77

BARD

FINAL REPORT
PROJECT NO. US-2006-91

**Carotenoid-Rich Algal Biomass for Aquaculture:
Astaxanthin Production by *Haematococcus pluvialis***

E. Gantt, A. Vonshak, S. Boussiba, Z. Cohen, A. Richmond

1996

BARD SCIENTIFIC REPORT: Final Report

DATE: August 18, 1996

**BARD
P.O. BOX 6
Bet Dagan, ISRAEL**

BARD project No. US-2006-91R

**Title: Carotenoid-Rich Algal Biomass for Aquaculture:
Astaxanthin Production by *Haematococcus pluvialis***

Investigators' Names

Investigators' Institutions

E. Gantt	University of Maryland, College Park
A. Vonshak	Ben Gurion University of the Negev
S. Boussiba	Ben Gurion University of the Negev
Z. Cohen	Ben Gurion University of the Negev
A. Richmond	Ben Gurion University of the Negev

Project's starting date: August 1, 1992

Type of Report: Final

Signatures

Principal Investigator

Elisabeth Gantt

Institution's Authorizing Official

**Erica Magrum, Director
Research Administration & Advancement**

TABLE OF CONTENTS

ABSTRACT.....	1
OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL.....	2
RELEVANT DATA SUMMARY.....	4
A. <i>Conditions and Physiology for Astaxanthin Accumulation</i>	4
1. Effect of temperature and irradiance.....	4
2. Changes in photosynthetic components and capacity during carotenoid accumulation.....	4
3. Evaluation of <i>Haematococcus</i> strains and mutants.....	6
4. Evaluating the photoprotective role of astaxanthin.....	7
B. <i>Biosynthetic pathway and genes encoding carotenoid enzymes</i>	8
1. Functional analysis of β -cyclase in cyanobacteria.....	8
2. Diphenylamine inhibition of astaxanthin synthesis.....	10
3. Functional analysis of cyclases in higher plants.....	11
4. Functional analysis of β -carotene hydroxylase	12
C. <i>Optimizing Cell Production in Photobioreactors</i>	13
DESCRIPTION OF THE COOPERATION.....	13
EVALUATION OF THE RESEARCH ACHIEVEMENTS.....	14
CONCLUSION AND FUTURE PROSPECTS.....	15
PUBLICATIONS RESULTING FROM RESEARCH.....	16
PATENTS.....	17

ABSTRACT

Carotenoid-Rich Algal Biomass for Aquaculture: Astaxanthin Production by *Haematococcus pluvialis*

The synthesis of carotenoids has been studied toward enhancing the production of keto-carotenoids, since fish and crustaceans raised by aquaculture require astaxanthin and other keto-carotenoids in their feed for desirable pigmentation. Notable progress has been made in attaining the goals of determining improved conditions for ketocarotenoid production in *Haematococcus pluvialis*, and in elucidating the carotenoid biosynthetic pathway. For production of astaxanthin a number of strains of the green alga *Haematococcus* were evaluated, and strain CCAG was found to be optimal for photoautotrophic growth. Of four mutants, selected for enhanced carotenoid production, two hold considerable promise because carotenoid accumulation occurs without encystment. The biosynthetic pathway of carotenoids was elucidated in photosynthetic organisms by characterizing novel genes encoding carotenoid enzymes and by examining the function of these enzymes in a bacterial complementation system. Two cyclases (β - and ϵ -) were cloned that are at a critical branch point in the pathway. One branch leads to the formation of β -carotene and zeaxanthin and astaxanthin, and the other to the production of α -carotene and lutein. Cyclization of both endgroups of lycopene to yield β -carotene was shown to be catalyzed by a single gene product, β -lycopene cyclase in cyanobacteria and plants. The formation of α -carotene was found to require the ϵ -cyclase gene product in addition to the β -cyclase. By cloning a β -hydroxylase gene we showed that a single gene product forms zeaxanthin by hydroxylation of both β -carotene rings. It is expected that a second hydroxylase is required in the synthesis of astaxanthin, since canthaxanthin rather than zeaxanthin is the precursor. Evidence, from inhibitor studies, suggests that astaxanthin is formed from canthaxanthin and that β -carotene is a major precursor. Feasibility studies with the photobioreactors have shown that a two-stage system is the most practical, where *Haematococcus* cultures are first grown to high cell density and are then switched to high light for maximal astaxanthin production. The basic knowledge and molecular tools generated from this study will significantly enhance *Haematococcus* as a viable model for enhanced astaxanthin production.

OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL:

Considerable interest has been developing in producing ketocarotenoid-containing algal biomass in large quantity to meet the need for natural aquaculture feed additives and food coloring agents. A limited understanding of the physiology of algal growth on a large scale, and a lack of detailed information on the influence of nutritional and environmental factors on accumulation of carotenoid pigments are impediments to the development of reliable and economical commercial production processes.

Carotenoids are important in absorbing light for photosynthesis, in protecting the photosynthetic apparatus from the overexcitation of light, as vegetable colorants, and in aquaculture of crustaceans and certain fishes. Carotenoids are ubiquitous and essential components of all known oxygenic photosynthetic organisms. The bicyclic hydrocarbon β -carotene is found closely associated with all oxygenic photosynthetic reaction centers where it performs the critical role of protecting against potentially lethal photo-oxidative damage. Zeaxanthin is thought to be involved in the nonradiative dissipation of light energy under conditions of excessive photon capture by the photosynthetic light-harvesting apparatus in green plants and algae. Several other carotenoids may serve as antenna pigments which enable plants to capture photons at wavelengths not efficiently absorbed by chlorophyll (Goodwin 1980, The Biochemistry of Carotenoids, Chapman and Hall), or provide precursors for the biosynthesis of the plant growth regulator abscisic acid, and are precursors for vitamin A in humans and animals.

Certain "secondary" or non-photosynthetic carotenoids can accumulate in a few green algae under stress conditions. The orange-red ketocarotenoids, in particular astaxanthin (Fig. 1) are an effective carotenoid colorant in fish and crustaceans. *Haematococcus* and some other algae will accumulate ketocarotenoids in the cells, which makes them of interest for mass culturing. Since the accumulation usually occurs under unfavorable conditions, cell division virtually ceases, thus making it difficult to maintain a continuous culture regimen and thereby

achieve continuous ketocarotenoid production.

Primary emphasis of our research has been to understand the physiological parameters associated with the accumulation of carotenoids in the unicellular green alga *Haematococcus pluvialis* so that mass production could be enhanced. Also, we concentrated on elucidation of the carotenoid biosynthetic pathway since it had been very poorly understood. Because this is the era of the genetic manipulation of plants, this work provides molecular tools for targeted improvement in carotenoid accumulation.

A major goal of the research has been to provide fundamental knowledge of cell physiology, carotenogenesis, and algal mass culture which is essential to the development of an economical commercial production process. Considerable progress has been made toward solving the original objectives which were to:

1. Optimize nutritional and environmental conditions for maximum yield of astaxanthin (a ketocarotenoid) in strains of *Haematococcus*.
2. Select mutants which accumulate greater amounts of astaxanthin.
3. Study the induction and developmental process of encystment which is intimately associated with astaxanthin accumulation.
4. Examine the expression of genes and ascertain the subcellular location of enzymes of the carotenoid biosynthesis pathway during the transition from vegetative cell to cyst.
5. Isolate and characterize one or more of the enzymes specific for the biosynthesis of astaxanthin and other ketocarotenoids.
6. Evaluate and optimize large-scale production in closed tubular photobioreactors.

Recognition of results from work supported by this grant was recently given. In a commentary in the latest issue of *The Plant Cell* (September 1996, C.B.Taylor, 8:1447-1450) one of the most highly regarded plant journals; much of our work on the molecular genetics of carotenoid biosynthesis was featured. The value of the heterologous expression system in *E. coli* in elucidation of the cyclization of lycopene and the control of carotenoid biosynthesis was

emphasized. Also, in 1993 a BARD Newsletter presented the efforts on the pilot studies for mass culturing for astaxanthin production. To date, 7 papers have been published in highly regarded journals, and another has been submitted.

