EFFECT OF LIGHT INTENSITY AND GLYCEROL ON THE GROWTH, PIGMENT COMPOSITION, AND ULTRASTRUCTURE OF *CHROOMONAS* SP.[1,2,3]

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SUMMARY

Growth of Chroomonas *sp. increased with light intensity (100, 1800, and 2700 μw/cm²) with a fivefold increase from the lowest to the highest intensity. Chlorophyll and phycocyanin content per cell were greater in cells grown at low light intensity, but the ratio of chlorophyll a and c did not vary appreciably. Cells grown at low light intensity had 30% more phycocyanin than cells grown at high intensities of light. The chloroplast of cells with the higher phycocyanin content had average intrathylakoidal widths of 300 Å, whereas those cells with the lower phycocyanin content had average intrathylakoidal widths of 200 Å. This result is compatible with the hypothesis that phycocyanin is located in the intrathylakoidal space in the cryptophyte algae. Of the various energy sources tested, only glycerol was able to support limited growth under nonphotosynthetic conditions. Under no condition was the chloroplast reduced to an etioplast or proplastid state. Starch accumulation was greatest in cells grown in continuous white light in glycerol. Eyespots were commonest in cells grown in darkness and interrupted every 24 hr by a few seconds of white light. It was concluded that this organism is an obligate phototroph.*

INTRODUCTION

The photosynthetic members of the cryptophyceae have long been recognized as possessing characteristics which make them a distinct group (*6*). They are unique in being the only group to possess chlorophyll *a* and *c* as well as phycobiliproteins (*1*). Our aim was to explore the possibility of using these organisms as an experimental system to determine the sequence of pigment synthesis and chloroplast development. One method of manipulating the chloroplast structure and pigments is by varying the light intensity to which an alga is exposed. Varying light intensity can induce changes in growth and respiration (*4*), pigment composition and membrane development (*16*), pigment ratios (*3*), and ultrastruc-

ture (*13,14*). Another approach is to use a developmental system beginning with a proplastid or etioplastid state. Such systems exist in *Euglena gracilis* var. *bacillaris* (*10,11*) and in *Ochromonas danica* (*8*) where the chloroplasts regress when cells are grown in the dark on a heterotrophic medium. Toward this end numerous energy sources were tested to determine their potential for supporting heterotrophic growth in *Chroomonas* sp. and to see if a reduction of the chloroplast could be obtained. We found that the chloroplast of *Chroomonas* sp. was not reduced to an etioplast or proplastid state. Although the cells were able to grow in the presence of glycerol at very low light intensities, they were unable to grow in absolute darkness. Furthermore, the best growth was obtained at the highest light intensity tested (2700 μw/cm²).

MATERIALS AND METHODS

The culture of *Chroomonas* sp. was originally obtained from Dr. L. Provasoli's laboratory. Axenic cultures were grown on DV medium, essentially as described by Provasoli & MacLaughlin (*15*) at room temperature, in Erlenmeyer flasks, and illuminated with daylight fluorescent lamps (Westinghouse F 40 D). All cultures prior to treatment were kept under continuous illumination at 270 μw/cm² (40 ft-c).

Compounds tested for heterotrophic growth. Compounds tested as possible energy sources for heterotrophic growth were the following: 2-deoxy-ribose, D-mannitol, D-sorbitol, fructose, galactose, glucose, glucoseamine, glycerol, sucrose, caproic acid, DL-alanine, DL-lactic acid, glycine, L-lactic acid, propionic acid, triacetin, valeric acid, sodium ammonium acetate, sodium citrate, DL-isocitrate, glyoxylate, L-malate, oxaloacetate, sodium pyruvate, sodium succinate, and sodium fumarate. The above compounds were added separately to the DV medium using aseptic technique at 0.0002, 0.002, 0.25, 0.5, 0.75, and 1.0 M concentrations. Complex media such as casamino acids, nutrient broth, peptone, tryptone, and yeast extract were also tested at concentrations of 0.1, 0.05%. Duplicate cultures were incubated, 1 set in light (at 1800 μw/cm²) and 1 set in darkness for 2–4 weeks. Growth was monitored by measuring turbidity at 600 nm with a Coleman spectrophotometer at weekly intervals.

