Does Astaxanthin Protect *Haematococcus* against Light Damage?

Lu Fan, Avigad Vonshak, Aliza Zarka and Sammy Boussiba

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

Z. Naturforsch. **53c,** 93–100 (1998); received October 28/November 7, 1997

Astaxanthin, *Haematococcus*, High Light, Reactive Oxygen Species (ROS)

The photoprotective function of the ketocarotenoid astaxanthin in *Haematococcus* was questioned. When exposed to high irradiance and/or nutritional stress, green *Haematococcus* cells turned red due to accumulation of an immense quantity of the red pigment astaxanthin. Our results demonstrate that: 1) The addition of diphenylamine, an inhibitor of astaxanthin biosynthesis, causes cell death under high light intensity; 2) Red cells are susceptible to high light stress to the same extent or even higher then green ones upon exposure to a very high light intensity (4000 μmol photon m^{-2} s^{-1}); 3) Addition of $^1O_2$ generators (methylene blue, rose bengal) under noninductive conditions (low light of 100 μmol photon m^{-2} s^{-1}) induced astaxanthin accumulation. This can be reversed by an exogenous $^1O_2$ quencher (histidine); 4) Histidine can prevent the accumulation of astaxanthin induced by phosphate starvation. We suggest that: 1) Astaxanthin is the result of the photoprotection process rather than the protective agent; 2) $^1O_2$ is involved indirectly in astaxanthin accumulation process.

Introduction

Carotenoids are widely distributed in various plants and animals (Weedon, 1971, Goodwin, 1976, 1980). In the plant kingdom they act as accessory, light-harvesting pigments, utilizing light over a wide spectral range (Cogdell, 1988; Cogdell *et al.,* 1994). They also act as photoprotective agents that protect plant cells from photooxidative damage caused by absorption of incident visible light (Burnett, 1976; Goodwin, 1980; Ben-Amotz *et al.,* 1989; Sandmann *et al.,* 1993; Hagen *et al.,* 1994), and as antioxidants (Krinsky, 1979; Schroeder and Johnson, 1993, 1995).

The green alga *Haematococcus pluvialis* Flotow (Volvocales) is green in color, in its vegetative stage. Upon exposure to stress conditions, such as high irradiance or nutrient starvation, the vegetative cells are transformed into red resting cysts, accumulating a massive amount of the ketocarotenoid astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) (Droop, 1954; Boussiba and Vonshak, 1991; Johnson and An, 1991; Kobayashi *et al.,* 1991). Most of the work published so far on *Haematococcus* has been focused on the growth of the alga, its physiology and carotenogenesis (Lu *et al.,* 1994, 1995; Boussiba and Vonshak, 1991; Kobayashi *et al.,* 1992a, 1992b, 1993). However the physiological function of the accumulated astaxanthin has not yet been clarified. Droop (1954) postulated that astaxanthin acted as a storage material. Some reports indicated that astaxanthin was involved in phototaxis (Litvin *et al.,* 1978; Angelini *et al.,* 1986; Braune and Ekelund, 1990). Yong and Lee (1991) suggested a photoprotective role for astaxanthin, and later Hagen *et al.* (1993, 1994) suggested that astaxanthin acts as a "sunshade". They proposed that the cells loaded with astaxanthin can adapt to extreme increase in radiation by lowering blue-light absorption of the photosynthetic apparatus.

In the present work we have questioned the photoprotective role of astaxanthin and provide evidence to support the hypothesis that this pigment is the result of the photoprotective process, rather than the protective agent, and for the involvement of $^1O_2$ in its biosynthesis.

Abbreviations: DPA, diphenylamine; ROS, reactive oxygen species; $^1O_2$, singlet oxygen; $O_2^-\cdot$, superoxide; $HO\cdot$, hydroxyl radical; HPFD, high photon flux density.

Reprint requests to Prof. Sammy Boussiba.
Fax: 00972-7-6596802.
E-mail: sammy@bgumail.bgu.ac.il.

Materials and Methods

Algal strain

Haematococcus pluvialis Flotow (formerly *H. lacustris*) was obtained from the Culture Collection of the University of Göttingen, Germany.