RELEVANT DATA SUMMARY

A. Conditions and Physiology for Astaxanthin Accumulation

1. Effect of temperature and irradiance

Toward enhancing cell growth and subsequent astaxanthin production, culture characteristics of *Haematococcus pluvialis* (Göttingen Culture Collection) were evaluated (batch culture) at various temperatures and light conditions (Boussiba *et al.* 1992, Fan *et al.* 1994: attached). Astaxanthin content was significantly affected by growth conditions. In mineral medium (BG-11) optimal growth occurred at 25-28°C. At higher temperature, no cell division was observed, and cell diameter increased from 5 to 25 μm . Optimal photosynthetic efficiency occurred at 80-90 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$. Astaxanthin production was enhanced by increasing light intensity, up to 400 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$, and by depletion of essential minerals. Among the nutrient elements tested, phosphate deficiency resulted in the greatest astaxanthin induction, with a 9-fold increase of astaxanthin content (as percent of dry weight). Deficiency of nitrogen, sulfate, potassium, or magnesium showed respective astaxanthin increases of 6, 5, 4, and 2.5-fold. Clearly, deficiency of any single nutrient element will disturb cellular processes, such as photosynthesis, respiration and probably the interaction between cellular compartments. However, the mechanism(s) by which these occur is yet to be elucidated.

2. Changes in photosynthetic components and capacity during carotenoid accumulation.

Photosynthetic activity, chloroplast enzymes, and polypeptides were compared in green and red (ketocarotenoid-containing) cultures of *H. pluvialis* (Tan *et al.* 1995: attached). Green cultures, grown at 80 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ in an acetate-containing medium, had a mean

generation time of 27 h. Ketocarotenoid accumulation was induced by transfer of green cultures to PO_4 -deficient medium and exposure to $250 \mu\text{mol photons m}^{-2}\text{s}^{-1}$. Under these conditions there was no increase in cell number, and the cultures produced substantial amounts of astaxanthin. Relative amounts of enzymes and thylakoid polypeptides in red and green cells were ascertained by immunoprobings with standardization on a Chl basis. In red cultures the level of cytochrome *f* was greatly decreased ($<1\%$ of green cell level), which is expected to greatly impair the linear electron flow from PS II to PS I. Also, the levels of apoproteins in red cells, namely of CPI, D2, CP47, LHCI and Rubisco, were reduced to 15%, 18%, 29%, 48%, and 80%, respectively, of those in green cells. Only ATP synthase exhibited no significant change in the two types of cultures. The respiration rate of red cultures was much higher ($100 \mu\text{moles O}_2\text{mg Chl}^{-1}\text{h}^{-1}$) than those of green cells ($16 \mu\text{moles O}_2\text{mg Chl}^{-1}\text{h}^{-1}$). Conversely, net O_2 -evolution (at P_{max}) in green cultures was $80 \mu\text{moles O}_2\text{mg Chl}^{-1}\text{h}^{-1}$, but was $-40 \mu\text{moles O}_2\text{mg Chl}^{-1}\text{h}^{-1}$ in red cultures. Photosystem II activity was demonstrated in broken cells of both green and red cultures, showing activity of 40 and $15 \mu\text{moles DCPIPmg Chl}^{-1}\text{h}^{-1}$ (with DPC as electron donor), respectively. In contrast, PS I activity measured by the Mehler reaction showed that red rather than green cells had a greater activity (64 vs. $46 \mu\text{moles O}_2\text{mg Chl}^{-1}\text{h}^{-1}$, respectively). Thus, in spite of the decline of O_2 -evolution in red cells, the photosystems were still functional, and the decline of O_2 -evolution in red cells is largely attributable to an increase in the respiration rate and the impairment of linear electron flow from PS II to PS I and, to some extent, to a decrease in components of the photosystems. The destabilization of the cyt. *b₆/f* complex during phosphate (also found with nitrate) deficiency, suggests that cells have an impaired carbon balance because CO_2 fixation is probably limited by reducing power. Carotenoid accumulation is considered to be a consequence of a general stress response, hence a secondary rather than a primary consequence. From these results it can be inferred that it might be possible to find conditions where photosynthetically fully active cells could be induced to accumulate astaxanthin and to continue cell division and growth.

