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On the Factors Which Determine Massive β-Carotene Accumulation in the Halotolerant Alga *Dunaliella bardawil*

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ABSTRACT

Dunaliella bardawil, **a β-carotene-accumulating halotolerant alga, has been analyzed for the effect of various growth conditions on its pigment content, and compared with *Dunaliella salina*, a β-carotene nonaccumulating species. In *D. bardawil*, increasing light intensity and light period or inhibiting growth by various stress conditions such as nutrient deficiency or high salt concentration caused a decrease in the content of chlorophyll per cell and an increase in the amount of β-carotene per cell. As a result, the β-carotene-to-chlorophyll ratio increased from about 0.4 to 13 grams per gram and the alga changed its visual appearance from green to deep orange. *D. salina* grown similarly decreased in content of both chlorophyll and β-carotene per cell and the culture turned from green to yellowish. Low chlorophyll-containing cells of *D. bardawil* or *D. salina* exhibit very high photosynthetic rates when expressed on a chlorophyll basis (~600 micromoles O_2 evolved per milligram chlorophyll per hour).**

Variation of pigment content in *D. bardawil* by a large variety of environmental agents has been correlated with the integral irradiance received by the algal culture during a division cycle. The higher the integral irradiance per division cycle, the lower the chlorophyll content per cell; the higher the β-carotene content per cell, and therefore the higher the β-carotene-to-chlorophyll ratio. The results are interpreted as indicating a protecting effect of β-carotene against injury by high irradiance under conditions of impairment in chlorophyll content per cell.

The algal genus *Dunaliella* contains species (4, 5) which possess the unique ability to accumulate large amounts of β-carotene both in nature (6, 14) or under some laboratory conditions (7, 11–13, 15, 17–19, 20). *D. bardawil* was recently shown to accumulate β-carotene to at least 8% of its dry weight when grown under defined growth conditions such as high light intensity, high salt concentration, extreme temperatures or nitrate deficiency (4). Most of the accumulated β-carotene was concentrated in intrachloroplastic lipoidal globules, which could be isolated and purified.

There is no agreement in the literature regarding the factors which control β-carotene synthesis in *Dunaliella* (3, 8, 9). Several observations point to nutritional imbalance, particularly lack of nitrogen and to a role of light intensity. The present communication presents data which support the suggestion that the extent of β-carotene accumulation in *Dunaliella* is a direct function of the integrated amount of light to which the algae are exposed during one division cycle. It is suggested that the accumulated β-carotene plays a role in protecting the β-carotene-accumulating species against damaging radiation. This may explain their observed ecological dominance in nature over other species of *Dunaliella* which are incapable of accumulating β-carotene.

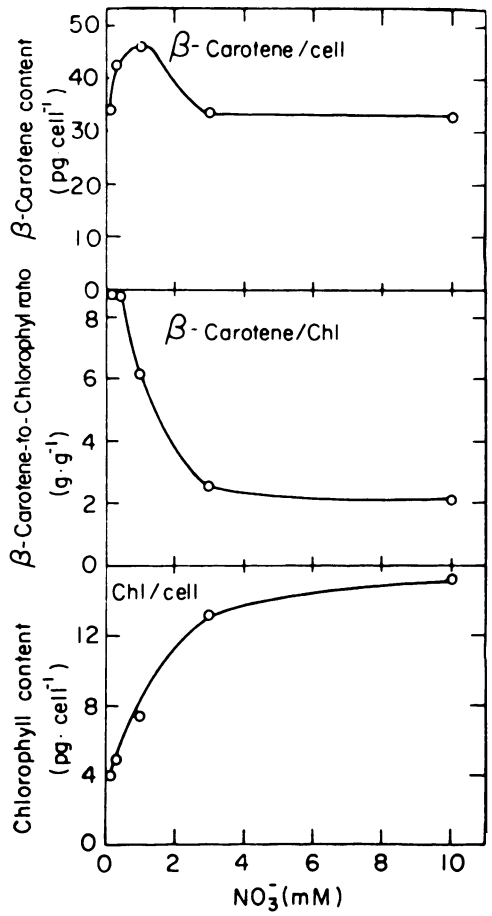

FIG. 1. Effect of nitrate concentration on Chl and β-carotene content of *D. bardawil*. Nitrate-starved algae were transferred to media containing 3.0 M NaCl at 25°C under continuous fluorescent irradiation of 10 kergs cm^{-2} s^{-1}. At the beginning and the end of the experiments, the cultures contained the following concentrations of nitrate, respectively: 10 → 4.1 mM; 3 → 0.14 mM; 1 → 0 mM; 0.3 → 0 mM; 0.1 → 0 mM; 0 → 0 mM. Data were derived from the early stationary phase of growth.