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Growth conditions

The composition of BG-11 medium used and growth conditions were as previously described (Boussiba and Vonshak, 1991). Astaxanthin content and growth parameters (chlorophyll content, turbidity, and cell number) were measured as described previously (Boussiba *et al.*, 1992). Cultures of green cells were maintained under astaxanthin-noninductive conditions, i.e., photon flux density (PFD) of 100 μmol photon m^{-2} s^{-1} and complete BG-11 medium. Red cells were obtained by exposing green cells (non flagellated) to astaxanthin-inductive conditions, i.e., PFD of 200 μmol photon m^{-2} s^{-1} and phosphate-free BG-11 medium for different periods of time (Lu *et al.*, 1995).

Inhibition of carotenoid biosynthesis

A stock solution (30 mM) of diphenylamine (DPA), a specific inhibitor of carotenoid biosynthesis (Lu *et al.*, 1995), was prepared by dissolving pure crystalline DPA (BDH Chemicals Ltd.) in 70% basic (pH 9.0) ethanol (Paerl 1984).

Photoinhibition experiments

Green and red cells, obtained as described above, were harvested by centrifugation (740×*g*, 2 min), and the pellets were resuspended in fresh BG-11 medium to obtain a turbidity of 2.8 O.D at 730 nm. Samples of these cell suspensions were placed in test tubes with an inner diameter of 2.2 cm and exposed to high PFD of 4,000 μmol photon m^{-2} s^{-1}, in a temperature-controlled water bath (25 °C) for up to 60 minutes. Light was provided by a Halogen-Superhot bulb (1,000 W, Osram, Germany). Samples were withdrawn at different time intervals for measurement of photosynthetic activities; oxygen evolution rates and chlorophyll *a* fluorescence ratio (Fv/Fm), at room temperature. The results were expressed as percentages of their respective initial rates.

Photosynthetic activities

O_2 evolution rate was measured as previously described (Vonshak *et al.*, 1988), using a Clark-type oxygen electrode (Yellow Springs, Ohio). Fv/Fm was measured with a Photosynthetic Efficiency Analyzer (Hansatech, UK) in a 1 ml sample which had been dark-adapted for 10 min.

Reactive oxygen species (ROS) generators

For superoxide ($O_2^{-\cdot}$) production, paraquat (1–100 μM) or duraquinone (2–5 μM) were used (Schroeder and Johnson, 1993); for $^1O_2$, methylene blue (1–3 μM), rose bengal (1–20 μM) or eosin (2 μM) were used at 100 μmol photon m^{-2} s^{-1} (Knox and Dodge, 1985; Jimenez and Pick, 1993); H_2O_2 (1 μM to 1 mM) was used alone or together with $FeSO_4$ (450 μM) for $HO^{\cdot}$ production.

Scavengers of Reactive Oxygen Species

Histidine (10, 25, 50 mM) was used as a quencher of $^1O_2$ (Mishra and Ghanotakis, 1994), mannitol (30 mM) for $O_2^{-\cdot}$ (Schroeder and Johnson, 1993), and KI (1 mM) for $HO^{\cdot}$ (Kobayashi *et al.*, 1993). Propyl gallate, epinephrine, butylcatechol, or Tiron, each at a concentration of 2 mM, were also used as scavengers of oxygen radicals (Chen *et al.*, 1992).

Results

Effect of DPA and light intensity on the growth of H. pluvialis

To test whether astaxanthin accumulated under high light intensity is involved in the photoprotection mechanism, we have used a specific inhibitor of astaxanthin biosynthesis – DPA. Green cells, inoculated at an initial concentration of 1 μg Chl ml^{-1} at which self shading is low, were grown at light intensity of 100 or 400 μmol photon m^{-2} s^{-1} in the presence or absence of 30 μM DPA. The effects of these treatments on culture turbidity, cell number, and astaxanthin content are shown in Fig. 1. Under light intensity of 100 μmol photon m^{-2} s^{-1} in the absence of DPA, culture turbidity (Fig. 1A) and cell number (Fig. 1B) increased at normal ratio, while astaxanthin content remained constant and at a low level (Fig. 1C). Addition of DPA did not have any significant effect on either parameters. Furthermore, we have shown previously (Lu *et al.*, 1995) that addition of 30 μM DPA under the above conditions did not affect cellular metabolism and carotenoid profile as well. Under the higher irradiance of 400 μmol photon m^{-2} s^{-1} in the absence of DPA, cell number re-

