Plant Physiol. (1990) 93, 389–395
0032-0889/90/93/0389/07/$01.00/0

Received for publication October 4, 1989
Accepted December 4, 1989

Photoinduction of Massive β-Carotene Accumulation by the Alga *Dunaliella bardawil*[1]

Kinetics and Dependence on Gene Activation

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ABSTRACT

The massive accumulation of β-carotene by the halotolerant micro alga *Dunaliella bardawil*, in response to high light intensity and several other environmental factors, has been studied so far under different sets of fixed conditions. To determine the kinetics and characteristics of the induction of β-carotene accumulation, cells continuously grown under white light of approximately 27 microeinsteins per square meter per second were exposed to light of approximately 1650 microeinsteins per square meter per second. The exposed cells accumulate β-carotene in two stages: the first stage, lasting for 24 hours, starts shortly after exposure, whereas the second stage starts concomitantly with the onset of the stationary phase and persists until the cells collapse. Actinomycin D, chloramphenicol, or cycloheximide added to low-illuminated cultures abolish the subsequent induction of β-carotene accumulation by high light intensity. These results, together with the early exponential kinetics of accumulation, point to the role of gene activation in the process. *In vivo* labeling of proteins and *in vitro* translation of poly(A)$^+$ mRNA revealed several pronounced differences between low-illuminated and high-illuminated cells. A strongly light-induced protein of approximately 55 kilodaltons, as well as other light-induced proteins could possibly fulfill a carotenogenic function.

Carotenes are widely occurring, natural pigments fulfilling diverse functions. In photosynthetic organisms, carotenes act as accessory light-harvesting pigments and provide protection against photo-oxidation. The regulation of carotene biosynthesis as a function of developmental stage or environmental changes has been studied in a host of organisms ranging from bacteria to higher plants. In most, although not all instances carotene biosynthesis has been found to exhibit a partial or complete dependence on light (for reviews see references 2, 8, 10, 15).

The exceptional ability of the micro algae *Dunaliella bardawil* to accumulate massive amounts of β-carotene, primarily in response to high light intensity (1–7 and references therein), makes it an interesting subject for studies of carotenogenesis regulation. The genus *Dunaliella* includes unicellular, halotolerant, motile, green algae which are devoid of a rigid cell wall and contain a single, large, cup-shaped chloroplast (2). All *Dunaliella* species synthesize β-carotene, but only a few isolates, *e.g. D. bardawil*, have the potential to accumulate the pigment to up to 10% of the algal dry weight. β-Carotene production is proportional to the light intensity with the effective range of light wavelengths overlapping the photosynthetically active radiation range (5). Pigment content is also enhanced under growth-limiting conditions including high salinity, or deprivation of mineral nutrients such as nitrate or sulfate (1, 3, 7 and references therein). The combined effects of light and growth limitations on β-carotene accumulation have led to the proposal that accumulation is directly proportional to the integral amount of light to which the *D. bardawil* cells are exposed during a division cycle (3). The accumulated β-carotene, mainly consisting of the 9-*cis* and all-*trans* isomers (4), is stored in the form of intrachloroplastic lipid globules and provides protection against photoinhibition by light of high intensity (6, 7).

In most studies so far, β-carotene levels were compared in *D. bardawil* cultures growing under different sets of fixed conditions, rather than during the transition from unfavorable to favorable conditions for pigment accumulation. The goal of the present study was to define conditions, kinetics, and characteristics of accumulation induction. For this purpose, cells containing low levels of β-carotene were exposed to conditions conducive to pigment accumulation, and the induction process was analyzed. The results reveal the existence of two stages of β-carotene accumulation and provide evidence for the involvement of gene activation in the process.