MATERIALS AND METHODS

Algae. *Dunaliella salina* was obtained from the culture collection of Dr. W. H. Thomas, La Jolla, CA. *Dunaliella bardawil*, a local isolated species, is deposited with the American Type Culture Collection, Rockville, MD (No. 30861). *D. salina*, which has been used in this study, is unable to accumulate β-carotene (4). *D. bardawil*, may be identical to one of the β-carotene-accumulating variants of *D. salina* Teod. which have been described previously (6, 14).

Growth Conditions. Algae were cultivated in a growth medium containing, unless indicated otherwise, 3 M NaCl, 5 mM $MgSO_4$, 0.3 mM $CaCl_2$, 5 mM KNO_3, 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 (2). Algae were cultivated in two different facilities: (a) a temperature-controlled (25°C) growth room under illumination with cool white fluorescent lamps at an intensity of 10 kergs cm^{-2} s^{-1} with slow shaking; (b) in a temperature-controlled Aminco photosynthetic Warburg apparatus (25°C) equipped for algal growth with tungsten lamps of different intensities (5 to 550 kergs cm^{-2} s^{-1}), light-dark periods, and shaking.

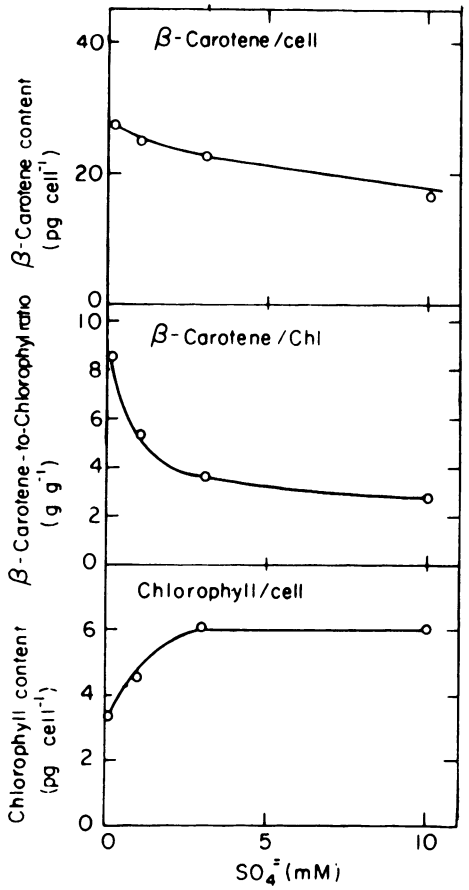

FIG. 2. Effect of sulfate concentration on Chl and β-carotene content of *D. bardawil*. Sulfate-starved algae were transferred to media containing 3 M NaCl, and the indicated sulfate concentrations and grown under the conditions specified for Figure 1. Data were derived from the early stationary phase of growth.

Table I. *Pigment Content of Continuous Light and Diurnal Cycle Grown Cells of D. bardawil*

Algae pregrown under light intensity of 120 kergs cm^{-2} s^{-1} were transferred to fresh medium containing 3 M NaCl in the Warburg apparatus under the indicated light intensities. Diurnal cycle was adjusted to 10 h light and 14 h dark. Data shown were derived from the fifth day of growth during the logarithmic phase at a cell concentration of about 5×10^5 cells ml^{-1}.

Growth Conditions	β-Carotene	Chl	β-Carotene/Chl
		pg cell^{-1}	
Continuous light			
24 kergs cm^{-2} s^{-1}	13.6	9.1	1.5
275 kergs cm^{-2} s^{-1}	25.7	4.6	5.6
Diurnal cycle			
24 kergs cm^{-2} s^{-1}	7.9	7.6	1.0
275 kergs cm^{-2} s^{-1}	16.5	4.8	3.4

Growth Parameters and Pigments. Cell number was determined in a Coulter Counter with a 100-μm orifice tube. Chl and β-carotene were extracted from an algal pellet with acetone, diluted to 80% acetone with water (v/v) and assayed according to Arnon (1) and Jensen (10). $E^{1\%}_{1\,cm}$ of 2273 at 480 nm has been used to calculate β-carotene concentration.