Glycerol treatment. Control cultures were grown in mineral medium (DV). A second set of cultures was grown on DV medium plus 1.0 M glycerol. The glycerol was added aseptically prior to incubation under the following conditions: (1) in continuous light (1800 μw/cm²); (2) in absolute darkness (as tested by placing a strip of Kodak Plus-X film in a companion flask, a clear strip after development indicated absence of exposure to light); or (3) in "relative darkness" (interruption of dark every 24 hr with a few seconds of white room light).
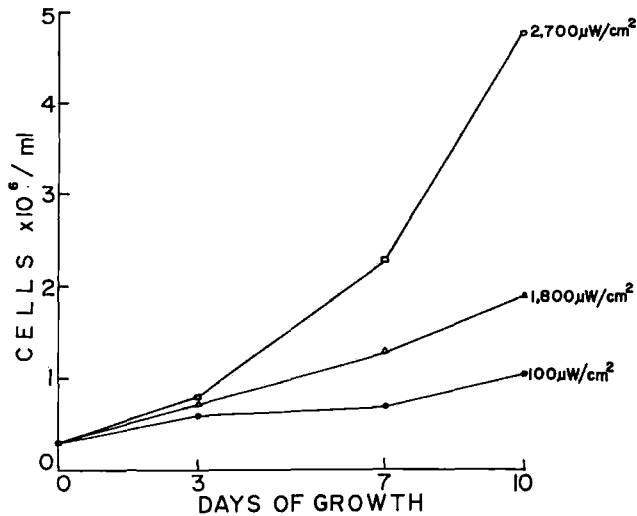
FIG. 1. Growth of *Chroomonas* sp. under continuous illumination at 3 light intensities, 100 (O), 1800 (△), and 2700 (□) μw/cm².

TABLE 1. *Chlorophyll a and c and phycocyanin content of cells grown at different light intensities after 7 days of growth.*

Irradiance, μw/cm²	μg Chl a / 10⁶ cells	μg Chl c / 10⁶ cells	Chl a / Chl c	μg Phycocyanin / 10⁶ cells	μg Phycocyanin / μg Chl a & c
100	0.448	0.178	2.51	1.62	2.5
1800	0.394	0.165	2.35	1.08	1.9
2700	0.377	0.168	2.20	1.05	1.9

width. The average width was determined from 100 measurements per treatment.

RESULTS

Effect of light intensity. Cultures exposed to a light intensity of 2700 μw/cm² had 5 times as many cells as those which were exposed at 100 μw/cm² (Fig. 1).

There was less total chlorophyll (Chl) per cell with increase in light intensity (Table 1). At the lowest light intensity the Chl *a* and *c* content was 0.62 picogram Chl/cell, which was slightly greater than the 0.54 picogram Chl/cell obtained at higher light intensity. The ratio of Chl *a* to *c* did not vary with the light intensity.

Phycocyanin (PC) content per cell also varied inversely with the light intensity from 1.05 to 1.62 picogram/cell (Table 1). Cells grown at the highest intensity contained 30% less PC than those grown at low light intensity. The ratio of PC to Chl declined with increased illumination.

Along with increased PC content, there was a concomitant increase in the intrathylakoidal width in the chloroplast of cells grown under low light intensities (Fig. 2). Cells with the higher phycocyanin content had an intrathylakoidal width of about 300 ± 20 Å, whereas cells with 30% less phycocyanin had an average width of 200 ± 20 Å (Fig. 3 and 4). Furthermore, cells grown at low light intensity had a proportionally greater total thylakoidal area than cells grown at high light intensity. The total chloroplast area (as determined by the cut and weigh method [see Materials and Methods]) was about the same under all light intensities. Small amounts of starch and rather closely packed thylakoids characterized the low intensity light grown cells (Fig. 2). At higher light intensities the thylakoids were more widely spaced (Fig. 3 and 4) and starch was considerably more abundant.