3. Evaluation of *Haematococcus* strains and mutants

For purposes of growing *Haematococcus* in a bioreactor it necessary to evaluate *Haematococcus* strains which have enhanced astaxanthin production, and/or cultures which produce extra ketocarotenoids in the active growth phase. Normally, cell division ceases under high astaxanthin accumulation conditions. Six *Haematococcus* isolates originating from different countries, regions, and culture collections were examined. Evaluation in autotrophic and heterotrophic media showed that growth of most strains was greatest in a heterotrophic medium. Per liter the medium contained 20 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 20 mg $\text{Ca}[\text{NO}_3]_2 \cdot 4\text{H}_2\text{O}$, 2 g yeast extract, and 14/6 mM sodium acetate, 2.7 mM L-asparagine. However, a heterotrophic medium is less suitable for outdoor bioreactors because the danger of bacterial and fungal contamination is much greater than with a mineral medium such as BG-11. In evaluating the results, we conclude that the *Haematococcus* strain CCAG on which we have done most of our studies (*H. pluvialis* Flotow from the Göttingen Culture Collection) is the strain best suited for photoautotrophic growth in bioreactors, compared to the five other wild-type strains evaluated.

A limitation to enhanced production of carotenoids in *H. pluvialis* is that cessation of active growth and formation of thick-walled cells are kinetically linked to pigment accumulation. Mutants were therefore sought in which carotenoid accumulation occurred in actively growing cultures. Vegetative cells were mutagenized with ethane methylsulfonate and exposed to continuous high blue-light irradiance ($330 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) for a time-period sufficient to kill the majority of cells. Surviving cells were spread on agar plants, and mutants (Car 1 - Car 4) were selected on the basis of their orange-red color. Subsequent subculturing has shown that the mutants are stable. Carotenoid accumulation in the mutants occurred earlier in the developmental cycle than in the wild type under medium light conditions ($150 \mu\text{mol photon} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). Car 2 forms very large cyst-type cells and was not found desirable for continuous culturing in bioreactors. The results of the mutant studies indicate that carotenoid accumulation can be uncoupled from cessation of cell division. Two of the mutants (Car 3 and Car 4) now

under investigation hold considerable promise for improving carotenoid production in bioreactors. Avoiding the encystment stage, with these type of mutants, can provide continuously growing cultures which accumulate astaxanthin in reasonable quantities.

4. Evaluating the photoprotective role of astaxanthin

A well recognized protective function of carotenoids is to prevent damage to the photosynthetic apparatus caused by excessive illumination by quenching of chlorophyll triplet states and of singlet oxygen radicals. Astaxanthin accumulation in *Haematococcus* has been regarded to serve a similar function, even though astaxanthin, rather than accumulating in the chloroplast, accumulates in the cytoplasm. To test whether astaxanthin could protect the photosynthetic apparatus by screening out excessive irradiance, photoinhibition experiments under high light ($450 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) were performed. Changes in photosynthetic activity, measured as oxygen evolution rates or variable fluorescence (F_v/F_m), of green (astaxanthin-poor) and red (astaxanthin-rich) demonstrated that both exhibited typical photoinhibition characteristics. Surprisingly, photosynthetic activity in green cells containing only trace amounts of astaxanthin decreased slowly, whereas, photosynthetic activity decreased faster in red cells (Fan *et al.* 1996: submitted). These experiments indicate that astaxanthin did not protect the photosynthetic reaction centers of *Haematococcus* from damage caused by excessive irradiance, and thus cannot be regarded as an overall screen. However, since the astaxanthin is in the cytoplasm and thus not within close contact of the photosynthetic systems in the thylakoids, or at the periphery of the cell, it would be difficult to understand by what known mechanism it could serve a protective function at considerable distance. Nevertheless, astaxanthin could serve other functions in protecting cells by serving as a screen to filter out excessive irradiance. By a series of experiments the role of astaxanthin as an exogenous scavenger of radical oxygen scavenger was also evaluated. Since astaxanthin production is induced by a multitude of stress factors, our collective data lead us to propose that it is part of a general system of antioxidants for destruction of singlet oxygen.