Photosynthetic O_2 Evolution. O_2 evolution was measured polarographically with a Rank Brothers O_2 electrode at a constant temperature of 25°C under various light intensities with white light of wavelength greater than 400 nm. Cells were suspended in a fresh medium and adjusted to the desired initial O_2 tension by bubbling N_2 prior to illumination.

Light Intensity Measurement. Illumination was measured with a Radiometer (Yellow Spring Instruments model 65). The thermistor was shielded against IR irradiation by an IR heat filter.

RESULTS

Effect of Nitrate and Sulfate Concentration. Figure 1 illustrates the effect of nitrate depletion on growth and pigment content of *D. bardawil* cultivated under relatively low light intensity. As expected, the algae cease to divide when the available nitrate is depleted. The amount of Chl per cell decreased with the decreased availability of nitrate. However, β-carotene content per cell does not change markedly with increased limitation by nitrate, leading to a marked increase in the β-carotene-to-Chl ratio in the nitrate-starved cells. This is mostly due to the fact that β-carotene synthesis continues during the early stationary phase when the cells do not produce additional Chl. Similar trends of Chl and β-carotene changes were noted under limiting concentrations of sulfate (Fig. 2).

Effect of Light Intensity and Salt Concentration. The effect of light intensity on growth and pigment content of *D. bardawil* and *D. salina* under conditions of continuous illumination in a medium containing 3.5 M NaCl is illustrated in Figure 3. Growth rate on the basis of cell number in both species was not affected by a wide range of nonlimiting light intensities between 5 and 550 kerg cm^{-2} s^{-1}. The Chl content per cell in both species showed an inverse dependence on light intensity, being lowest at the highest light intensity. The β-carotene content per cell on the other hand, increased sharply with light intensity in *D. bardawil* but decreased slightly in *D. salina*. A maximal β-carotene-to-Chl ratio of 13 was observed in high light-grown *D. bardawil*, whereas *D. salina* grown under the same conditions had a ratio of only 0.8.

Diurnally grown cells of *D. bardawil* had a lower β-carotene content and β-carotene-to-Chl ratio than that of cells growing similarly but under continuous light (Table I). This was true even though growth rates, expressed as a cell basis, were nearly identical under the conditions specified in Table I.

The interaction between salt concentration and light intensity on the β-carotene-to-Chl ratio of *D. bardawil* and *D. salina* is illustrated in Figures 4 and 5. Minimal Chl content, maximal β-carotene content, and maximal β-carotene-to-Chl ratios are observed on exposure of *D. bardawil* to the combination of the highest light intensity and NaCl concentration (Fig. 4). *D. salina* also exhibited an increase in β-carotene-to-Chl ratio under these conditions (Fig. 5) but that increase was derived mostly from the more marked decrease in Chl content per cell without any extra synthesis of β-carotene (not shown). When grown above 4 M NaCl and under continuous illumination with light intensity of 550 kergs cm^{-2} s^{-1}, *D. salina* died within 2 to 4 d but growth of *D. bardawil* was not affected. Also, no significant changes in average cell volume were detected between cells grown under the different experimental conditions employed.

Light Intensity and Photosynthesis. Inasmuch as growth under low and high light intensity produces cells with markedly different Chl and carotene content (Figs. 4 and 5), it was of interest to test

