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Stereoisomers of β-Carotene and Phytoene in the Alga *Dunaliella bardawil*

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ABSTRACT

***Dunaliella bardawil*, a halotolerant green alga, was previously shown to accumulate high concentrations of β-carotene when grown outdoors under defined conditions. The β-carotene of algae cultivated under high light intensity in media containing a high salt concentration is composed of approximately 50% all-*trans* β-carotene and 40% 9-*cis* β-carotene. We show here that the 9-*cis* to all-*trans* ratio is proportional to the integral light intensity to which the algae are exposed during a division cycle. In cells grown under a continuous white light of 2000 microeinsteins per square meter per second, the ratio reached a value of around 1.5, while in cells grown under a light intensity of 50 microeinsteins per square meter per second, the ratio was around 0.2. As previously shown, algae treated with the herbicide norflurazon accumulate phytoene in place of β-carotene. Electron micrographs showed that the phytoene is accumulated in many distinct globules located in the interthylakoid spaces of the chloroplast. Here too, two isomers are present, apparently all-*trans* and 9-*cis* phytoene, and their ratio is dependent upon the integral light intensity to which the algae are exposed during a division cycle. In the presence of norflurazon, *Dunaliella bardawil* grown under a light intensity of 2000 microeinsteins per square meter per second contained about 8% phytoene with a 9-*cis* to all-*trans* ratio of about 1.0. This ratio decreased to about 0.1 when the light intensity was reduced to 50 microeinsteins per square meter per second. These data suggest that the isomerization reaction which leads to the production of the 9-*cis* isomer occurs early in the path of carotene biosynthesis, at or before the formation of all-*trans* phytoene. The presence of the 9-*cis* isomer of β-carotene and the dependence of its preponderance on light intensity seem to be a common feature of many plant parts. Thus carrots which are exposed to minimal light contain no 9-*cis* isomer while sun-exposed leaves, fruits, and flowers contain 20 to 50% of the 9-*cis* isomer.**

Two isolates of halotolerant algae, *Dunaliella bardawil* Ben-Amotz & Avron and *D. salina* Teod., possess the ability to accumulate very large amounts of β-carotene (more than 10% of the algal dry weight) under defined conditions (3, 13). The extent of β-carotene accumulation was shown to be a direct function of the integral amount of light to which the algae are exposed during a division cycle (2). Thus, maximal accumulation of β-carotene is observed when the cells are grown under growth limiting conditions, such as limiting nitrogen or high NaCl concentrations, while exposed to high light intensity (2, 5, 9, 13–16).

We (4) have recently shown that in the presence of norflurazon, a herbicide which blocks the conversion of phytoene to β-carotene, *Dunaliella bardawil* accumulates massive amounts of phytoene in place of β-carotene. The β-carotene in *D. bardawil* was previously shown to be composed of approximately equivalent amounts of the all-*trans* and 9-*cis* isomers (3). The presence and importance of β-carotene isomers in plants and algae has been a subject of controversy (10). Earlier studies suggested that the presence of carotenoid isomers may be due to isomerization during the then prevalent long extraction and purification procedures (17). However, recently developed rapid HPLC analysis has provided clear evidence for the natural occurrence of different xanthophyll and carotenoid isomers in plants and algae (1, 3, 7, 8, 12). Also, the natural occurrence of 15-*cis* phytoene as the major isomer has been reported in higher plants, algae, fungi, and photosynthetic bacteria (10). This communication addresses three main issues: (a) What is the physiological control of the ratio of 9-*cis* to all-*trans* β-carotene in *D. bardawil*? (b) Under conditions which enhance the formation of 9-*cis* β-carotene, and in the presence of norflurazon, is 9-*cis* phytoene production also apparent and enhanced? (c) Is the formation of 9-*cis* β-carotene in plants ubiquitous, or is it unique to *D. bardawil*?

MATERIALS AND METHODS

Algae. *Dunaliella bardawil* Ben-Amotz & Avron, a local isolated species, is deposited with the American Type Culture Collection, Rockville, MD, No. 30861. *D. salina* was obtained from the culture collection of Dr. W. H. Thomas, La Jolla, CA. This *D. salina* is unable to accumulate β-carotene.

Leaves, Fruit, Roots, and Flowers. *Eriobotrya japonica* (loquat), *Prunus armenica* (apricot), *Prunus persica* (peach), *Capsicum annum* (pepper), *Latuca sativa* (lettuce), *Daucus carota* (carrot), and *Acacia decurrens* (flower) were obtained locally and extracted fresh.