B. Biosynthetic pathway and genes encoding carotenoid enzymes

The possible pathway of astaxanthin production in *Haematococcus* is outlined in Fig. 1 and represents a composite primarily of results from plants and other algae (key references in Cunningham *et al.* 1996: attached). Since carotenoids are tetraterpenes, they share a common pathway with other terpenoids until the production of the symmetrical 40 carbon phytoene from geranylgeranyl diphosphate. Phytoene undergoes a series of desaturations to form lycopene. At the time the initial proposal was written phytoene synthase (converts GGDP to phytoene) and phytoene desaturase (converts phytoene to ζ -carotene) were the only carotenoid synthesizing enzymes for which the genes in plants or algae had been identified. Since then, we have cloned the genes and described the molecular structure and enzymatic function of three more enzymes for carotenoid biosynthesis (Cunningham *et al.* 1994, 1996, Sun *et al.* 1996: attached) and furthermore provide evidence that β -carotene is a required precursor for astaxanthin production (Fan *et al.* 1995: attached).

1. Functional analysis of β -cyclase in cyanobacteria

A key branch point in carotenoid synthesis of photosynthetic organisms occurs at lycopene. The pathway branches with the cyclization of lycopene to form two different types of bicyclic compounds essential for the photosynthetic apparatus. Compounds with two β rings, including β -carotene, zeaxanthin and violaxanthin, derive from one branch. A second branch (not shown in Fig. 1), whereby the carotenoids contain one β and one ϵ ring, leads to lutein. Previous to our recent work, no cyclase had been cloned from a photosynthetic organism. We selected a cyanobacterium as a model for plants in cloning the first cyclase since it was already known that cyanobacterial enzymes were homologous with those in plants. The first lycopene cyclase from an oxygenic photosynthetic organism was cloned by using a transformable cyanobacterium (*Synechococcus* strain PCC7942) (Cunningham *et al.* 1994: attached). This gene, which we named *crtL*, was expressed in strains of *E. coli* that were genetically engineered to accumulate lycopene, neurosporene, and ζ -carotene (see Fig. 1). The *crtL* gene product

FIGURE 1. PROPOSED CAROTENOID BIOSYNTHETIC PATHWAY LEADING TO ASTAXANTHIN PRODUCTION IN *Haematococcus*

converts the acyclic lycopene into the bicyclic β -carotene, an essential component of the photosynthetic apparatus in oxygen-evolving organisms and a source of vitamin A in animal nutrition. The enzyme also converts neurosporene to the monocyclic β -zeacarotene, but does not cyclize ζ -carotene. The bleaching herbicide 2-(4-methylphenoxy)triethylamine hydrochloride effectively inhibits both cyclization reactions. A mutation that confers resistance to this herbicide in *Synechococcus* PCC7942 was identified as a mutation in the promoter region of *crtL*. The deduced amino acid sequence of lycopene cyclase specifies a polypeptide of 411 amino acids with a molecular weight of 46,125. DNA gel blot hybridization analysis indicated a single copy of *crtL* in this cyanobacterium. Except for an FAD binding motif, the cyanobacterial lycopene cyclase bears little resemblance to two known lycopene cyclase enzymes from nonphotosynthetic bacteria. Results from DNA gel blot hybridization suggested that the cyanobacterial gene is homologous to plant and algal genes encoding β -lycopene cyclase, which we and others (cf. Cunningham *et al.* 1996:attached) subsequently cloned.