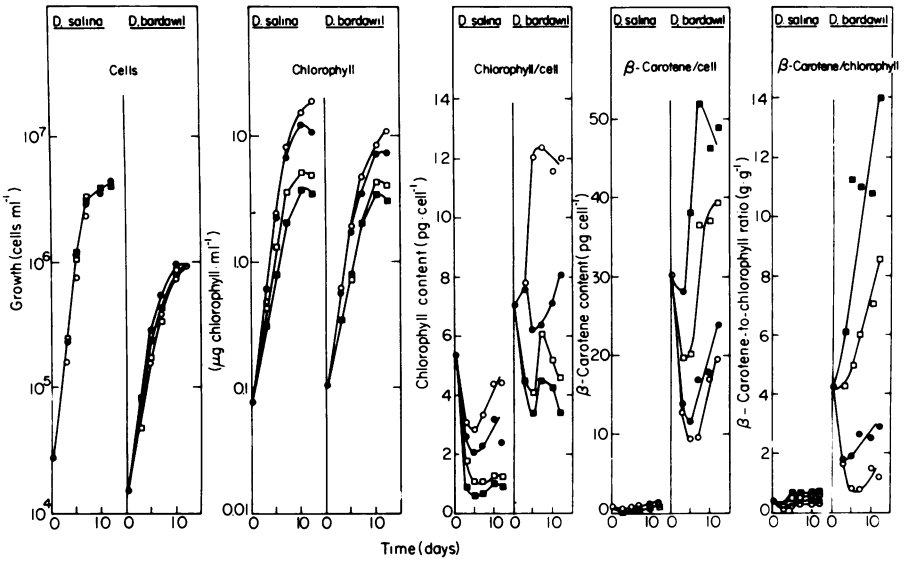

FIG. 3. Growth and pigment content of *D. bardawil* and *D. salina* grown under different light intensities. Algae grown at a high light intensity of 120 kergs cm^{-2} s^{-1} were diluted with fresh media containing 3.5 M NaCl and cultivated in the Warburg apparatus under continuous illumination at 25°C. Light intensities were: (○), 5; (●), 45; (□), 120; (■), 550 kergs cm^{-2} s^{-1}.

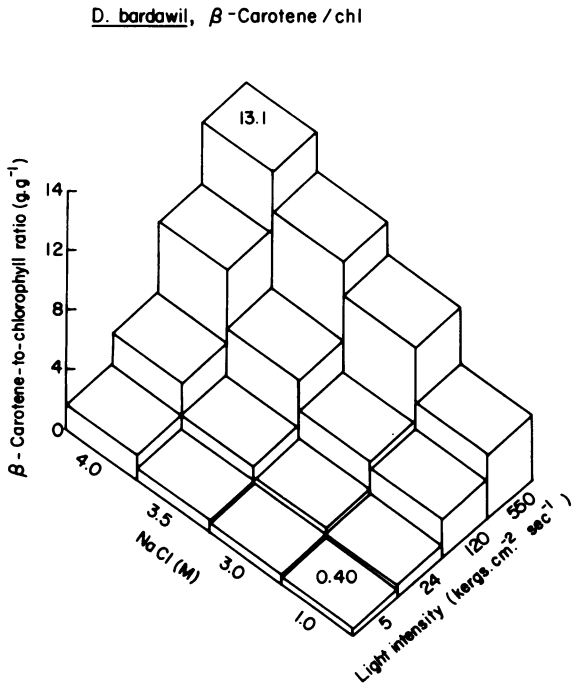

FIG. 4. Interaction between salt concentration and light intensity on the β-carotene-to-Chl ratio in *D. bardawil*. *D. bardawil*, preadapted to the indicated NaCl concentration at light intensity of 120 kergs cm^{-2} s^{-1}, was transferred to fresh media with the same NaCl concentrations and the indicated irradiation in the Warburg apparatus. Data shown were derived from the fifth day (during the logarithmic phase) at cell concentrations of around 5×10^5 cells ml^{-1}.

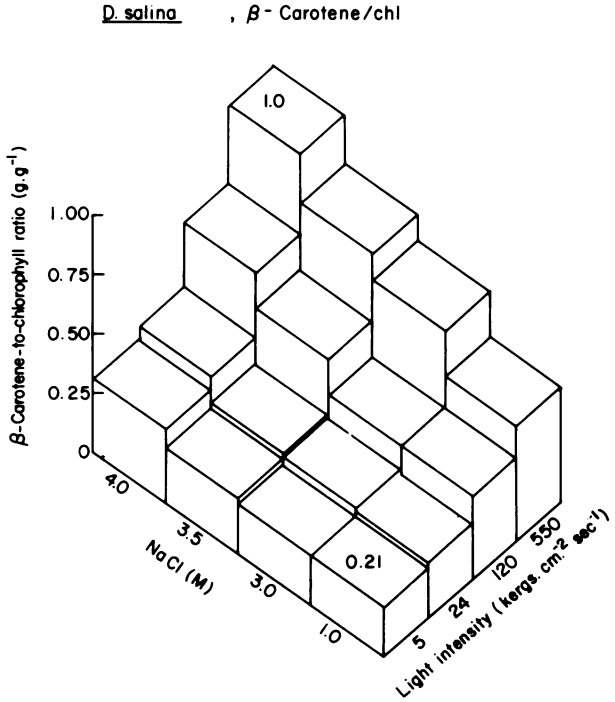

FIG. 5. Interaction between salt concentration and light intensity on β-carotene-to-Chl ratio in *D. salina*. *D. salina* was preadapted, grown, and assayed under the same conditions as described for *D. bardawil* in Figure 4, except that cell concentrations were around 3×10^6 cells ml^{-1}.