Growth Conditions. The algae were cultivated in a growth medium containing 2 M NaCl, 5 mM $MgSO_4$, 0.3 mM $CaCl_2$, 0.75 mM KNO_3 (unless indicated otherwise), 0.2 mM KH_2PO_4, 1.5 μM $FeCl_3$, 6 μM EDTA, 50 mM $NaHCO_3$, and a trace metal mix (pH 8.0). Algae were cultivated at 25°C with continuous light and shaking in two different facilities: (a) a temperature controlled Aminco Warburg photosynthetic apparatus equipped with halogen lamps of varying intensities (200–2000 μE m^{-2} s^{-1}, 400–700 nm at the flask level); (b) a growth room illuminated with cool white fluorescent lamps at different intensities (50–200 μE m^{-2} s^{-1}).

Growth Parameters and Pigments. Cell number was determined in a Coulter Counter model ZM with a 100 μm orifice. Chl and β-carotene were extracted from an algal pellet with acetone, diluted with water to 80% acetone (v/v), assayed as described (2). Phytoene was similarly extracted, the pigments transferred to hexane with the addition of water, and the hexane phase dried completely under N_2 and redissolved in hexane (4).

HPLC Analysis. *Extraction.* Samples of the algae were centrifuged shortly before the analysis and the pellets were extracted with acetone. Pigments were transferred to hexane with the addition of water, dried completely under N_2, and redissolved in methylene chloride. The same extraction was applied to the other plant parts' analyses, but grinding was necessary for complete pigment extraction. The preparative time for pigment analysis by HPLC did not exceed 15 min. Synthetic β-carotene (Hoffman La Roche) was dissolved prior to chromatography in methylene chloride.

Columns and Solvents. A stainless steel column of 25 cm × 4.6 mm i.d. packed with C18 reversed phase material of 5 μm particle size (Vydac TP201 54) was used for pigment analysis (11). Elution was performed with isocratic solvent of 1 ml/min methanol:acetonitrile (9:1 v/v). This column provided excellent separation of the β-carotene isomers. A better separation of the more polar pigments (xanthophylls and Chl) could be obtained with a gradient of methanol:acetonitrile (9:1 v/v)—water. An increase of retention time after about 20 injections of samples could be reversed by washing the column with methanol:acetonitrile:methylene chloride (8:1:1 v/v/v).

HPLC Equipment. HPLC was performed by using a Waters system equipped with 510 and 501 pumps, U6K injector, 490 Programmable Multiwavelength Detector and 420 Fluorescence Detector. The Programmable Multiwavelength Detector was set at 450 nm for the detection of carotenoids, 436 nm for the detection of Chl, and 287 nm for the detection of phytoene. The fluorometer was equipped with an S-20 photomultiplier tube and excitation and emission filters to detect Chl fluorescence. The four outputs, three absorption and one fluorescence, were read simultaneously by using a Waters System Interface Module and a Waters 840 Data and Chromatography Control Station on a Digital 350 Professional Computer. The unit provides maxplotting for the determination of maximum absorbance. The HPLC system was attached to a flow-through Hewlett Packard 8452A Diode Array spectrophotometer, and each eluted peak of identification interest was assayed spectrally.

Electron Microscopy. Electron microscopy of *D. bardawil* was performed as previously described (3).

Phytoene Identification. The phytoenes in the algal extract were further separated for preparative identification on a column of aluminum oxide (30 × 1 cm) (Woelm neutral alumina, Brockmann activity grade I) which was developed with light petrol 40 to 60°C. Fractions were collected, concentrated under N_2, and monitored for total phytoene content and for purity by HPLC coupled to a Diode Array spectrophotometer. Both 9-*cis* phytoene and all-*trans* phytoene were eluted from the alumina column by the light petrol with no addition of diethyl ether; 9-*cis* phytoene was eluted first, and the all-*trans* closely after. The UV absorption spectra in light petrol had a sharp peak at 286 nm with inflections at 276 and 297 nm. The 9-*cis* isomer shoulder at 276 nm was lower than that of the all-*trans*, with a relative absorbance ratio to the peak at 286 of 0.81 and 0.84, respectively. The mass spectrum of the 9-*cis* phytoene showed a molecular ion peak at m/e 544 with a strong fragment ion at M-205 (m/e 339, $C_{25}H_{39}$) and two weak fragments at M-137 (m/e 407, $C_{10}H_{17}$) and at M-71 (m/e 473, C_5H_{11}). These results, and the kinetic analogy to the formation of 9-*cis* β-carotene, support the identification as 9-*cis* phytoene.