MATERIALS AND METHODS

Algae and Growth Conditions

Dunaliella bardawil Ben Amotz and Avron is a locally isolated species (American Type Culture Collection, Rockville, MD, No 30861). *D. salina*, a strain unable to accumulate β-carotene, was obtained by A. Ben Amotz and M. Avron from the culture collection of Dr. W. H. Thomas, La Jolla, CA. The algae were normally grown in the medium described previously (3) with 3 M NaCl. The axenic cultures studied were derived from single colonies grown from individual cells on agar plates as follows: 100 to 200 cells were suspended in 6 mL melted 0.6% Bacto-agar (Difco) in growth medium, at 37 to 40°C, and overlayed on solid 1.5% agar in the same

[1] Supported in part by the Leo and Julia Forchheimer Center for Molecular Genetics, the Weizmann Institute of Science.

medium. The Petri plates were sealed with Parafilm to minimize evaporation, and incubated in an incubator-shaker growth chamber (New Brunswick Scientific), at 26°C, under continuous illumination with cool white fluorescent lamps. The intensity of the light reaching the cultures was approximately 110 μE m^{-2} s^{-1}. (Light intensities were determined by a wavelength independent radiometer [YSI-Kettering, model 65A].) Colonies of *D. salina* became visible after 7 to 10 d of incubation, and colonies of *D. bardawil*, after 14 to 17 d. Cells from single colonies were first transferred to 5 to 10 mL of liquid growth medium as used in the plates, and incubated under the same conditions as the plates, but with continuous, slow rotation. After the culture reached a density of about 10^5 cells/mL, it was diluted with 90 to 240 mL of fresh medium. These suspensions were the source of all the cells used. All the media used were sterile and all manipulations were carried out under aseptic conditions.

Low-Illuminated Cells and Light Induction

Low-illuminated cells were grown in 1 L cultures placed in 2 L Erlenmeyer flasks covered with two layers of lignin sheets to reduce the intensity of the light to approximately 27 μE m^{-2} s^{-1}. Under these conditions, the generation time of the algae was 48 to 72 h, as compared to a generation time of 24 to 36 h of cells grown in uncovered flasks. For light induction, 100 mL of the low-illuminated culture, containing 4 to 5 × 10^5 cells/mL, were transferred to a 250 mL Erlenmeyer flask which was placed in a temperature-controlled photosynthetic Warburg apparatus (Aminco), set at 26°C, equipped with three continuously-lit 500 W halogen lamps. The light intensity reaching the cells was approximately 1650 μE m^{-2} s^{-1}.

Incubation with Inhibitors

Cycloheximide and chloramphenicol were added to low-illuminated cells 3 to 4 h before exposure to light of high intensity, and actinomycin D was added 12 h before exposure. Culture flasks containing chloramphenicol or actinomycin D, and corresponding control cultures without inhibitors, were covered with yellow cellophane sheets (16).

Cell Growth and Pigment Determination

Cell number was determined in a Coulter Counter model ZM with a 100 μm orifice (5). Chl and β-carotene determinations were as described (3, 5).

In Vivo Protein Labeling

A sample of 0.5 mL from a 3 to 6 × 10^5 cells/mL culture was centrifuged, the cell pellet was resuspended in growth medium without sulfate and incubated for 1 h, as described above. To this suspension was added 15 to 25 μL of [^{35}S] sodium sulfate (350–400 mCi/mmol; 12–20 mCi/ml; New England Nuclear), and incubation was continued for 1 h. Cells were collected by centrifugation, resuspended in loading buffer (11), boiled for 3 min and the proteins were resolved by electrophoresis on a 12.5% polyacrylamide-SDS gel (11).

Poly(A)$^+$ mRNA Purification and *in Vitro* Translation

Total RNA was extracted essentially as described (12) with a final additional step of LiCl precipitation (14). Poly(A)$^+$ mRNA was purified by two cycles of fractionation on oligo(dT)-cellulose. The final yield was 1 to 1.5% of the total RNA. *In vitro* translation by a reticulocyte lysate (Promega) was according to the manufacturer's instructions, with 0.5 μg mRNA and 30 μCi of [^{35}S]methionine (1130 Ci/mmol, Amersham) added per reaction. The translation products were resolved on a 7.5 to 15% polyacrylamide gradient-SDS gel (11).

Electron Microscopy

The procedures used were as described previously (7).

RESULTS

Induction of β-Carotene Accumulation by High Light

The first objective of this study was to characterize conditions under which *Dunaliella bardawil* cells contained minimal levels of β-carotene, and then to light-induce these cells to accumulate the pigment. As the cellular contents of β-carotene increased with the salinity of the medium (3), and to avoid osmotic changes during light induction, the algae were continuously grown in media containing 3 M NaCl. Illumination conditions were then established for minimizing the β-carotene level, but still permitting photoautotrophic growth of the algae. When the light intensity was reduced to 27 μE m^{-2} s^{-1}, the culture became green in appearance, and contained, per cell, 2 to 5 pg β-carotene and 10 to 12 pg Chl. The weight ratio of β-carotene in Chl in the low-illuminated cells was 0.3, lower than previously reported values for *D. bardawil*, and approaching the ratio determined for *D. salina* (5, 7).