2. Diphenylamine inhibition of astaxanthin synthesis

To obtain an idea of the pathway of synthesis from β -carotene to astaxanthin, cells in the astaxanthin accumulation phase were analyzed. Diphenylamine (DPA), which was known to affect carotenoid accumulation in some algae and in yeast, was used with *Haematococcus*. Diphenylamine (30 μ M), when added to growing green cultures had no major effect on cell division, chlorophyll content, carotenoid composition, and oxygen evolution (photosystem II indicator) and oxygen uptake (photosystem I indicator) (Fan *et al.* 1995: attached). However, when cultures were placed under astaxanthin accumulating conditions, i.e. phosphate deficiency and high light, DPA affected the carotenoid composition. Examination by electron microscopy of treated and control cells indicated that the cells had common organelles, but the chloroplast was greatly reduced and most of the cell volume was occupied by electron dense material, i.e. putative carotenoids. Without DPA, astaxanthin and its esters were the primary carotenoids produced after 5 days along with lesser amounts of canthaxanthin. Interestingly, when treated

with the DPA inhibitor, β -carotene was primarily accumulated and astaxanthin was not detectable. Upon removal of DPA, by washing the cells, astaxanthin then accumulated and the amount β -carotene declined. These results suggest that β -carotene is the primary precursor of astaxanthin, and it is very likely that canthaxanthin is an intermediate. Canthaxanthin is presumably hydroxylated in the process of astaxanthin formation, but this remains to be elucidated. An alternate pathway from β -carotene via zeaxanthin is suggested in Fig. 1, although it is not considered likely that the zeaxanthin is an intermediate in the formation of astaxanthin since it was not detectable with or without DPA. Evidence for canthaxanthin as intermediate was recently reported by Lotan and Hirschberg (1995, FEBS Letts. 364:125) who cloned a gene encoding β -C-4-oxygenase from *Haematococcus* that converts β -carotene to canthaxanthin.

3. Functional analysis of cyclases in higher plants

The biosynthesis of carotenoids occurs within the chloroplasts of plants and algae. Several sites of carotenoid synthesis in the cytoplasm of *Haematococcus* remain as possibilities, although there is no conclusive evidence at this time. Carotenoids with cyclic end groups are essential components of the photosynthetic membranes in all plants, algae, and cyanobacteria. These lipid-soluble compounds protect against photooxidation, harvest light for photosynthesis, and dissipate excess light energy absorbed by the antenna pigments. The cyclization of lycopene is a key branch point in the pathway of carotenoid biosynthesis, and as noted above two types of cyclic end groups are found in eukaryotic photosynthetic organisms: those with β and ϵ rings. Carotenoids with two β rings such as β -carotene are ubiquitous, and also include astaxanthin (Fig. 1). Allocation of precursor substrates at key branch points likely influences the synthesis and accumulation of carotenoids later in the pathway, such as astaxanthin. It was thus of interest to explore the formation of the ϵ -ring since carotenoids with β,ϵ -rings are produced in significant quantities (the β,ϵ -carotenoid lutein may account for more than 50% of the total carotenoid pigments in plant and algae). We identified and sequenced cDNAs that encode the enzymes catalyzing the formation of these two rings in *Arabidopsis* (Cunningham *et al.*, 1996: attached).

These β and ϵ cyclases are encoded by related, single-copy genes, and both enzymes use the linear, symmetrical lycopene as a substrate. However, the ϵ cyclase adds only one ring, to form the monocyclic δ -carotene, whereas the β cyclase introduces a ring at both ends of lycopene to form the bicyclic β -carotene. When combined, the β and ϵ cyclases convert lycopene to α -carotene, a carotenoid with one β and one ϵ ring. The inability of the ϵ -cyclase to catalyze the introduction of a second ϵ ring reveals a mechanism by which production and proportions of the two types of rings may be controlled and adjusted in plants and algae. It is possible that transcription and/or translation of the ϵ -cyclase is turned off, when *Haematococcus* is placed under stress conditions, and substrate flow may therefore be directed primarily to β -carotene which serves as the precursor for astaxanthin accumulation.