β-Carotene Inhibitor. Norflurazon (4-Chl-5-(methyl-amino)-2-(α,α,α-trifluro-*m*-tolyl)-3 (2H)-pyridazinine, San 9789) (4).

RESULTS

Effect of Light Intensity on the Content of β-Carotene Stereoisomers. The effect of light intensity on the isomer composition of β-carotene in *D. bardawil* is illustrated in Figure 1. The pigments were eluted in the order xanthophylls (peaks 1, 2, 4, 5, 6), Chl *b* (peak 3), Chl *a* (peak 7), α-carotene (peak 8), all-*trans* β-carotene (peak 9), and 9-*cis* β-carotene (peak 10). As observed earlier (3), the β-carotene content increased sharply and the Chl content decreased with increasing light intensity, with the former reaching a maximum of above 10% of the algal dry weight (see also Fig. 2). Figure 1 illustrates that the increase in total β-carotene is associated with an increase in the ratio of 9-*cis* to all-*trans* β-carotene from 0.21 to 1.3.

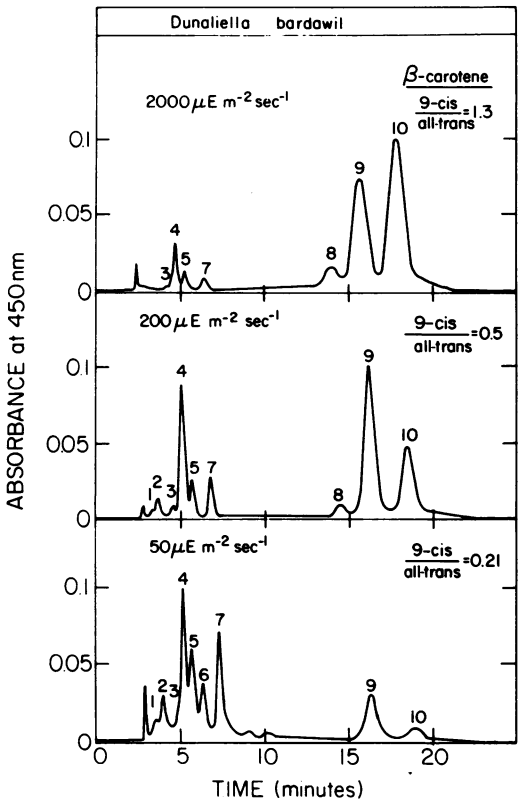

FIG. 1. Effect of light intensity on the ratio of 9-*cis* to all-*trans* β-carotene in *D. bardawil*. Algae were grown under different light intensities. Pigments were extracted from algal pellets and samples injected into a Vydac TP201 TP54 column as described under "Materials and Methods." The peak numbers correspond to the following pigments: 1, violaxanthin; 2, neoxanthin; 3, Chl *b*; 4, lutein; 5, zeaxanthin; 6, antherxanthin; 7, Chl *a*; 8, α-carotene; 9, all-*trans* β-carotene; 10, 9-*cis* β-carotene.

It was previously shown (2) that the β-carotene to Chl ratio of *D. bardawil* increased under a great variety of conditions, as a function of the integral amount of light to which the algae were exposed during a division cycle (*i.e.* irradiance × doubling time). As can be seen in Figure 2, the increase in the 9-*cis* to all-*trans* β-carotene ratio was also correlated with the integral irradiance received by the algal culture during a division cycle. Thus, changing the light intensity, or varying the growth rate by increasing salinity or varying temperature, all led to accumulation of β-carotene and to changes in the 9-*cis* to all-*trans* ratio.

Effect of Light Intensity on Content of Phytoene Stereoisomers. The effect of light intensity on the pigment content of *D. bardawil* in the presence of the bleaching herbicide norflurazon was also studied. Figure 3 confirms that with 0.1 μM norflurazon, which inhibited β-carotene production by around 80% (4), there was a great increase in phytoene formation. Furthermore, the amount of phytoene accumulated was also proportional to the integral irradiance to which the cells were exposed during a division cycle (Fig. 4).