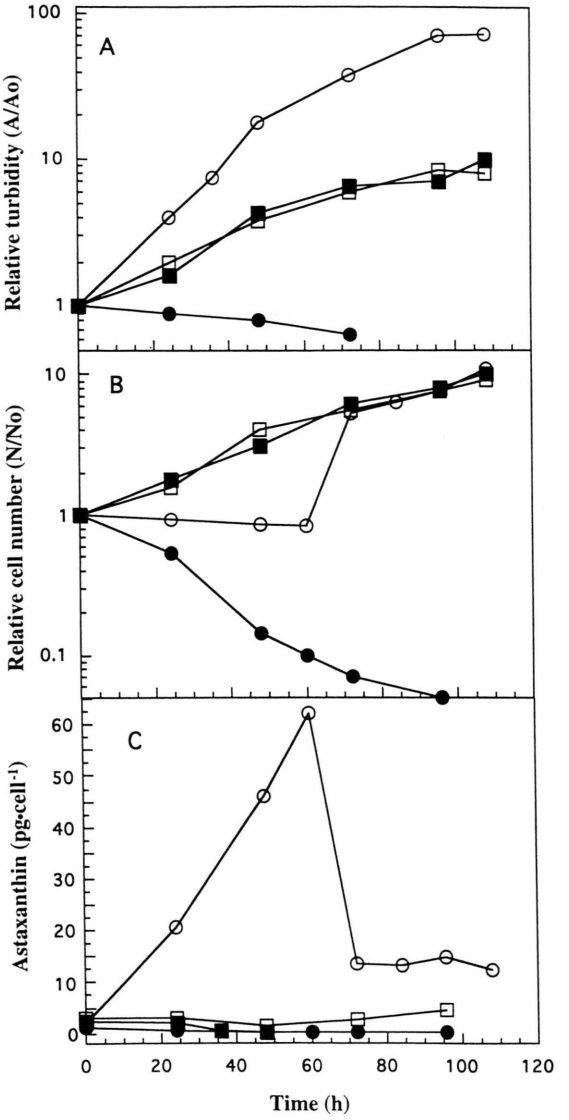

Fig. 1. Effect of high irradiance and DPA (30 μM) on turbidity (A), cellular growth (B) and astaxanthin content (C) in *H. pluvialis*. Green cells were harvested and resuspended in fresh BG-11 medium to a final concentration of 10^5 cells ml^{-1}. Cell suspensions were then incubated at the following conditions: 100 μmol photon m^{-2} s^{-1} without DPA (□); 100 μmol photon·m^{-2}·s^{-1} with DPA (■); 400 μmol photon·m^{-2}·s^{-1} without DPA (○); 400 μmol photon·m^{-2}·s^{-1} with DPA (●).

mained constant at the first 60 hours (Fig. 1B), during which astaxanthin content increased, and cells were transformed to red cysts (Fig. 1C). At the same time, culture turbidity increased (Fig. 1A) due to an increase in cell size (as noted by microscopic observations). After the first 60 hours all the red cysts started dividing simultaneously, releasing 4, 8, 16 or sometimes 32 daughter cells (flagellate) from each cyst. As a consequence, cell number increased sharply, and astaxanthin content per cell declined (Fig. 1C). Addition of DPA under the high irradiance (400 μmol photon m^{-2} s^{-1}) prevented astaxanthin accumulation and caused a drastic decline in cell number finally leading to cell death (Fig. 1 A,B).

Effect of high light intensity on photosynthetic activities

The possibility that astaxanthin protects the cells by acting as a filter, screening excessive irradiance was tested by exposing green and two types of red cells of *H. pluvialis* containing different amounts of astaxanthin (Table I) to high photon flux density (4,000 μmol photon m^{-2} s^{-1}). The changes in the photosynthetic activities of the cells, measured as light-limited oxygen evolution rates, and in the maximal efficiency of PSII photochemistry, measured as Fv/Fm, indicated that a typical photoinhibition of photosynthesis is taking place following the exposure to the high light stress (Fig. 2). The photosynthetic activities of the green cells, decreased at a lower rate than those of the red cells. Among the red cells, the photosynthetic activities decreased faster in cells containing a large amount of astaxanthin than in cells containing a smaller amount of astaxanthin. This trend was observed when either Fv/Fm or O_2 evolution were measured.

Role of ROS in astaxanthin accumulation

The involvement of ROS in carotenoid biosynthesis in *D. bardawil* (Shaish *et al.*, 1993) and in *H. pluvialis* (Kobayashi *et al.*, 1993) has been suggested. We have tested the effect of various exogenous ROS generators and scavengers on astaxanthin accumulation. Upon addition of a $^1O_2$ generator, methylene blue (2 μM), to cultures grown at 100 μmol photon m^{-2} s^{-1} (carotenoid-non-inductive conditions), cell division stopped for 40 h (Fig. 3A) during which time astaxanthin accumulation took place (Fig. 3B). Cell division was resumed only after that time, followed by a decline in the cellular content of astaxanthin. The pigment accumulation could be partially repressed

Table I. Pigment content of green and two types of red cells used in the photoinhibition experiment.