Effect of energy sources. Chroomonas sp., as most other cryptophytes tested, grows slowly in comparison with some dinoflagellates or euglenoids. Addition of 0.0002 M of glycine, DL-alanine, DL-lactic acid, DL-malate, or sodium acetate to DV medium, enhanced growth only in cultures incubated at light intensities of 1800 μw/cm² and above. Only glycerol was able

Light intensity treatments. The following irradiances were used: 100 μw/cm² (about 15 ft-c), 1800 μw/cm² (about 300 ft-c), and 2700 μw/cm² (about 1000 ft-c). Light intensities were determined with an Eppley Pyranometer with a GG 400 (Schott glass) filter dome. The sensitivity of the instrument was 9.95 μwcm²/μv.

Growth and pigment determination. Cell counts were made on duplicate samples of each culture with a haemocytometer. Cells were immobilized by the addition of a drop of 0.1% glutaraldehyde. Ten fields were counted from each sample and expressed as number of cells × 10⁶/ml.

Chlorophyll extracts were made in 90% acetone (v/v) for 30 min. The extracts were clarified by centrifugation and their absorbance determined with a Cary model 14 spectrophotometer. Concentrations of chlorophyll *a* and *c* were calculated (17) by using the following simultaneous equations:

$$Chl\ a\ (\mu g/ml) = 11.64\ (A_{663}\ nm) + 0.1\ (A_{630}\ nm)$$
$$Chl\ c\ (\mu g/ml) = -5.53\ (A_{663}\ nm) + 54.22\ (A_{630}\ nm)$$

Phycocyanin determinations were made simultaneously on duplicate samples. Extracts were prepared by ultrasonic disruption of whole cells in 0.05 M phosphate buffer pH 6.8, followed by centrifugation at 200,000 g for 1 hr. The absorption spectrum of the clarified blue supernatant was determined spectrophotometrically. Phycocyanin content was calculated from the absorption at 645 nm using an extinction coefficient of 11.4 mg/ml (1.0 cm pathway) according to MacColl *et al.* (to appear in *J. Biol. Chem.* 1973).

Electron microscopy. Cells were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer (pH 6.8 and with 0.2 M sucrose), postfixed in 1% osmium tetroxide, and embedded in Epon as described previously (7). Thin sections were stained with lead citrate or uranyl acetate plus lead citrate, and examined in a Philips 300 electron microscope.