Figures 3 and 4 also illustrate that 9-*cis* phytoene was produced

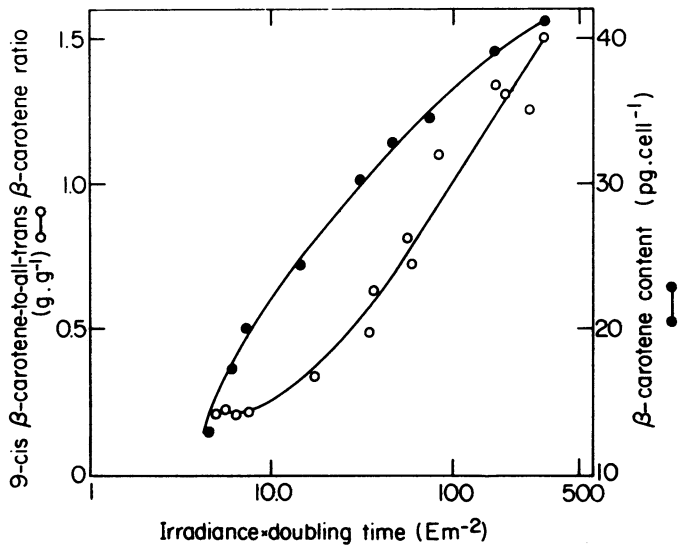

FIG. 2. Dependence of β-carotene content and the 9-*cis* to all-*trans* ratio in *D. bardawil* on the integral irradiance per division cycle. Light intensity was varied between 50 to 2000 μE m^{-2} s^{-1}. Data are also plotted from experiments where the salt content of the medium and the temperature of growth were varied. Pigments were analyzed by HPLC as described in Figure 1.

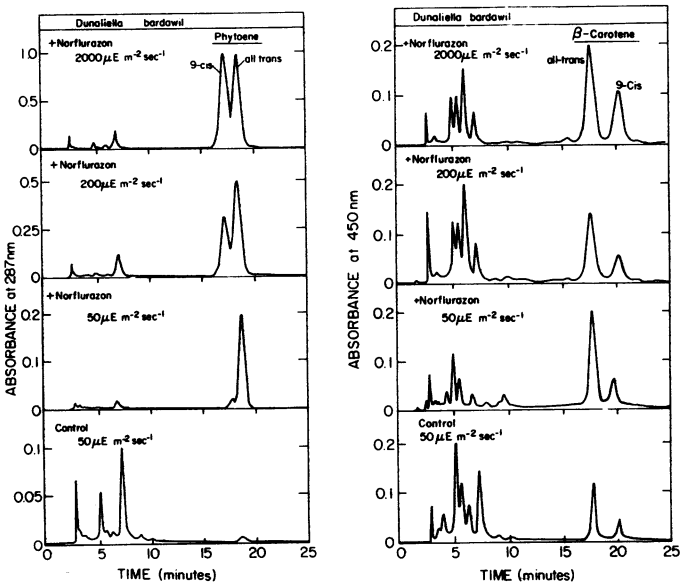

FIG. 3. Effect of light intensity in the presence of norflurazon on the pigment content of *D. bardawil*. Samples from the pigment extract of algae grown at the indicated light intensities were fractionated by HPLC and read simultaneously at 287 nm for phytoene and phytoene isomers (left), and at 450 nm for β-carotene and β-carotene isomers (right), as detailed under "Materials and Methods." Norflurazon was added at 0.1 μM.

alongside the all-*trans* iosmer, and that the ratio of 9-*cis* to all-*trans* phytoene was also a function of the light intensity and the growth rate. When algae were grown under a high light intensity of 2000 μE m^{-2} s^{-1}, a maximal ratio of 9-*cis* to all-*trans* phytoene of about 1.0 was observed. When algae were grown under high light intensity with norflurazon, the cultures progressively turned from pale green to white and eventually collapsed. As previously demonstrated (4), these cells lost their resistance to high light and were thus severely photoinhibited. The high light norflurazon-treated cells had only minute amounts of Chl as detected by HPLC with the sensitive Chl fluorescence assay during the HPLC separation.

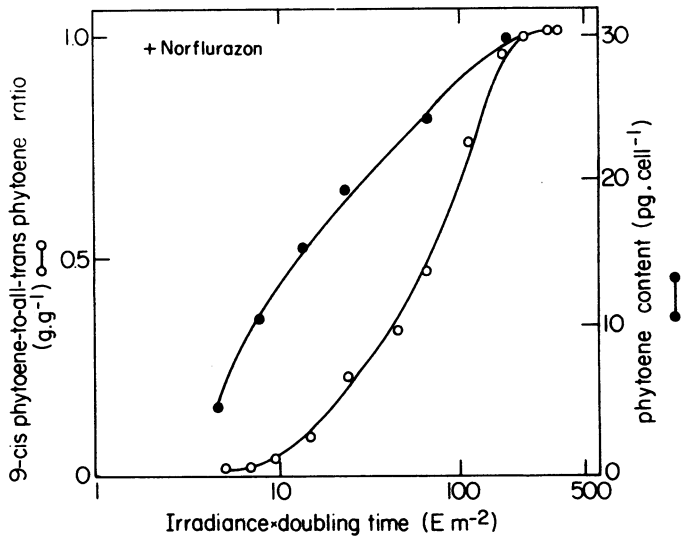

FIG. 4. Dependence of phytoene content and the 9-*cis* to all-*trans* ratio in *D. bardawil* on the integral irradiance per division cycle. Conditions were as described under Figure 2, except that norflurazon was added at 0.1 μM. Pigments were analyzed by HPLC as described in Figure 3.