To induce β-carotene accumulation, the low-illuminated cells were exposed to light of approximately 1650 μE m^{-2} s^{-1}. This exposure caused approximately 5% cell death and elicited a transient stress response in the surviving population including rounding of the cells, decreased motility and cessation of cell division. Recovery occurred 4 to 6 h after exposure. Cells exposed for 24 h started to turn orange and their chlorophyll contents dropped to approximately 9 pg/cell.

Cells after 24 h of high illumination were further compared to the low-illuminated, starting cells by electron microscopy (Fig. 1). β-Carotene globules, which are very few in the starting cells, are greatly increased in number already after 24 h of illumination. In addition, the thylakoid membranes of the exposed cells appear less condensed and the starch bodies less defined than those in the starting cells.

To establish the detailed kinetics of the induction of β-carotene accumulation, cell density and β-carotene accumulation were determined after different times of exposure of low-illuminated cultures to light of high intensity. The results (Fig. 2) show an exponential, up to 10-fold increase in the cellular content of β-carotene during the first 24 h after exposure followed by a period of 3 to 4 d, during which the β-carotene level remained nearly constant, or rose slowly. Ensuing this period is a second stage of increased β-carotene

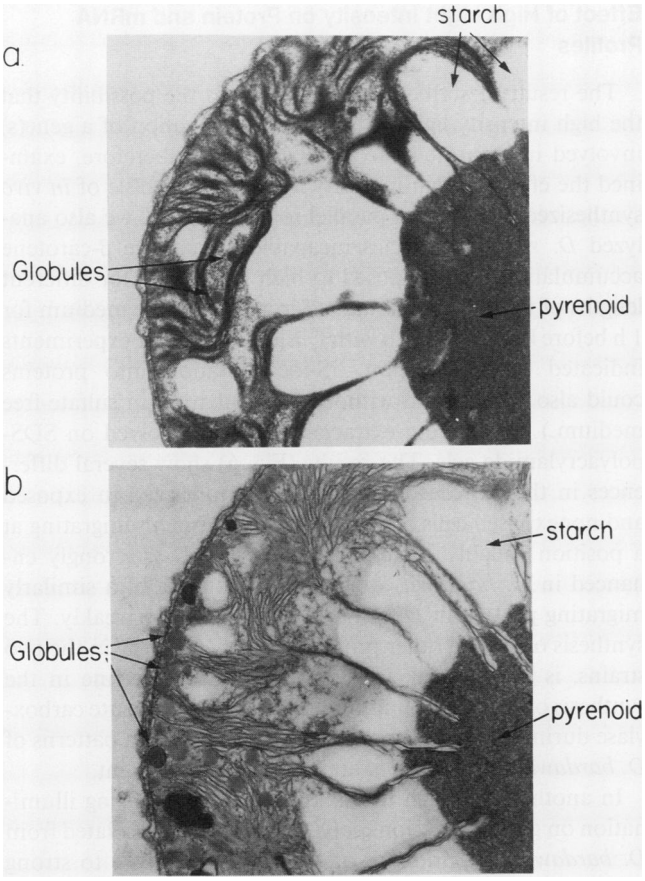

Figure 1. Electron micrographs of (a) low-illuminated *D. bardawil* cells and (b) cells after 24 h of exposure to high light intensity. Only some of the β-carotene globules are indicated.

accumulation as the culture approaches and reaches the stationary phase. During the entire period, the cells undergo one to two divisions. The results shown in Figure 2, as well as the results of two other independent experiments, were also plotted to show changes in the rate of β-carotene accumulation at different time periods after induction (Fig. 3). The two stages of accelerated β-carotene accumulation were repeatedly observed in all three experiments. However, the early stage shows better reproducibility than the late phase in both the timing and extent of rate increase.