Longitudinal sections of twenty randomly selected cells were photographed at a magnification of 27,000 ×. Chloroplasts were then cut out from the resulting prints and separated by further cutting into thylakoids, stroma, starch, and pyrenoid. The cut-outs were then weighed and the mean and standard deviation determined. Thylakoid widths were measured on enlarged photographic prints (96,000 ×) in cross sectional areas where the thylakoid membrane pairs had the sharpest images. The distance between the darkly stained outer edges of the photosynthetic lamellae was taken to represent the thylakoid
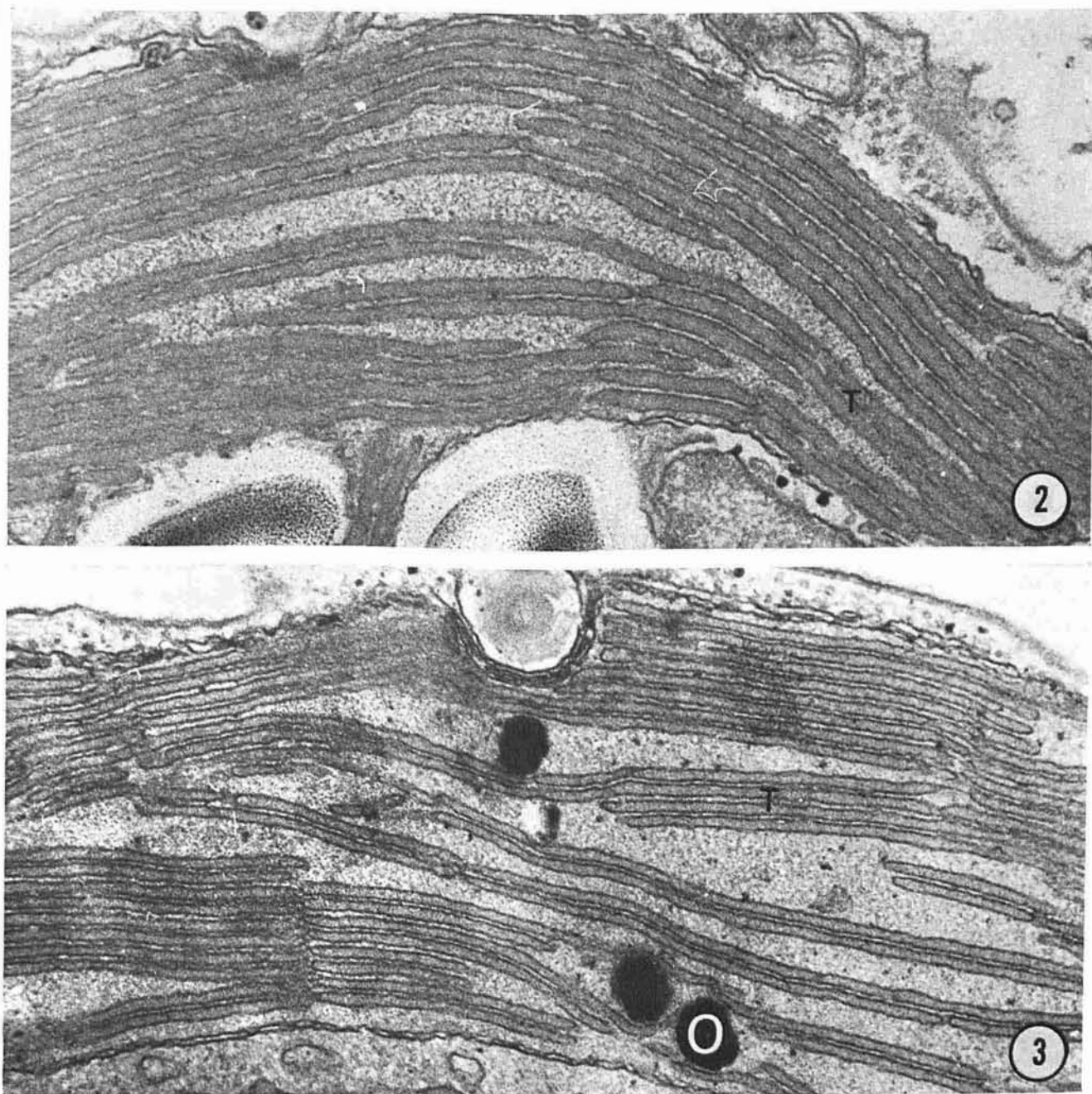
FIG. 2, 3. Sections of cells grown at 100 μw/cm^2 (Fig. 2) and 2700 μw/cm^2 (Fig. 3). Note the greater intrathylakoidal width in the top figure than in the lower one. The cells grown at low light intensity had also a higher phycocyanin content than cells grown at high light level. T = thylakoids, O = osmophylic body. $\times$ 96,000.

to support growth in light (at 1800 μw/cm^2) as well as in "relative darkness," but only for a short time, at high glycerol concentration. None of the complex media tested enhanced growth in light (at 1800 μw/cm^2) or in the dark.

Effect of glycerol. According to Antia *et al.* (2) glycerol was able to support growth and pigment synthesis in darkness of the red pigmented *Chroo-*