It should be noted that the identification of the high light induced phytoene isomer (Fig. 3) as 9-*cis* phytoene is based on analogy with the 9-*cis* β-carotene and on the typical absorption and mass spectra of phytoene. An authentic 9-*cis* phytoene sample was not available.

Distinctive Globules of β-Carotene and Phytoene in *Dunaliella bardawil*. In contrast to high light grown algae (3), *D. bardawil* grown under low light does not contain β-carotene globules in its chloroplast (Fig. 5A). When the algae are transferred to conditions which enrich β-carotene or phytoene formation, a large number of intraplastidic globules of β-carotene or of phytoene are accumulated (Fig. 5, B and C). Under the preparative techniques employed, the β-carotene and the phytoene globules are distinctive in both shape and intensity, the latter being larger and paler than the former.

β-Carotene Stereoisomers in Other Plants. A few representative plants were selected for analysis by HPLC of their content of β-carotene isomers in an attempt to correlate the extent of light exposure of the plant tissue to its β-carotene isomerization. As can be seen (Fig. 6; Table 1), the tested leaves, fruits, flowers, and algae, all grown outdoors, contained 17 to 43% of their total β-carotene in the form of the 9-*cis* isomer. The total β-carotene in lettuce was around 13% of the total extracted pigments, smaller than the content of Chl and xanthophyll and much smaller than in *D. bardawil* (around 90% of the total pigments). The extract from the carrot root, which is not exposed to light during growth, contained about 60% all-*trans* β-carotene, 30% α-carotene and 4% lutein, but no 9-*cis* β-carotene.

DISCUSSION

The unique ability of *D. bardawil* to synthesize very large amounts of β-carotene makes it a choice eukaryotic cell for studies of the path of biosynthesis of β-carotene, and for evaluation of the effect of metabolic inhibitors on carotene biosynthesis.

Using an alumina column, the β-carotene of *D. bardawil* was previously shown (3) to be composed of approximately equivalent amounts of 9-*cis* and all-*trans* β-carotene. With the intro-