Parameter	Green cells	Red cells containing low[a] astaxanthin	Red cells containing high[b] astaxanthin
Chlorophyll [pg cell^{-1}]	12.4	11.2	10.7
Astaxanthin [pg cell^{-1}]	3.7	30.4	87.8
Astaxanthin/chlorophyll ratio [g g^{-1}]	0.3	2.7	8.2

[a] Exposed for 3 days to inductive conditions (see Materials and Methods).
[b] Exposed for 8 days to inductive conditions (see Materials and Methods).

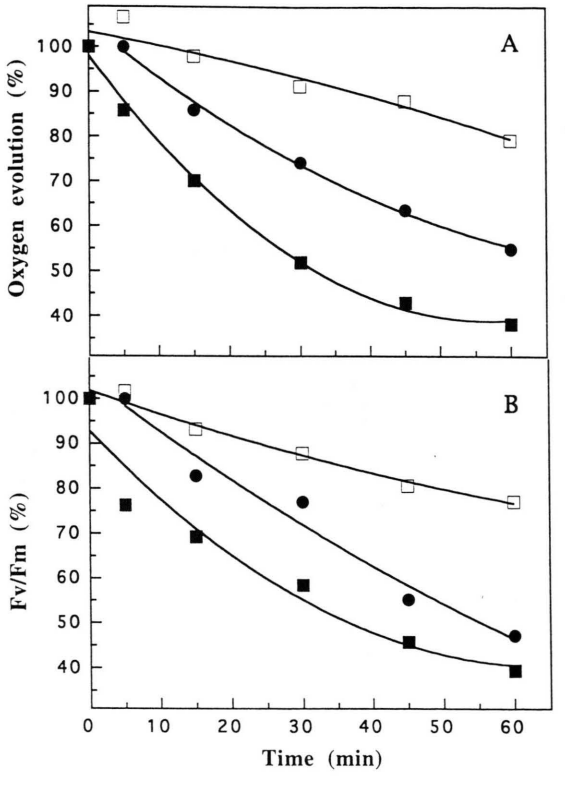

Fig. 2. Effect of extremely high irradiance of 4000 μmol photon m^{-2} s^{-1} for 60 min on the photosynthetic activities of *H. pluvialis*. Green cells (□) and two types of red cells: Red I (●), Red II (■) , containing low and high amounts of astaxanthin respectively (characterized in Table I) were harvested and resuspended in fresh BG-11 medium, to obtain suspensions with the same turbidity (2.81 O. D.). Samples were withdrawn at different time intervals for measuring photosynthetic activities (oxygen evolution and Fv/Fm). The results are expressed as the percentage of their respective initial values. Values for O_2 evolution are 190, 165, 65 (μmol mg chl^{-1} h^{-1}) for green, Red I and Red II, respectively. Values for the Fv/Fm parameter were 0.7, 0.61 and 0.28 for green, Red I and Red II, respectively.

by addition of the $^1O_2$ quencher histidine, at a concentration of 25 mM.

When methylene blue and histidine were added together, a smaller accumulation of astaxanthin took place and cell division proceeded, although at a lower rate then in the control culture (Fig. 3A,B). Similar results were obtained (inhibition of cells division accompanied with astaxanthin accumulation) with 20 μM rose bengal or 2 μM eosin (data not shown). Methylene blue, rose bengal and eosin are known to generate $^1O_2$ in the presence of light. Their effects may therefore suggest that $^1O_2$ is required for astaxanthin accumulation, while the cell number remained constant. Other ROS, such as O_2^{-}·, H_2O_2 and HO·, apparently are not involved in this process, as the addition of their respective generators (see Materials and Methods) did not exert the same effect (data not shown). While under non-inductive-conditions histidine alone had no effect on algal growth or astaxanthin accumulation (Fig. 3A,B), it was found to inhibit astaxanthin accumulation under carotenoid-inductive conditions, i.e. phosphate starvation (Fig. 3C) (Boussiba *et al.*, 1992).