Induction of β-Carotene Accumulation by Nutrient Depletion

The β-carotene level of *D. bardawil* has been previously shown to increase under conditions of nutritional deficiency. We have examined the β-carotene accumulation kinetics in cells continuously grown in low light upon transfer to sulfate-depleted medium (Fig. 4). In this medium, the cells stop dividing and start to accumulate β-carotene. The rate of pigment accumulation is, however, considerably slower than that observed under strong light. Even 90 h of sulfate starvation were insufficient to bring the β-carotene level to that reached after 24 h of intense illumination in nondepleted medium.

Transcriptional and Translational Inhibitors Block Accumulation

For further characterization of the regulation of β-carotene accumulation, we chose to study the early phase of induction immediately following exposure to high light. Within this phase, a reproducible, large increase in β-carotene level occurs over a low background in algae capable of cell division.

As a first step toward the elucidation of the induction mechanism, transcriptional (actinomycin D) and translational (chloramphenicol or cycloheximide) inhibitors were used to evaluate the role of gene activation in the process. A serious difficulty stemmed from the high light intensity used for the induction which caused photolysis of actinomycin D into a nontoxic product(s), and of chloramphenicol into a toxic product(s) (16, and references therein). The use of yellow filters, which partially screened light of wavelengths absorbed

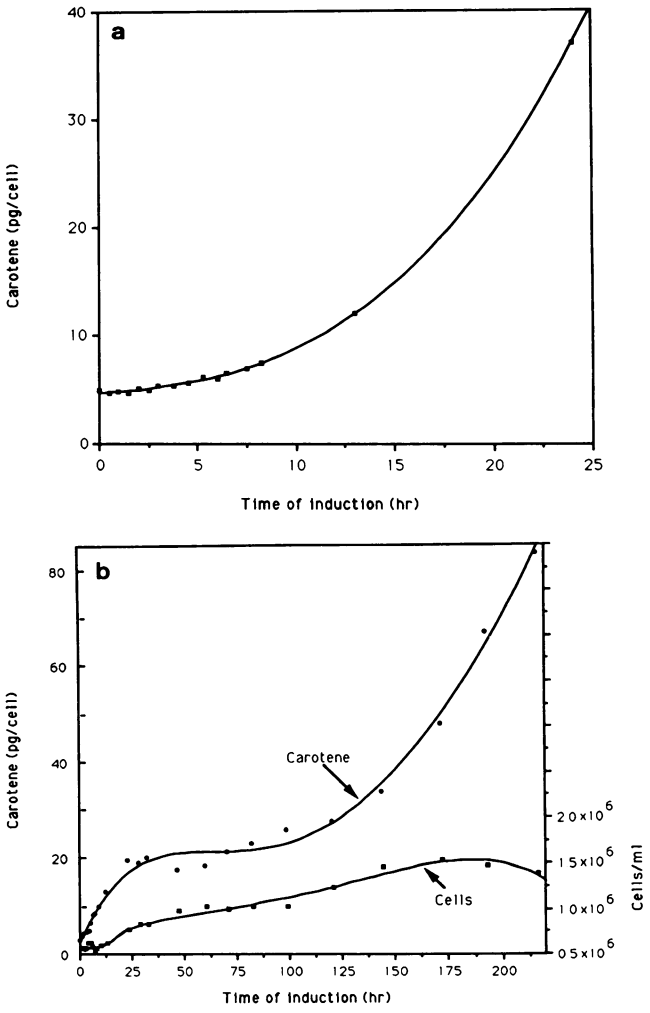

Figure 2. Course of β-carotene accumulation following exposure of low-illuminated *D. bardawil* cells to high light intensity. (a) Experiment 1, β-carotene contents was determined during the first 24 h after exposure. The initial level of β-carotene was 5 pg/cell. (b) Experiment 2, β-carotene contents and cell density were followed for 10 d after exposure to high light intensity. The initial β-carotene level was 2.5 pg/cell.