whether such cells are different in their photosynthetic activity on a Chl basis. Figure 6 shows light saturation curves of photosynthetic O_2 evolution for *D. bardawil* and *D. salina* cells grown under high (550 kergs cm^{-2} s^{-1}) and low (5 kergs cm^{-2} s^{-1}) intensity. As can be seen, maximal photosynthetic rates were considerably higher, on a Chl basis, for the high light (low Chl per cell)-grown cells. Rates of up to 600 μmol O_2 mg^{-1} Chl h^{-1} were measured. The differences in maximal rate of photosynthesis essentially disappeared when the data were expressed on a cell rather than a Chl basis. The relative quantum yield on a Chl basis (*i.e.* initial slope of the light saturation curves in Fig. 6) did not vary markedly between the differently grown cells.

The respiration rates of high light-grown *D. bardawil* and *D. salina* were 109 and 80 μmol O_2 mg^{-1} Chl h^{-1}, and of low light grown *D. bardawil* and *D. salina* were 24 and 30 μmol O_2 mg^{-1} Chl h^{-1}, respectively. Similar observations were noted in *Scenedesmus obliquus* grown on strong and weak light conditions (21).

DISCUSSION

Previously published data on *D. bardawil* (and the closely related β-carotene-accumulating strain of *D. salina* Teod.) indi-

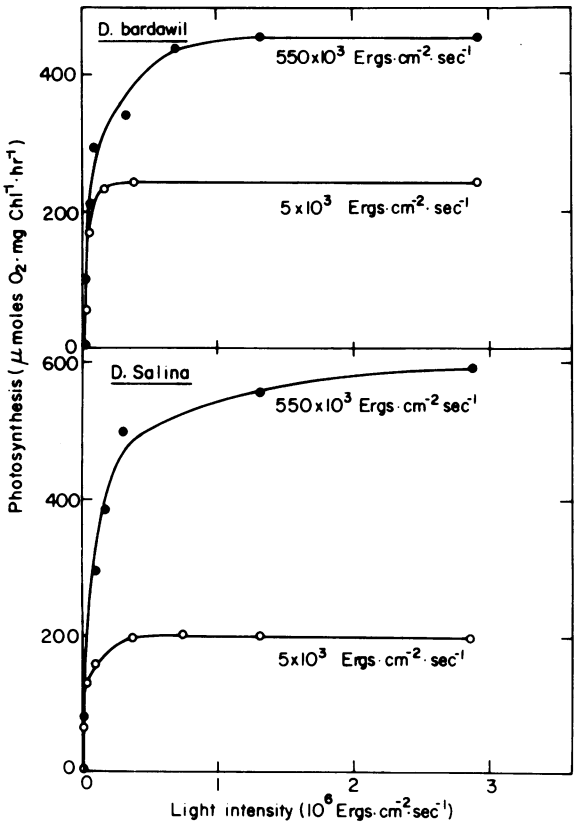

FIG. 6. Photosynthetic O_2 evolution of cultures of *D. bardawil* and *D. salina* grown under high and low light intensities. Cultures of algae were grown under the indicated continuous light intensities in media containing 3 M NaCl at 25°C. At the end of the logarithmic phase, algae were centrifuged, diluted in fresh media to about 20 μg Chl ml^{-1}, and assayed for O_2 evolution under various intensities of white light (>400 nm). At 20 μg Chl ml^{-1}, the cell suspension contained: (a) for *D. bardawil* grown under low light intensity, 1.6×10^6 cells ml^{-1} and 13.1 μg β-carotene ml^{-1}; (b) for *D. bardawil* grown under high light intensity, 5.2×10^6 cells ml^{-1} and 178 μg β-carotene ml^{-1}; (c) for *D. salina* grown under low light intensity, 4.4×10^6 cells ml^{-1} and 4.92 μg β-carotene ml^{-1}; and (d) for *D. salina* grown under high light intensity, 22.9×10^6 cells ml^{-1} and 15.7 μg β-carotene ml^{-1}. The O_2 evolution rates are corrected for O_2 uptake, as measured in the dark following an illumination period (see text).