Discussion

The nature and mechanisms by which carotenoids provide protection to cells against photooxidative damage have continuously been pursued by many researchers. To date, there are three well-accepted mechanisms. The first, suggested by Krinsky (1971, 1979, 1994), proposes that carotenoids prevent damage of excessive irradiance by directly quenching triplet chlorophyll (^{3}Chl) or $^1O_2$ produced during photodynamic reactions. This quenching mechanism requires close proximity between the quencher (carotenoids) and the photosensitized molecules (chlorophyll). Hence, this

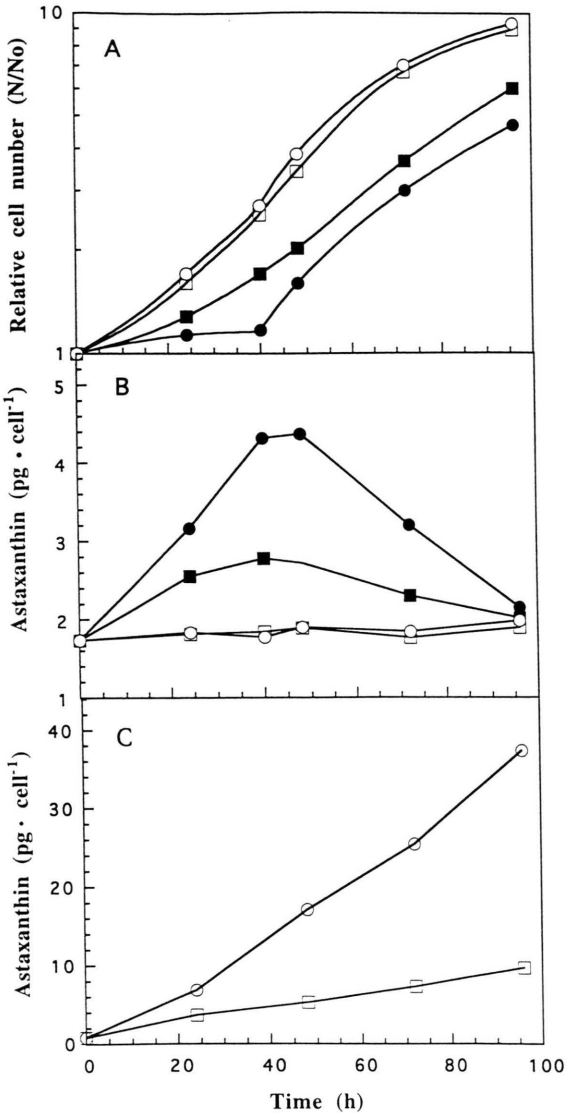

Fig. 3. Effect of methylene blue and histidine on cell number (A) and astaxanthin content (B & C) of *H. pluvialis*. Green cells were incubated in full BG-11 medium at 100 μmol photon m^{-2} s^{-1} (A & B) or in phosphate-free BG-11 medium at 200 μmol photon m^{-2} s^{-1} (C). (□) 25 mM histidine; (●) 2 μM methylene blue; (■) 25 mM histidine plus 2 μM methylene blue; (○) control.

type of photoprotection can only be provided by those carotenoids which are integrated in the two photosystems. The second mechanism of carotenoid photoprotection was proposed by Ben-Amotz *et al.* (1989). According to Ben-Amotz, the large amount of β-carotene accumulated in the inter-thylakoid space, in the chloroplast of the green alga *Dunaliella bardawil*, acts as a screen that prevents excessive irradiance of blue light from reaching the antenna chlorophylls. This screening effect is mainly due to the large overlap between the absorption spectrum of these two pigments in the blue light region. The third mechanism is the xantophyll cycle (Demmig-Adams and Adams III, 1992) which consists of light-dependent conversions of three xanthophylls (oxygenated carotenoids) in a cyclic reaction involving a de-epoxidation sequence from the diepoxide violaxanthin via the monoepoxide antheraxanthin to the epoxide-free form from zeaxanthin, and an epoxidation sequence in the reverse direction. This xanthophyl cycle is present in the chloroplasts of higher plants and several algal groups including green algae.

The massively accumulated astaxanthin in *H. pluvialis* is located in the cytoplasm (Lang, 1968; Santos and Mesquita, 1984). Therefore, it is unlikely that astaxanthin exerts a photoprotective role in this alga by quenching excited triplet chlorophylls (^{3}Chl) due to the distance between the two. In addition, the overlap between the absorption spectra of astaxanthin and chlorophyll is rather small; hence, it is difficult to imagine how astaxanthin can act as a screen filtering excessive blue light absorbed by chlorophylls. At this stage, we do not posses evidence for the existence of the xantophyll pathway in *Haematococcus* and it is difficult to speculate the involvement of astaxanthin precursors in this cycle.