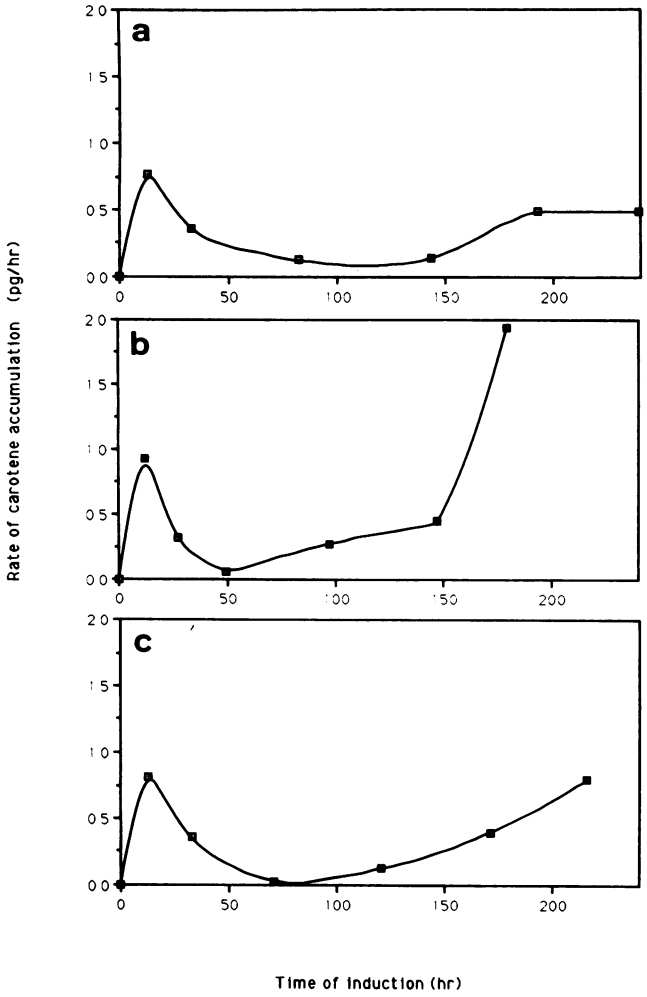

Figure 3. Changes in the rate of β-carotene accumulation by *D. bardawil* with time of exposure. Average rates of β-carotene accumulation for given periods after exposure were calculated by dividing the increment of β-carotene (pg/cell) between two consecutive time points by the time (h) between these points. These values were plotted against the end time point of each interval. (The zero time point was set at zero to reflect the constant β-carotene contents of the cells prior to their exposure to light of high intensity.) a, b, and c represent three independent experiments. c, data from the experiment shown in Figure 2b.

by the inhibitors, prevented the photolysis of both compounds during the duration of the experiment without considerably affecting the induction process. The effective range of concentrations and time of addition of actinomycin D were determined by using cell growth inhibition as a criterion. The conditions for chloramphenicol and cycloheximide inhibition were based on pulse-labeling of inhibitor-treated cultures with [^{35}S]sulfate, as was previously determined for *D. salina* (16).

The results (Fig. 5) clearly show that light-induction of β-carotene overproduction is inhibited by the transcriptional inhibitor, as well as by the organellar and cytoplasmic protein synthesis inhibitors. Hence, both transcription and *de novo* protein synthesis are required for at least the early phase of β-carotene accumulation induction.

Effect of High Light Intensity on Protein and mRNA Profiles

The results described above pointed to the possibility that the high intensity light brought about activation of a gene(s) involved in β-carotene accumulation. We, therefore, examined the effect of high intensity light on the profile of *in vivo* synthesized proteins. In parallel to *D. bardawil*, we also analyzed *D. salina*, a species incapable of massive β-carotene accumulation. Cells exposed to high light levels for different lengths of time were maintained in a sulfate-free medium for 1 h before labeling for 1 h with [^{35}S]sulfate. (Later experiments indicated that significant ^{35}S-incorporation into proteins could also be achieved without preincubation in sulfate-free medium.) The protein extracts were then resolved on SDS-polyacrylamide gels. The results (Fig. 6) show several differences in the pattern of the proteins synthesized in exposed and nonexposed cells. The synthesis of a protein migrating at a position roughly corresponding to 55 kD is strongly enhanced in *D. bardawil*, whereas the synthesis of a similarly migrating protein in *D. salina* is increased only weakly. The synthesis of several other proteins, mostly differing in the two strains, is also altered, including a transient decline in the synthesis of the large subunit of ribulose bisphosphate carboxylase during illumination. As a whole, the protein patterns of *D. bardawil*, and *D. salina* are significantly different.

