	0.8	1.1	6.3	2.3	—
	6	30.0	0.4	3.3	0.5	5.9	3.9	0.7	6.4	15.4	6.3	12.1	4.9	0.4	0.8	0.7	6.2	2.0	—
	12	28.9	0.4	2.3	0.4	7.1	4.2	0.4	3.6	13.7	5.8	19.0	5.7	0.3	0.8	0.4	5.1	1.6	—
	24	28.5	—	1.1	tr	4.8	2.6	0.6	7.1	16.9	4.7	19.5	4.7	0.8	1.6	0.5	4.8	1.5	—
	48	32.9	—	1.1	tr	3.9	2.3	0.3	7.2	17.2	5.5	13.9	5.7	0.3	1.5	0.4	5.0	1.8	—
	72	34.5	—	1.3	tr	4.3	3.1	0.2	6.6	14.5	4.5	14.8	6.4	0.2	1.4	0.6	4.8	2.5	—
	96	28.8	—	1.6	0.2	3.2	3.0	0.3	9.3	15.7	4.4	12.5	6.6	0.2	1.7	0.7	6.2	3.6	—

[a] Traces (less then 1%) of 20:0 and of an unidentified fatty acid were also present.
TAG, triacylglycerols; GL, glycolipids; PL, phospholipids; tr, traces.

In *Haematococcus*, astaxanthin appears mostly as mono- and di-esters of various fatty acids and constitutes up to 95% of total secondary carotenoids in the cells (Lee and Zhang 1999). These pigments are present in lipid globules outside the chloroplast (Sun et al. 1998, Grünewald et al. 2001). The cellular function of astaxanthin is not clear. It was suggested to have a role in protection from photodamage by reducing the amount of light available to the light harvesting pigment–protein complex (Bidigare et al. 1993). Other researchers suggested that it may act as an antioxidant, inhibiting lipid peroxidation (Hagen et al. 1993). Under stress conditions, such as high light irradiance or nitrogen limitation, *Haematococcus lacustris* formed clusters of globules containing carotenoids, mostly astaxanthin, at the cell center (Yong and Lee 1991). After exposure to high light intensities, these clusters underwent a reversible spreading so as to shield a larger surface area of the chloroplast (Yong and Lee 1991). Similarly, under high light conditions, cells of the unicellular alga *Dunaliella bardawil* overproduce β-carotene. However, the pigment is accumulated in the plastids, in newly formed lipid droplets that are predominantly made of TAG (Rabbani et al. 1998).

Within 6 h after transfer to high light, cells of *H. pluvialis* ceased to divide and transformed into nonmotile spherical red cells (aplanospores). Cell weight and astaxanthin and fatty acid content increased. The newly formed fatty acids were predominated by the presence of 18:1, 16:0, and 18:2. The former are the end products of the *de novo* pathway of fatty acid biosynthesis. Under optimal conditions, most of the fatty acid flux is esterified into phospholipids and galactolipids for further desaturation, whereas the rest is transferred, together with PUFAs provided by phospholipids, to the fatty acid pool that supply acyl groups for the production of TAG. However, under stress conditions, TAGs become the major lipid class, using most of the 18:1 and 16:0 produced by the *de novo* pathway. Similarly, in *D. salina*, after an increase in irradiance, the proportion of 16:0 and 18:1 increased at the expense of the PUFAs 16:2, 16:4, 18:3ω6, and 18:3ω3 (Mendoza et al. 1999). The increase in the 16:0/16:4 ratio was related to the change in the balance between storage and photosynthetic related fatty acids during the adaptation to high light. The content of 18:1 was positively correlated with the cellular content of carotenes and with irradiance.

The absolute increase in the fatty acid content under high light was significantly lower than that obtained under nitrogen starvation, 12.4% ± 2.8 versus 39.8% ± 1.1 (of dry weight), respectively. The finding in both cases that accumulation of fatty acids was linearly correlated with that of astaxanthin strongly suggests that these processes are interrelated. Because astaxanthin is not water soluble, the TAG, which forms large globules, can serve as a depository where the pigment could be dissolved. The hydrocarbon skeleton of astaxanthin is responsible for its hydrophobic nature, rendering it water insoluble, yet its hydroxy groups significantly reduce its solubility in the oil globule, which is predominantly made of TAG. The monoesterification of the hydroxy groups increases its hydrophobicity and therefore its solubility in TAG. Under the conditions of our experiments, astaxanthin was almost entirely monoesterified (data not shown), and the molar ratio of TAG to the monoester was as high as 1:1. However, to reach such a high concentration in oil, the fatty acid composition of TAG needs to be tailor-made. Our results indicate that the accumulation of astaxanthin is accompanied and perhaps preceded by that of oleate-rich TAG. Presumably, oleic acid, whose structure is almost linear but is still unsaturated, would be more appropriate for dissolving the all *trans* astaxanthin than either saturated or *cis* PUFAs. Another possibility is that these TAGs serve as a reservoir of oleate for the esterification of astaxanthin that would take place on the interface of the globule. The fatty acid composition of the astaxanthin esters is very close to that of TAG, oleic acid being the predominant fatty acid (data not shown). Similarly, Bidigare et al. (1993) showed that the proportion of oleic acid in *Chalmydomonas* spp. increased from 11% in green cells to 59% in red cells. Concurrently, oleic acid constituted 51% of the fatty acids of astaxanthin esters. Recently, Grünewald et al. (2001) found β-carotene oxygenase activity both in the plastid and in the lipid globuli, suggesting that the lipid vesicles are involved also in the biosynthesis of astaxanthin. In such case, there is a great likelihood that the esterification would also take place in the globuli. The ability to fit the composition of astaxanthin esters with that of TAG is one of the reasons for *H. pluvialis* being the richest natural source of this pigment.

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