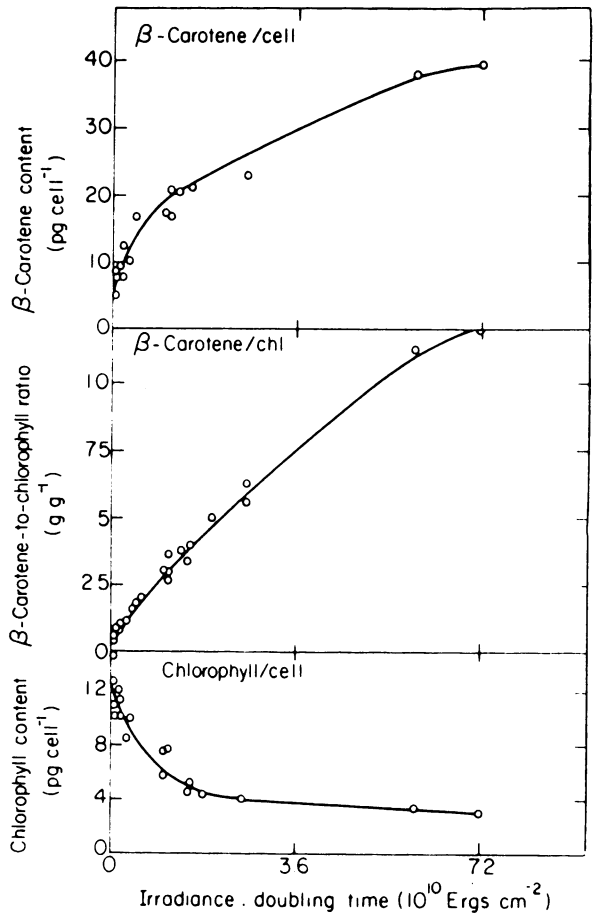

FIG. 7. Dependence of pigment content in *D. bardawil* on the integral irradiance per division cycle. Doubling time has been varied by growth at different salinities (1–4 M NaCl), different temperatures (15–40°C), different light intensities (5–550 kergs cm^{-2} s^{-1}), or continuous light *versus* diurnal cycle (10 h light, 14 h dark). Data were derived from cultures of about 5×10^5 cells ml^{-1} at the logarithmic phase of growth.

cated that these algae accumulate β-carotene under conditions which retard cell division. Thus, environmental stress conditions such as high NaCl concentration, extreme temperatures, extreme pH values or nutrient deficiency (11–13, 15, 18–20) increase the β-carotene-to-Chl ratio in inverse relation to the specific growth rate (4). As was shown here, the increase in β-carotene-to-Chl ratio in *D. bardawil* can be due to increased net synthesis of β-carotene, a decrease in Chl, or both. Under conditions of nutrient limitation, the ratio remains low as long as the growth rate is not affected, but rises dramatically as soon as growth and Chl synthesis slow down due to nutrient depletion. The typical orange color of the growth-retarded culture represents accumulation of β-carotene coupled with a decreased content of Chl.

An additional major inducing factor of β-carotene accumulation coupled with Chl depletion in *D. bardawil* is light intensity. The higher the intensity to which the algae are exposed, the higher is their β-carotene content and β-carotene-to-Chl ratio during logarithmic growth. Growth under conditions of high light intensity and reduced growth rate due to environmental stress result in cultures of minimal content of Chl and maximal content of β-carotene per cell, with a maximal β-carotene-to-Chl ratio of around 13.

On analyzing the data, a consistent correlation seems to exist between the β-carotene-to-Chl ratio and the integral quantity of light to which the culture is exposed during a division cycle. Thus, the longer the doubling time and the higher the intensity, the higher was the β-carotene-to-Chl ratio. Figure 7 illustrates plots of β-carotene and Chl content per cell and the β-carotene-to-Chl ratio as a function of this integral intensity per division (expressed as light intensity, in ergs cm^{-2} s^{-1}, multiplied by the doubling time). As can be seen, the large variety of data obtained from the logarithmic phase of algal growth ((4) and the present communication), where pigment concentration was varied by either light intensity, diurnal growth, temperature, or salt concentration, fall on the same curve in agreement with the proposed relationship between the integrated irradiance per division cycle and pigment content in *D. bardawil*.

The proposed relationship is in agreement with the previously suggested function of β-carotene accumulation in *D. bardawil* (4) as a protection of the cell against injury by high intensity irradiation. It is further supported by the observations that *D. salina* dies when exposed to very high light intensities under relatively slow growth conditions (>6×10^{10} ergs cm^{-2}, <0.5 pg Chl $cell^{-1}$), whereas *D. bardawil* survives and maintains a Chl level above 2 pg $cell^{-1}$. This may explain the predominance of *D. bardawil* over *D. salina* in saline lakes in nature, where the algae are exposed to high solar irradiation under relatively slow growth conditions.

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