In another approach to assess the effects of strong illumination on gene expression, poly(A)$^+$ mRNA was isolated from *D. bardawil* cells prior to, or 24 h after exposure to strong light, and translated *in vitro* by a reticulocyte lysate. The results (Fig. 7) show several distinct differences in the translation products of the two mRNAs compatible with both suppression and induction of genes by the intense light. A translational product specific to the mRNA from the highly illuminated cells resembles in electrophoretic mobility the major light-induced, *in vivo* synthesized protein.

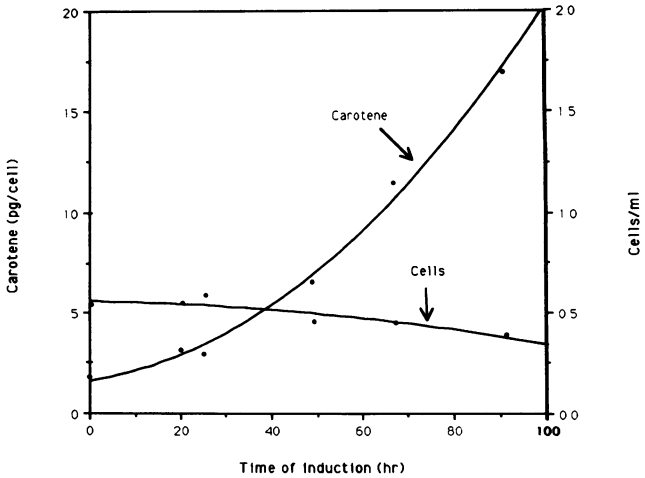

Figure 4. Course of β-carotene accumulation by *D. bardawil* during sulfate starvation under low illumination. β-Carotene and cell density were followed for 5 d after transfer of the cells to sulfate-free medium. The initial level of β-carotene was 2 pg/mL.