The exposure of *Haematococcus* cells to high light intensity as reported by several workers resulted in the accumulation of astaxanthin (Yong and Lee, 1991; Kobayashi *et al.*, 1992a; Boussiba *et al.*, 1992; Hagen *et al.*, 1994). In order to evaluate if this pigment provides cells protection against light damage as suggested by Hagen *et al.* (1994) and Yong and Lee (1991), we have used a specific inhibitor of astaxanthin biosynthesis DPA, which prevents the conversion of β-carotene to astaxanthin precursor echinenone (Lu *et al.*, 1995). This inhibitor when added to a culture exposed to high light intensity caused its death (Fig. 1 A,B), omitting of the inhibitor under this condition permitted astaxanthin accumulation and prevented cell death, indicating the possible involvement of astaxanthin biosynthesis in the protection mechanism.

To further question the role of astaxanthin in providing cells protection against light damage, we have induced green cells to accumulate astaxanthin, and their resistance to HPFD exposure was compared to that of non-induced cells. The damage to the PS II activity as an outcome of this exposure was evaluated by measuring both oxygen evolution activity and Fv/Fm. The results obtained indicated that astaxanthin does not protect the cells (Fig. 2). They also show that a higher amount of astaxanthin per cell does not give further advantage to the cell in respect to protection against light damage. This rules out the possibility that large amounts of astaxanthin could somehow overlap with the absorbance in the blue region resulting in some photoprotection on the photosynthetic machinery, as suggested previously (Hagen *et al.,* 1994). We suggest therefore that the pigment itself is not the protecting agent, and the photoprotection is provided only if astaxanthin biosynthesis is not interrupted.

What is then the trigger for astaxanthin accumulation when *Haematococcus* cells are exposed to stress conditions such as high light intensity? Kobayashi *et al.* (1993) have demonstrated that different reactive oxygen species are capable of activating astaxanthin biosynthesis. They have also suggested that it is very likely that $^1O_2$ may be the most affective ROS for enhanced carotenogenesis. Our results support this suggestion (although we have not found evidence to support the involvement of other ROS in the carotenogenesis process) and indicate that under non-inductive conditions (100 μmol photon m^{-2} s^{-1}) the addition of $^1O_2$ generator stimulated astaxanthin production, while the addition of the appropriate quencher inhibited this process (Fig. 3B). The effect of methylene blue on cell division and astaxanthin accumulation (Fig. 3A,B) was similar to that of exposing the cells to an irradiance of 400 μmol photon m^{-2} s^{-1} (Fig. 1B). Therefore, we propose that under high light intensity, astaxanthin accumulation is triggered by the overproduction of $^1O_2$. Also, in conditions other than high light intensity which cause astaxanthin accumulation (Boussiba *et al.,* 1992), such as phosphate starvation, the addition of histidine completely inhibited astaxanthin accumulation (Fig. 3C). We suggest that in response to $^1O_2$ formation, cells of *H. pluvialis* initiate astaxanthin biosynthesis which is an antioxidant defense system that discharges this toxic molecules. It is most likely that $^1O_2$ acts as both a messenger and an excessive energy carrier which inhibits photosynthesis and cell division (Fig. 3A), initiates the expression of carotenoid biosynthesis genes (Tonkyn *et al.,* 1992), and drives carotenogenesis processes (Fig. 3B).

Since this ROS is produced within the chloroplast, it is difficult to speculate its direct reaction with components involved in astaxanthin production within the cytoplasm. The mechanism by which $^1O_2$ activates asatxanthin biosynthesis is under investigation.

Acknowledgments

We are grateful to Prof. Norman Krinsky for valuable comments during preparation of this manuscript. We thank also Ms. Dorot Imber for editing the manuscript. This research was supported by Grant No. US-2006–91R from the United States-Israel Binational Agriculture Research and Development Fund (BARD) and by Rashi Foundation.

Publication No. 81 of the Microalgal Biotechnology Laboratory.

Angelini, F., Ascoll, C., Frediani, C. and Petracchi, D. (1986), Transient photoresponses of a phototactic microorganism, *Haematococcus pluvialis*, revealed by light scattering. Biophys. J. **50,** 929–936.