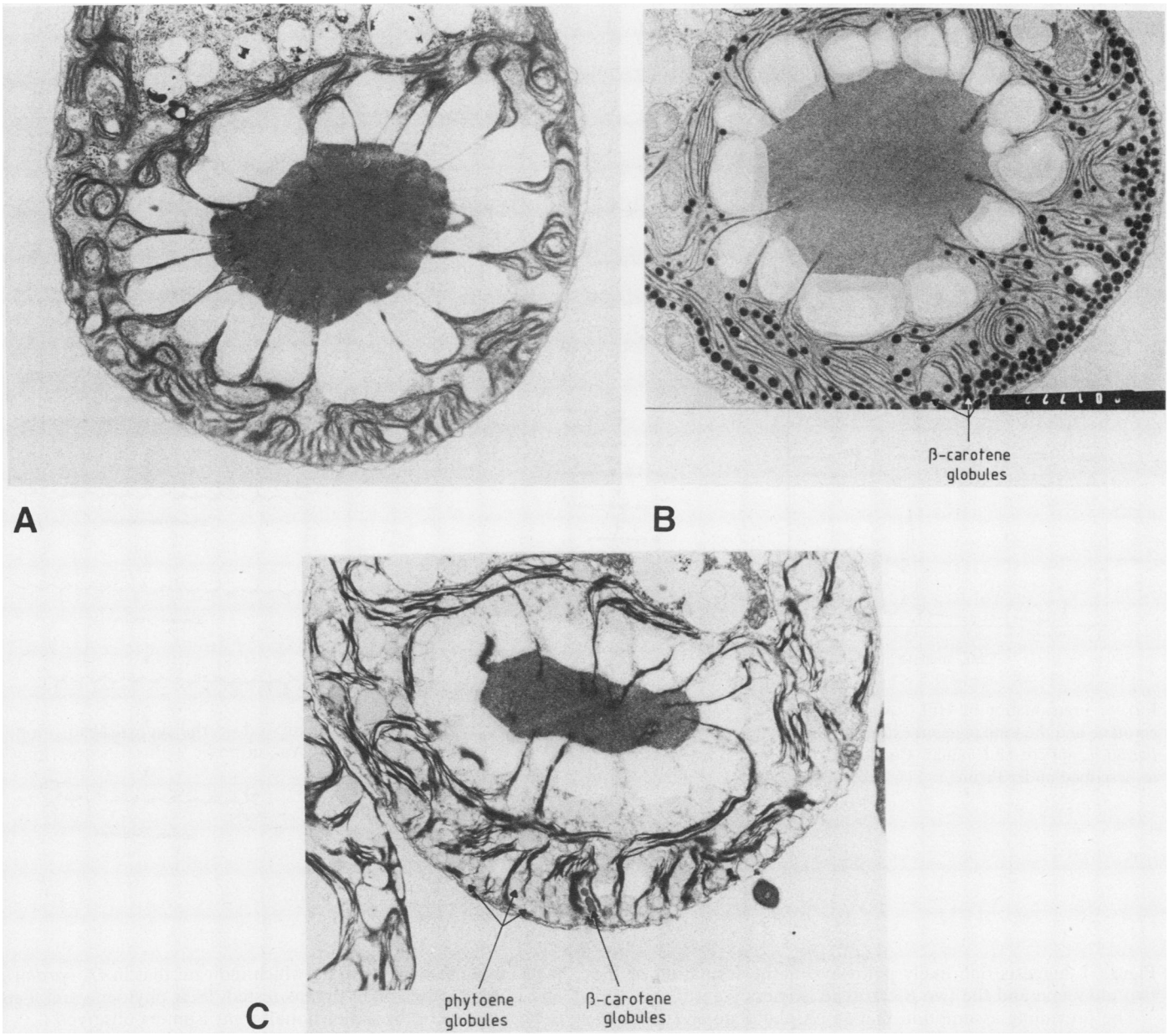

FIG. 5. Electron micrographs of sections of the algal chloroplasts from *D. bardawil* grown under low light (A), high light (B), and high light in the presence of 0.1 μM norflurazon (C). Enlargement $\times$50,000. Experimental conditions were as described under "Materials and Methods."

duction of the high capacity and stable C18 reversed phase columns, like the Vydac TP, such separation became considerably simpler, since many technical aspects such as stability and peak resolution of the alumina and calcium hydroxide columns (1) were no longer a problem. With these new columns, runs may be repeated on the same columns and high accuracy can be obtained in peak identification. Since the same isomers are seen in the same proportion in the new techniques, the earlier suggestion that the presence of the isomers may be due to significant isomerization which occurs during extraction and/or analysis (1) is no longer tenable.

The evidence presented is consistent with the suggestion that light plays a major role in the biosynthesis of 9-*cis* β-carotene. Both the increase in total β-carotene and the increase in the relative fraction of 9-*cis* β-carotene are proportional to the integral irradiance to which the culture is exposed during a division cycle. This seems to be a common feature to all plants and algae. Thus, parts of the plant which are not exposed to light, such as the carrot root, contain only all-*trans* β-carotene. In contrast, fruits, leaves, and flowers contain both the all-*trans* and the 9-*cis* isomers.

In the presence of norflurazon, phytoene is accumulated, and the proportion of 9-*cis* phytoene is increased in a manner resembling the accumulation of β-carotene and its 9-*cis* isomer. This provides a new insight into the proposed pathway of carotene synthesis in plants. Classically (6) (Fig. 7), all-*trans* phytoene (or 15-*cis* phytoene) is considered to be produced from 2-geranylgeranyl pyrophosphate in the path to all-*trans* β-carotene. Since isomerization to 9-*cis* phytoene or 9-*cis* β-carotene is induced by a similar set of conditions, it seems reasonable to assume that the isomerization occurs, in both cases, at or before the formation of the all-*trans* phytoene. Thus, the possibility that there is a light dependent conversion of all-*trans* β-carotene to the 9-*cis* isomer becomes unlikely. The biochemical pathway drawn in

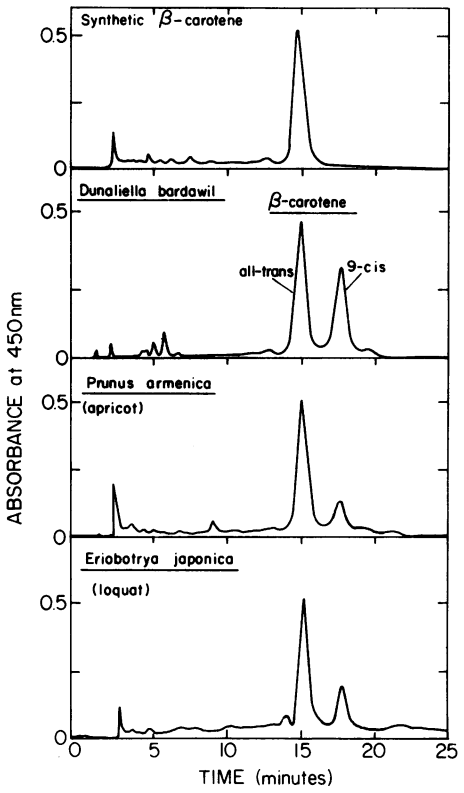

FIG. 6. Fractionation by HPLC of synthetic β-carotene and the total pigment extracts from *D. bardawil*, apricot and loquat. Conditions were as described under Figure 1.