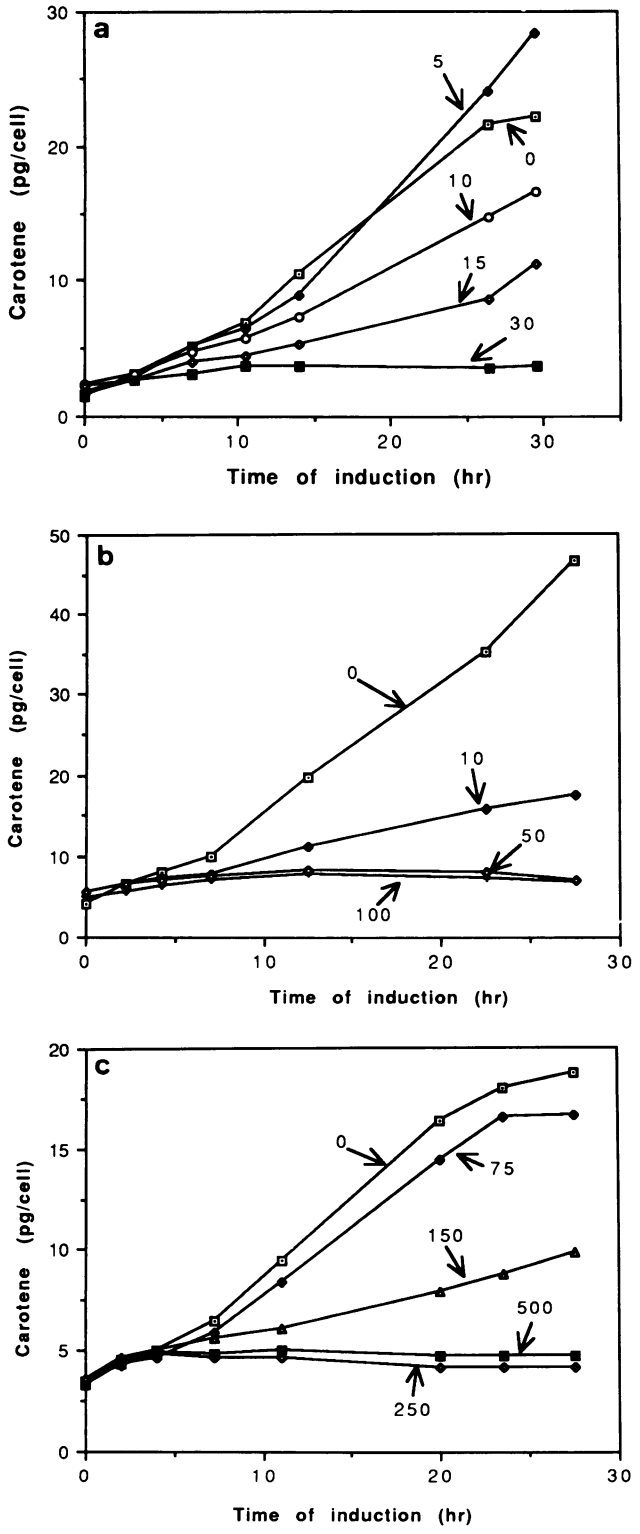

Figure 5. Effect of transcriptional and translational inhibitors on the light induction of β-carotene accumulation by *D. bardawil*. Detailed procedures are described in "Materials and Methods." (a) Actinomycin D; (b) chloramphenicol; (c) cycloheximide. The concentrations used are indicated next to the relevant curves: actinomycin D, chloramphenicol: μg/mL; cycloheximide: ng/mL.

DISCUSSION

Dunaliella bardawil cells exposed to high light accumulate β-carotene in two stages. The first stage starts shortly after exposure and lasts for approximately 24 h. Coincident with the rise in β-carotene contents the cells start to recover from the initial adverse effects of intensive illumination. These results are in keeping with the photo-protective role previously assigned to the carotene globules in *D. bardawil* (6). Once elevated, the β-carotene contents remain nearly constant until it rises again with the onset of the stationary phase.

The increase in the cellular level of β-carotene could be accounted for by several, not necessarily mutually exclusive mechanisms: (a) an accelerated rate of biosynthesis due to enhanced activity or *de novo* synthesis of carotenogenic enzymes; (b) continued β-carotene synthesis in nondividing cells, or a reduced rate of its degradation or secretion. Different mechanisms were previously found to operate in light induction of carotenogenesis in other systems. For example, a light-mediated change in enzyme activity has been concluded to be responsible for photo-induced carotene forma-

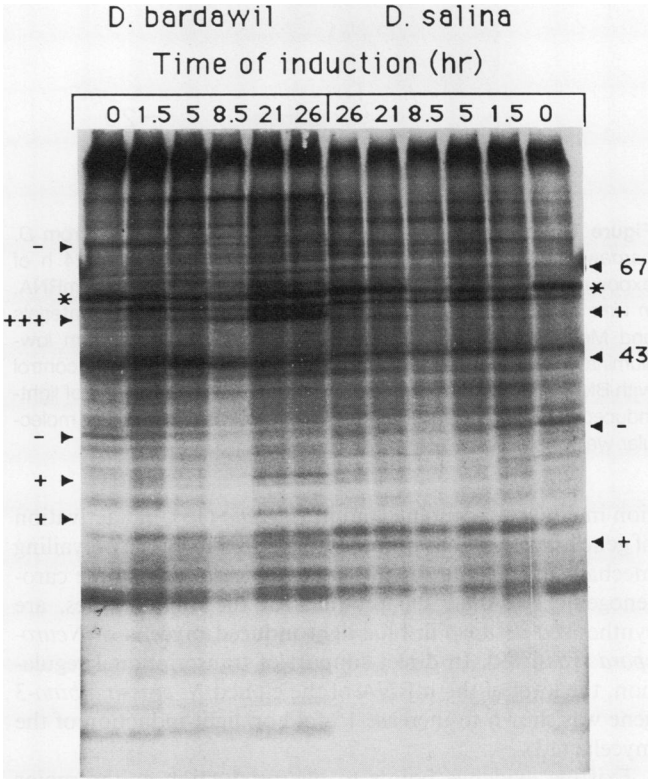

Figure 6. *In vivo* labeling of proteins synthesized in low-illuminated cells and after various times of exposure to high light intensity. Low-illuminated *D. bardawil* and *D. salina* cells were exposed to high light intensity for the time periods indicated on top of the corresponding lanes. Labeling and analytical procedures were as described in "Materials and Methods." Asterisk, position of the large subunit of ribulose bisphosphate carboxylase (determined by comparison with purified enzyme from *D. salina*); arrowheads, position of protein bands showing a decreased (−) or increased (+) intensity with time of exposure, or position of mol wt markers in kD.