4. Functional analysis of β -carotene hydroxylase

Zeaxanthin, a common carotenoid for xanthophylls required in photosynthetic tissues, is formed by the hydroxylation of both rings of β -carotene and was considered as a potential precursor for astaxanthin. Thus, it was of interest to take a molecular genetic approach, wherein we employed the previously used color screening system for carotenoid genes to identify a gene encoding β -carotene hydroxylase. An *Arabidopsis thaliana* cDNA encoding the enzyme β -carotene hydroxylase was identified by functional complementation in *E. coli*. It is important to note that an essential feature of the plasmid construct used for the color screening was a cDNA coding for isopentenyl diphosphate (pyrophosphate) isomerase from *Haematococcus* (Sun *et al.* 1996:attached). The product of the *Arabidopsis* cDNA was found to add hydroxyl groups to both β rings of β -carotene to form zeaxanthin. It also converts the monocyclic β -zeacarotene to hydroxy- β -zeacarotene. However, substrates containing ϵ rings were poor substrates, suggesting that a separate hydroxylating enzyme is required. The predicted amino acid sequence of the β -hydroxylase clone had only 31-37% identity with the functionally comparable hydroxylases in bacteria. The *Arabidopsis* gene is the first to be identified for a hydroxylase in a photosynthetic organism. The *Arabidopsis* polypeptide had a lengthy (> 130 amino acids) N-terminal extension,

not present in bacteria. Removal of the first 129 N-terminal amino acid residues resulted in the production of the monohydroxy carotenoid β -cryptoxanthin with β -carotene as the substrate. Thus, it appears that the N-terminal extension is required for full functioning of the enzyme in the higher plant (Sun *et al.*, 1996, J. Biol. Chem.: attached). It is of special interest that expression of isopentenyl diphosphate isomerase, from *Haematococcus*, resulted in substantial enhancement of zeaxanthin and its intermediate in the *E. coli* system. This suggests that this enzyme, although very early in the isoprenoid pathway (Fig. 1), plays a key role in regulating the production of carotenoids. In view of the results indicating that canthaxanthin, and not zeaxanthin is the precursor for astaxanthin, we expect that another hydroxylase, specific for canthaxanthin rather than zeaxanthin, is probably required for astaxanthin formation.

C. Optimizing Cell Production in Photobioreactors

A pilot system was developed whereby *Haematococcus pluvialis* can be effectively grown outdoors in a closed two-stage bioreactor system. A closed system is required for growing this alga in a simple mineral medium (like BG-11) in the normal pH range, since the cultures are readily susceptible to contaminants if open to the air. In the first stage, cultures are grown to a high cell density in multiliter quantities in a tubular bioreactor. Through tubular connections the cultures are subsequently transferred to plexiglass chambers (mounted like solar panels) that have a high surface to volume ratio to provide maximal exposure to sunlight. Under these conditions the cells rapidly begin the production of astaxanthin as they also change to the cyst stage. These pilot studies have shown that mass production is feasible.

DESCRIPTION OF THE COOPERATION

The cooperation between the laboratories in Israel and the U.S. has occurred on multiple levels on the research efforts with *Haematococcus pluvialis*. We cooperated in the production (in the U.S. lab.) of two astaxanthin accumulating mutants and in testing (in the Israeli lab.) their suitability for outdoor bioreactor studies. The second set of mutants (Car 3 and 4) will be

similarly tested.

Evaluation by electron microscopy of the effect on diphenylamine treatment on astaxanthin accumulation involved direct cooperation, with cell treatment being done in Israel and the electron microscopy in the U.S. laboratory.

Both laboratories compared the results on the elucidation of the biosynthetic pathway of carotenoids on a regular basis by mail, e-mail, FAX and in person.

Similarly, both laboratories compared the results seeking optimal conditions for growth, and results on important physiological parameters of astaxanthin production.