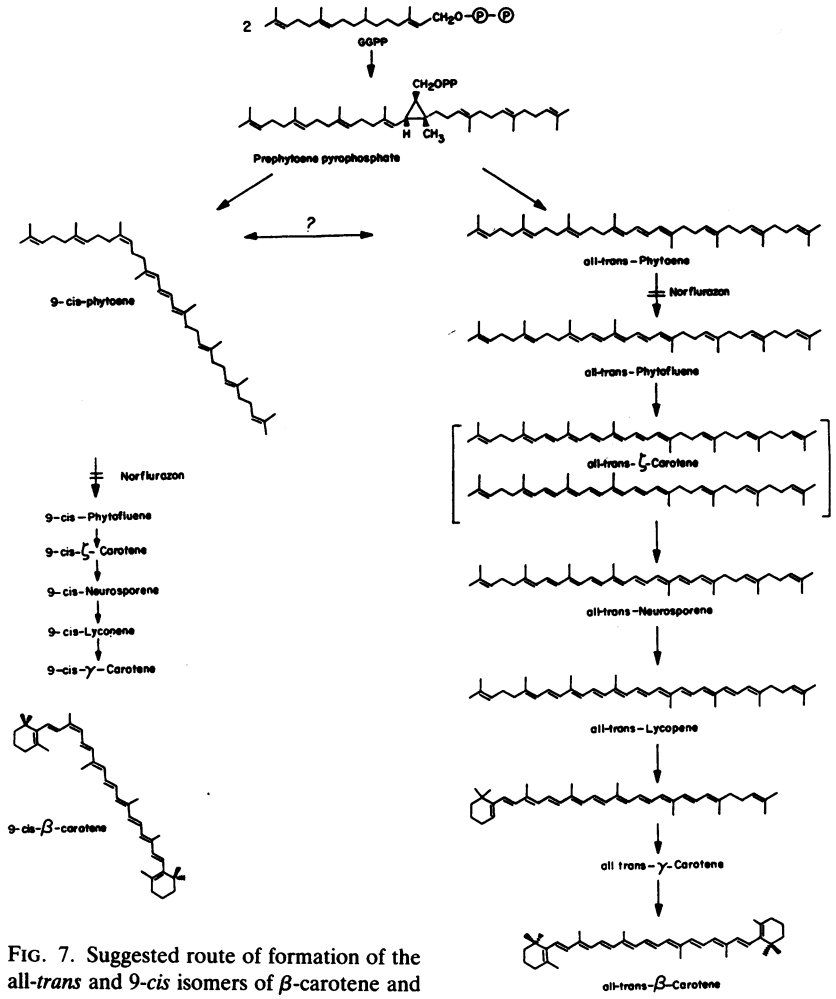

FIG. 7. Suggested route of formation of the all-*trans* and 9-*cis* isomers of β-carotene and phytoene.

Figure 7 suggests one likely pathway for the formation of the two phytoene and the two β-carotene isomers.

The prevailing assumption that 15-*cis* phytoene and 15-*cis* β-carotene are the major nonall-*trans* isomers in plants (6, 10, 12) is inconsistent with our data which indicate that in *D. bardawil* and in most other plant organs tested, 9-*cis* phytoene and 9-*cis* β-carotene were the major nonall-*trans* isomers observed.

Since 9-*cis* β-carotene is induced by exposure to light, its major

Table I. *Distribution of α-Carotene, All-trans β-Carotene, and 9-cis β-Carotene in Plant Tissues*

Fruits, leaves, flower, and carrots were cropped fresh from the field and extracted within a few hours for pigment determination. *D. salina* (LL) and *D. bardawil* (LL) were cultivated in the growth room under continuous low light (LL) intensity of about 100 μE m^{-2} s^{-1}. *D. bardawil* (outdoor) was grown with natural illumination (3).