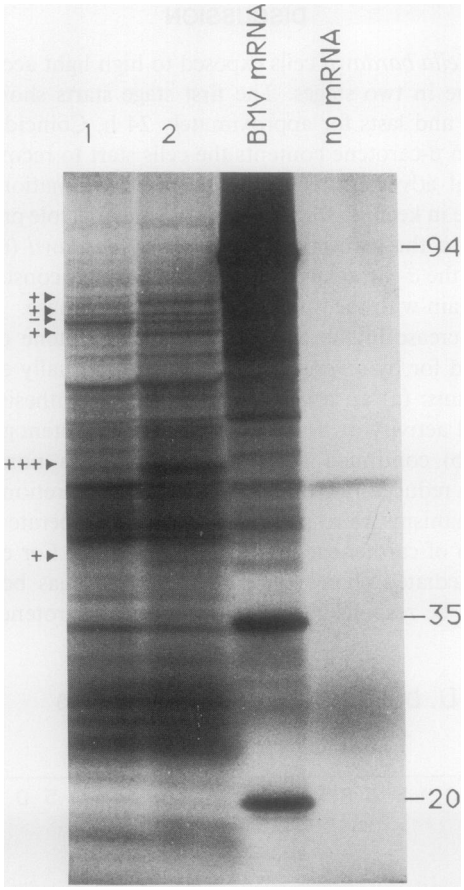

Figure 7. *In vitro* translation products of poly(A)$^+$ mRNA from *D. bardawil* grown under low illumination or from cells after 24 h of exposure to high light intensity. Procedures for purification of mRNA, *in vitro* translation and product analysis are described in "Materials and Methods." Lane 1, translation products of mRNA from low-illuminated cells; lane 2, from exposed cells; BMV mRNA, a control with BMV RNA. Arrowheads, position of translation products of light-induced (+), or light-suppressed (−) mRNAs; lines, position of molecular weight standards.

tion in *Chlorella* mutants and in *Euglena* (15), but activation of gene expression has been demonstrated to be the prevailing mechanism in some fungi (8, 15). Thus, at least three carotenogenic enzymes, the products of the albino genes, are synthesized *de novo* in blue light induced mycelia of *Neurospora crassa* (9). In direct support of transcriptional regulation, the level of the mRNA of the cloned *N. crassa albino*-3 gene was shown to increase 15-fold on light-induction of the mycelia (13).

Existing evidence points to overproduction as the major cause for β-carotene accumulation in *D. bardawil*. Norflurazon, a bleaching herbicide, blocks the conversion of the biosynthetic precursor phytoene to β-carotene. The massive accumulation of phytoene in norflurazon-treated, light-induced cells (4, 6) points to enhanced biosynthesis, rather than slowed turnover of β-carotene as the principal source of the accumulated pigment.

The early stage of β-carotene accumulation constitutes an immediate response to light, most probably mediated by photo-induced activation of a gene(s) encoding a β-carotene biosynthetic enzyme(s), or controlling factor(s). This conclusion is based on the exponential nature of the early kinetics of β-carotene accumulation and, more directly, on the dependence of accumulation on transcriptional activation and *de novo* protein synthesis. Inhibition by cycloheximide was previously reported for the induction of β-carotene accumulation in *D. salina* strain 9 by transfer to suboptimal temperature (17). The light-induced protein of approximately 55 kD observed in our *in vivo* labeling experiments, and its possible equivalent synthesized *in vitro*, as well as other light-induced proteins are potential candidates for inducible carotenogenic enzymes, or controlling factors.

The second stage of accumulation may partly result from the continued, nonaccelerated synthesis of β-carotene in cells which ceased to divide. However, this mechanism cannot solely account for the high rate of late accumulation observed in some experiments (for example, the experiment shown in Fig. 3b). Hence, activation and/or *de novo* synthesis of enzymes, or controlling factors, involved in β-carotene biosynthesis are also likely to underly the late phase of accelerated pigment accumulation. Unlike the first stage of induction, the second stage does not constitute an immediate response to high intensity light, but to another yet unknown trigger. Possibly, a similarity might exist between the mechanism of β-carotene overproduction in high-illuminated, stationary phase cells and in low-illuminated cells whose growth was limited by nutritional deficiencies, or other environmental stresses.

ACKNOWLEDGMENTS

The authors thank Drs. M. Avron and A. Ben Amotz for advice and discussions and Mr. Arie Segal for assistance in the growth of the algae.

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