monas salina. Conditions similar to those described by Antia *et al.* (2) were used to grow *Chroomonas* sp. cultures except that defined seawater medium (DV) was substituted for enriched seawater medium. Cultures of *Chroomonas* sp. were incubated as described in Materials and Methods. Further incubation conditions are referred to by the following abbreviations: L = continuous light at 1800 μw/cm^2;
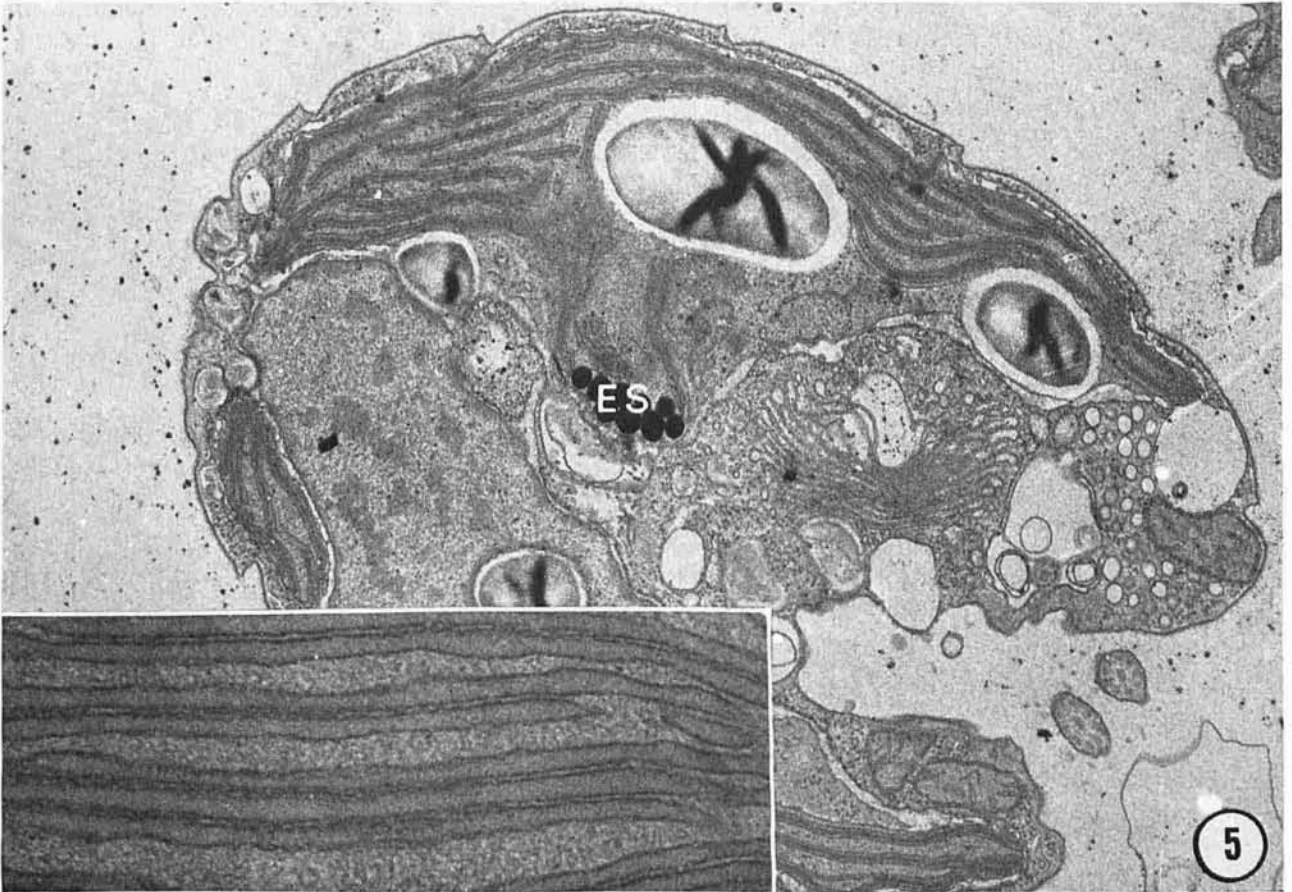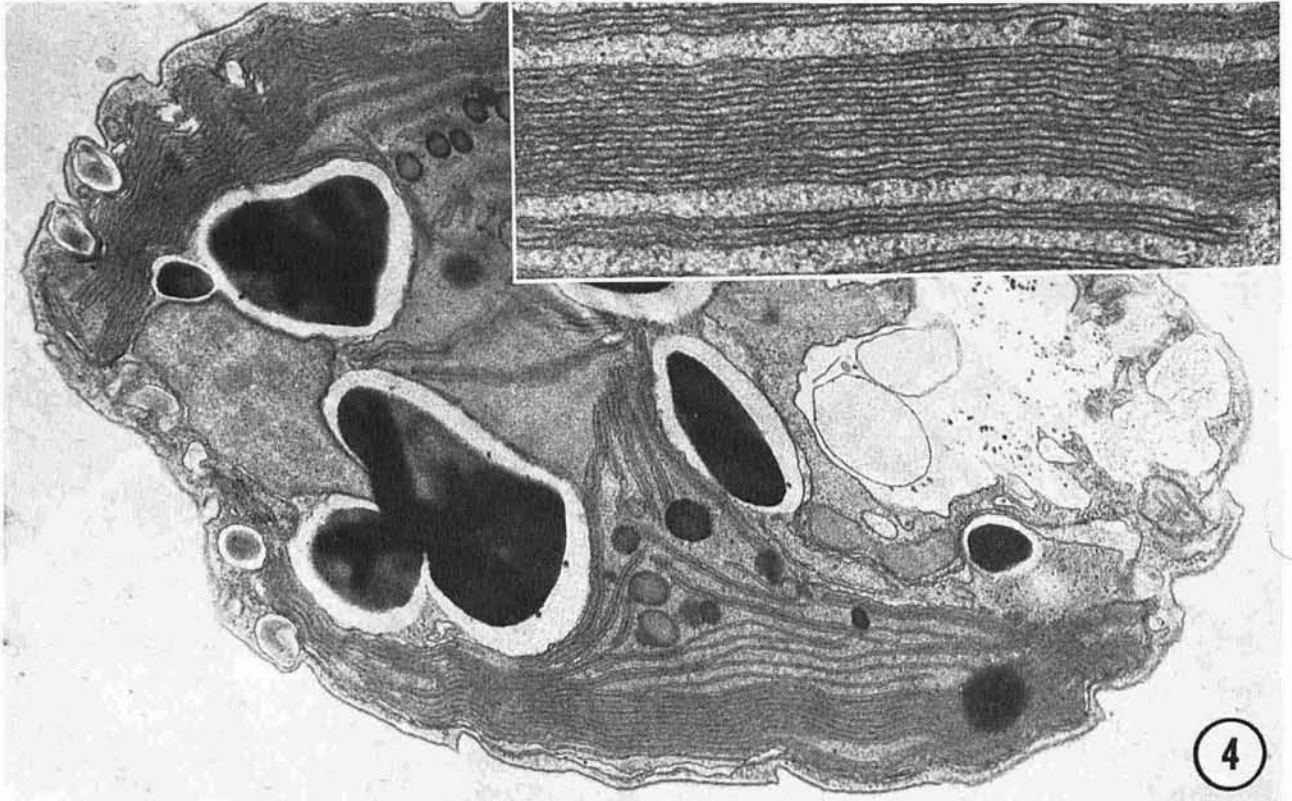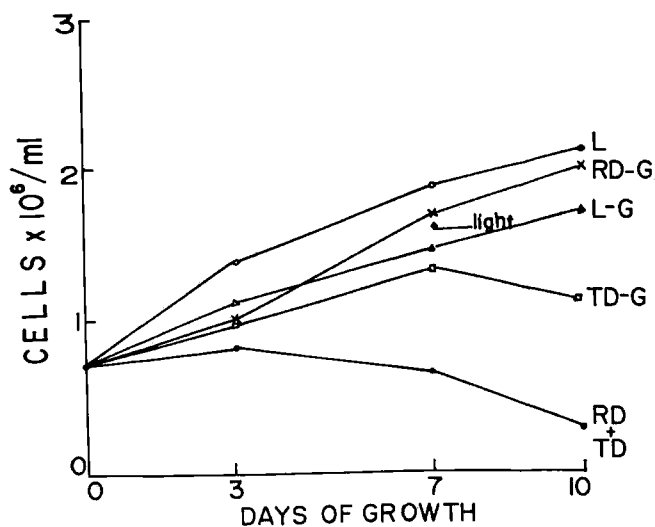
Fig. 6. Growth of *Chroomonas* sp. under the following conditions: L = continuous white light at 1800 $\mu w/cm^2$; L-G = continuous light, medium supplemented with glycerol 1.0 M; TD = total darkness; TD-G = total darkness, plus glycerol; RD = "relative darkness," darkness interrupted every 24 hr with few seconds of room light; RD-G = "relative darkness" plus glycerol. RD-G cells attained the growth at day 10 only when exposed to continuous light after day 7 (as marked by arrow).