Species	α-Carotene	β-carotene		β-Carotene 9-*cis*/all-*trans*
		all-*trans*	9-*cis*	
	% of total extracted pigments			
Prunus armenica (apricot)	<0.1	70.0	17.0	0.24
Prunus persica (peach)	<0.1	31.0	9.5	0.31
Eriobotrya japonica (loquat)	3.7	64.0	19.0	0.30
Capsicum annum (green pepper)	0.7	12.0	5.0	0.42
Latuca sativa (lettuce)	<0.1	10.5	2.5	0.24
Dunaliella salina (LL)	<0.1	10.0	3.2	0.32
Dunaliella bardawil (LL)	<0.1	9.5	2.0	0.21
Dunaliella bardawil (outdoor)	2.5	50.0	38.0	0.76
Daucus carota (carrot root)	31.0	63.0	0.0	
Acacia decurrens (flower)	<0.1	52.0	43.0	0.83

function may be related to light photoprotection. This may be related to the physical properties of 9-*cis* β-carotene. All-*trans* β-carotene is known to crystalize relatively easily from concentrated solutions, while 9-*cis* β-carotene tends to stay in solution under the same conditions (17). At least in *D. bardawil*, where β-carotene is highly concentrated in its β-carotene globules, the presence of the 9-*cis* isomer may help maintain an oily rather than a cyrstalline globule structure. The combination in the oily droplets of 9-*cis* and all-*trans* β-carotene may provide a more efficient photoprotective device.

LITERATURE CITED

1. ASHIKAWA I, A MIYATA, H KOIKE, Y INOUE, Y KOYAMA 1986 Light induced structural change of β-carotene in thylakoid membranes. Biochemistry 25: 6154–6160
2. BEN-AMOTZ A, M AVRON 1983 On the factors which determine massive β-carotene accumulation in the halotolerant alga *Dunaliella bardawil*. Plant Physiol 72: 593–597
3. BEN-AMOTZ A, A KATZ, M AVRON 1982 Accumulation of beta-carotene in halotolerant algae: purification and characterization of β-carotene-rich globules from *Dunaliella bardawil* (Chlorophyceae). J Phycol 18: 529–537
4. BEN-AMOTZ A. J GRESSEL, M AVRON 1987 Massive accumulation of phytoene induced by norflurazon in *Dunaliella bardawil* (Chlorophyceae) prevents recovery from photoinhibition. J. Phycol 23: 176–181
5. BOROWITZKA L, MA BOROWITZKA, TP MOULTON 1984 The mass culture of *Dunaliella salina* for fine chemicals: from laboratory to pilot plant. Hydrobiologia 116/117: 115–134
6. BRITTON G 1982 Carotenoid biosynthesis in higher plants. Physiol Veg 20: 735–755
7. BUSHWAY RG 1986 Determination of α- and β-carotene in some raw fruits and vegetables by high performance liquid chromatography. J Agric Food Chem 34: 409–412
8. CHANDLER LA, SJ SCHWARTZ 1987 HPLC separation of *cis-trans*-carotene isomers in fresh and processed fruits and vegetables. J Food Sci 52: 669–672
9. DROKOVA IH 1961 The alga *Dunaliella salina* as a source of β-carotene. Ukr Bot Zh 18: 110
10. GOODWIN TW 1980 The Biochemistry of Carotenoids, Ed 2, Vol 1. Chapman and Hall, London
11. JENSEN CD, TW HOWES, GA SPILLER, TS PATTISON, JH WHITTAM, J SCALA 1987 Observations on the effect of ingesting *cis*- and *trans*-β-carotene isomers on human serum concentrations. Nutr Rep Int 35: 413–422
12. KHACHICK F, GR BEECHER, NF WHITTAHOR 1986 Separation, identification and quantitation of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography. J Agric Food Chem 34: 603–616
13. LOEBLICH LA 1982 Photosynthesis and pigments influenced by light intensity and salinity in the halophilic *Dunaliella salina* (Chlorophyta). J Mar Biol Assoc UK 62: 493–503
14. MASYUK NP 1969 New species of the genus *Dunaliella salina* Teod. Ukr Bot Zh 26: 87–90
15. MILKO FS 1963 The effect of various environmental factors upon pigments formation in the alga *Dunaliella salina*. Mikrobiologiya 32: 299–307
16. SEMENENKO VE, AA ABDULLAEV 1980 Parametric control of beta-carotene biosynthesis in *Dunaliella salina* cells under conditions of intensive cultivation. Sov Plant Physiol 27: 22–30
17. ZECHMEISTER L 1962 *cis-trans*-Isomeric Carotenoids, Vitamin A and Arylpolyenes. Springer-Verlag, Vienna