Asada, K. and Takahashi, M. (1987), Production and scavenging of active oxygen in photosynthesis. In: Photoinhibition (D. J., Kyle, C. B. Osmond and C. J. Arntzen, eds.). Elsevier Science Publishers B. V. pp. 227–287.

Ben-Amotz, A., Shaish, A. and Avron, M. (1989), Mode of action of the massively accumulated β-carotene of *Dunaliella bardawil* in protecting the alga against damage by excess irradiation. Plant Physiol. **91,** 1040–1043.

Boussiba, S., Lu, F. and Vonshak, A. (1992), Enhancement and determination of astaxanthin accumulation in green alga *Haematococcus pluvialis*. Meth. Enzymol. **213,** 386–371.

Boussiba, S. and Vonshak, A (1991), Astaxanthin accumulation in the green alga *Haematococcus pluvialis*. Plant Cell Physiol. **32,** 1077–1082.

Braune, W. and Ekelund, N. G. A. (1990), Phototactic responses in *Haematococcus lacustris* and its modification by light intensity and the carotenoid biosynthesis inhibitor Norflurazon. Arch. Microbiol. **154,** 448–452.

Burnett, J. H. (1976), Functions of carotenoids other than in photosynthesis. In: Chemistry and Biochemistry of Plant Pigments, (T. W. Goodwin, ed.) 2 Vol. **1,** Academic Press, London, pp. 655–680.

Chen, G. X., Kazimir, J. and Cheniae, G. M. (1992), Photoinhibition of hydroxylamine-extracted photosystem II membranes: studies of the mechanism. Biochem. **31,** 11072–11083.

Cogdell, R. J. (1988), The function of pigments in chloroplasts. In: Plant Pigments (T. W. Goodwin, ed.). Academic Press, London, pp. 183–230.

Cogdell, R. J., Gillbro, T., Anderson, P. O., Liu, R. S. H. and Asa, A. E. (1994), Carotenoids as accessory light-harvesting pigments. Pure Appl. Chem. **66,** 1041–1046.

Dample, B. (1991), Regulation of bacterial oxidative stress genes. Ann. Rev. Genet. **25,** 315–337.

Demmig-Adams, B. and Adams III, W. W. (1992), Photoprotection and other responses of plants to high light stress. Ann. Rev. Plant Physiol. Plant Mol. Biol. **43,** 599–626.

Droop, M. R. (1954), Conditions governing haematochrome formation and loss in the alga *Haematococcus pluvialis* Flotow. Arch. Mikrobiol. **20S,** 391–397.

Goodwin, T. W. (1976), Distribution of carotenoids. In: Chemistry and Biochemistry of Plant Pigments (T. W. Goodwin, ed.), Vol. **1,** Academic Press, London, pp. 225–261.

Goodwin, T. W. (1980), *The Biochemistry of Carotenoids*. Vol. **1,** Plants. Chapman and Hall, New York, pp. 377.

Hagen, C., Braune, W. and Björn, L. O. (1993), Functional aspects of secondary carotenoids in *Haematococcus lacustris* (Girod) Rostafinski (Volvocales). IV. Protection from photodynamic damage. J. Photochem. Photobiol. **20,** 153–160.

Hagen, C., Braune, W. and Björn, L. O. (1994), Functional aspects of secondary carotenoids in *Haematococcus lacustris* (Volvocales). III. Action as a "sunshade". J. Phycol. **30,** 241–248.

Jimenez, C. and Pick, U. (1993), Differential reactivity of β-carotene isomers from *Dunaliella bardawil* toward oxygen radicals. Plant Physiol. **101,** 385–390.

Johnson, E. A. and An, G. H. (1991), Astaxanthin from microbial sources. Critical Rev. Biotechnol. **11,** 297–326.

Knox, J. P. and Dodge, A. D. (1985), The photodynamic action of eosin, a singlet-oxygen generator. The inhibition of photosynthetic electron transport. Planta **164,** 30–34.

Kobayashi, M., Kakizono, T. and Nagai, S. (1991), Astaxanthin production by a green alga, *Haematococcus pluvialis* accompanied with morphological changes in acetate media. J. Ferment. Bioeng. **71,** 335–339.

Kobayashi, M., Kakizono, T., Nishio, N. and Nagai, S. (1992a), Effect of light intensity, light quality and illumination cycle on astaxanthin formation in a green alga *Haematococcus pluvialis*. J. Fermentation Bioengineering **74**(1), 61–63.