EVALUATION OF RESEARCH ACHIEVEMENTS

Considerable progress has been made in the elucidation of the carotenoid biosynthetic pathway. Four novel genes were cloned and sequenced. Two β -cyclases, which convert lycopene to β -carotene, have been characterized from a cyanobacterium and from a higher plant. An ϵ -cyclase, which competes for the same lycopene substrate, was characterized from a higher plant, as was a hydroxylase which is responsible for zeaxanthin formation. Cloning of these genes provides useful probes to be used in studying the expression and regulation of carotenoid genes in *Haematococcus* and in plants. That isopentenyl diphosphate isomerase enhances carotenoid accumulation may be particularly useful toward enhancing astaxanthin production. The demonstration that cantathaxanthin is the intermediate in the pathway to astaxanthin production advances our knowledge for future developmental approaches.

The pilot studies with the photobioreactors show the feasibility of mass producing the algae for enhanced carotenoid production. Significant improvement can also be expected from a continuous culture system using some of the mutants where enhanced astaxanthin production has been dissociated from cyst formation which has been an impediment.

The original objectives of the grant have been attained. Furthermore, eight publications (including one submitted) in highly regarded journals have resulted from support by this grant.

CONCLUSIONS AND FUTURE PROSPECTS

The use of *Haematococcus* as an alga for mass production of astaxanthin looks very favorable. With much of the baseline data provided from this study, the Israel laboratory is going forth with improving the bioreactor efforts. The U.S. laboratory is using the techniques developed and genes obtained from the molecular genetic studies to study the regulation of carotenoid biosynthesis in *Haematococcus*. We have produced antisera to several of the carotenoid enzymes and are attempting to develop this alga into a transformable system to better serve as a model system for carotenoid studies.

PUBLICATIONS RESULTING FROM RESEARCH

- Boussiba S, Fan L, Vonshak A (1992)** Enhancement and determination of astaxanthin accumulation in the green alga *Haematococcus pluvialis*. *Methods in Enzymology* 213: 386-371
- Cunningham FX, Sun Z, Chamovitz D, Hirschberg J, Gantt E (1994)** Molecular structure and enzymatic function of lycopene cyclase from the cyanobacterium *Synechococcus* sp strain PCC7942. *The Plant Cell* 6: 1107-1121
- Cunningham FX, Pogson B, Sun Z, McDonald, DellaPenna D, Gantt E (1996)** Functional analysis of the β and ϵ lycopene cyclase enzymes of *Arabidopsis* reveals a mechanism for control of cyclic carotenoid formation. *The Plant Cell* 8:1613-1626
- Fan L, Vonshak A, Boussiba S (1994)** Effect of temperature and irradiance on growth of *Haematococcus pluvialis* (Chlorophyceae). *J Phycol* 30: 829-833
- Fan L, Vonshak A, Gabbay R, Hirschberg J, Cohen Z, Boussiba S (1995)** The biosynthetic pathway of astaxanthin in a green alga *Haematococcus pluvialis* as indicated by the inhibition with diphenylamine. *Plant Cell Physiol* 36: 1519-1524
- Fan L, Vonshak A, Boussiba S (1996)** A novel photoprotective mechanism for the ketocarotenoid astaxanthin *Haematococcus pluvialis*. *Plant Physiology* (submitted)
- Tan S, Cunningham FX, Youmans M, Grabowski B, Sun Z, Gantt E (1995)** Cytochrome *f* loss in astaxanthin-accumulating red cells of *Haematococcus pluvialis* (Chlorophyceae): comparison of photosynthetic activity, photosynthetic enzymes, and thylakoid membrane polypeptides in red and green cells. *J Phycol* 31: 897-905
- Sun Z, Gantt E, Cunningham FX (1996)** Cloning and Functional analysis of the β -carotene hydroxylase of *Arabidopsis thaliana*. *J Biol Chem* 271: 24349-24352

PATENTS

From work supported by this grant a patent applications has been submitted by the Office of Technology Liaison of the University of Maryland:

Docket No. 2747-063-27 *Genes of Carotenoid Biosynthesis and Metabolism and a System for Screening such Genes*