Kobayashi, M., Kakizono, T., Yamaguchi, K., Nishio, N. and Nagai, S. (1992b), Growth and astaxanthin formation of *Haematococcus pluvialis* in heterotrophic and mixotrophic conditions. J. Fermentation Bioengineering **74**(1), 17–20.

Kobayashi, M., Kakizono, T. and Nagai, S. (1993), Enhanced carotenoid biosynthesis by oxidative stress in acetate-induced cyst cells of a green unicellular alga, *Haematococcus pluvialis*. Appl. Environ. Microbiol. **59,** 867–873.

Krinsky, N. I. (1971), Function. In: Carotenoids (O. Isler, ed.) Birkhauser Verlag Basel, Stuttgart, pp. 669–716.

Krinsky, N. I. (1979), Carotenoid protection against oxidation. Pure Appl. Chem. **51,** 649–660.

Krinsky, N. I. (1994), The biological properties of carotenoids. Pure and Appl. Chem. **66**(5), 1003–1010.

Lang, N. J. (1968), Electron microscopic studies of extraplastidic astaxanthin in *Haematococcus*. J. Phycol. **4,** 12–19.

Lichtenthaler, H. K. (1988), Applications of Chlorophyll Fluorescence in Photosynthesis Research, Stress Physiology, Hydrobiology and Remote Sensing. Kluwer Academic Publishers, Dordrecht, pp. 366.

Litvin, F. F., Sineshchekov, O. A. and Sineshchekov, V. A. (1978), Photoreceptor electric potential in the phototaxis of the alga *Haematococcus pluvialis*. Nature **271,** 476–478.

Lu, F., Vonshak, A. and Boussiba, S. (1994), Effect of temperature and irradiance on growth of *Haematococcus pluvialis* (Chlorophyceae). J. Phycol. **30,** 829–833.

Lu, F., Vonshak, A., Gabbay, R., Hirschberg, J. and Boussiba, S. (1995), The biosynthetic pathway of astaxanthin in a green alga *Haematococcus pluvialis* as indicated by inhibition with diphenylamine. Plant Cell Physiol. **36,** 1519–1524.

Mishra, N. P. and Ghanotakis, D. F. (1994), Exposure of a photosystem II complex to chemically generated singlet oxygen results in D1 fragments similar to the

ones observed during aerobic photoinhibition. Biochim. Biophysic. Acta **1187,** 296–300.

Paerl, H. W. (1984), Cyanobacterial carotenoids: their roles in maintaining optimal photosynthetic production among aquatic bloom forming genera. Oecologia **61,** 143–149.

Sandmann, G., Kuhn, M. and Böger, P. (1993), Carotenoids in photosynthesis: Protection of D1 degradation in the light. Photosynthesis Research **35**, 185–190.

Santos, F. M. and Mesquita, J. F. (1984), Ultrastructural study of *Haematococcus lacustris* (Girod.) Rostafinski (Volvocales) 1. Some aspects of carotenogenesis. *Cytologia* **49,** 215–228.

Schroeder, W. A. and Johnson, E. A. (1993), Antioxidant role of carotenoids in *Phaffia rhodozyma*. J. General Microbiol. **139,** 907–912.

Schroeder, W. A. and Johnson, E. A. (1995), Singlet oxygen and peroxyl radicals regulate carotenoid biosynthesis in *Phaffia rhodozyma*. J. Biol. Chem. **270,** 18374–18379.

Shaish, A., Avron, M., Pick, U. and Ben-Amotz, A. (1993), Are active oxygen species involved in induction of β-carotene in *Dunaliella bardawil?* Planta **190,** 363–368.

Tonkyn, J. C., Deng, X. W. and Gruissem, W. (1992), Regulation of plastid gene expression during photooxidative stress. Plant Physiol. **99,** 1406–1415.

Vonshak, A., Guy, R., Poplawsky, R. and Ohad, I. (1988), Photoinhibition and its recovery in two strains of the cyanobacterium *Spirulina platensis*. Plant Cell Physiol. **29,** 721–726.

Weedon, B. C. L. (1971), Occurrence. In: Carotenoids (O. Isler ed.). Birkhauser Verlag Basel, Stuttgart, pp. 29–60.

Yong, Y. Y. R. and Lee, Y. K. (1991), Do carotenoids play a photoprotective role in the cytoplasm of *Haematococcus lacustris* (Chlorophyta)? Phycologia **30,** 257–261.