L-G = continuous light, medium supplemented with glycerol 1.0 M; TD = total darkness; TD-G = total darkness, medium supplemented with glycerol 1.0 M; RD = "relative darkness," darkness interrupted every 24 hr with a few seconds of room light; RD-G = "relative darkness," medium supplemented with glycerol 1.0 M.

No growth occurred on mineral medium alone without continuous illumination (Fig. 6). Glycerol was able to support growth in TD-G and RD-G cultures up to 7 days. After an initial doubling, the cell number began to decline and continued to do so (when followed for over 20 days). When RD-G cultures after 7 days were exposed to a light intensity of 1800 $\mu w/cm^2$, growth became equal to L cultures by day 10. In continuous light glycerol did not enhance growth. However, it is assumed that glycerol was taken up because the starch content (as determined by the cut and weigh method) of these cells was much greater than in cells grown in light without glycerol.

The Chl content per cell was essentially the same in L-G and L cells and ranged from 0.66 to 0.58 g Chl a and $c/10^6$ cells (Fig. 7A). However, in RD-G
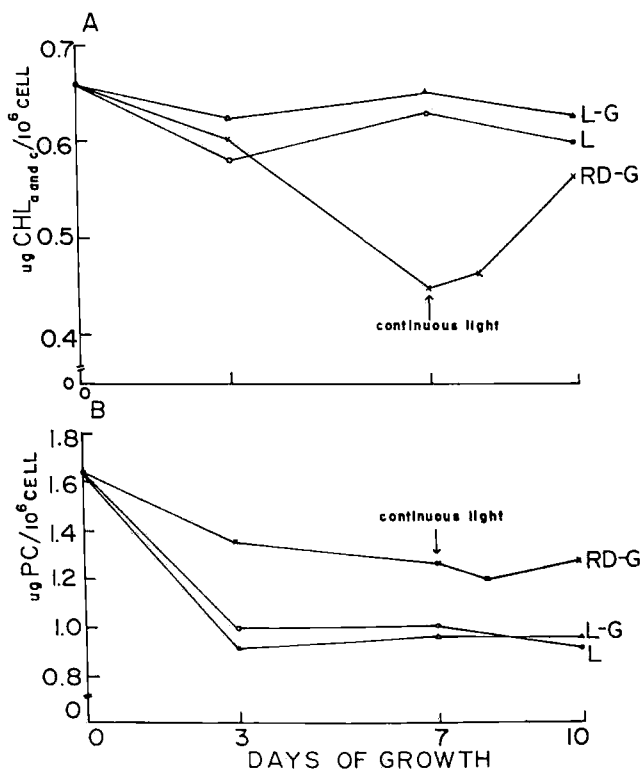

Fig. 7A, B. Pigment composition of *Chroomonas* sp. cells grown in continuous white light (at 1800 $\mu w/cm^2$) and in "relative darkness." A and B show chlorophyll a and c content per cells and phycocyanin content per cells, respectively. See Fig. 6 for conditions.

cells there was a considerable reduction in Chl content by the seventh day of growth. When illumination was restored, the Chl content per cell increased to levels comparable to those in L and L-G cells within 72 hr. Phycocyanin content was little affected by this treatment (Fig. 7B). The only change in this pigment occurred in the initial period before the third day of growth. The drop in PC content (seen in Fig. 7B) is attributed to the increase in light intensity because cells had been maintained prior to the experiment at 270 $\mu w/cm^2$, but were transferred to 1800 $\mu w/cm^2$ upon starting the experiment.

The morphology of 7-day L and L-G cells was the same, except that L-G cells contained about twice the amount of starch. In RD-G cells (Fig. 5), where the chloroplast and thylakoid area was reduced and PC content per cell was higher, the average intrathylakoidal width was 300 Å (Fig. 5 and 7); but in L (Fig. 4) and L-G cells, which had a lower phyco-

Fig. 4, 5. This section is typical of a cell grown at 1800 $\mu w/cm^2$ light intensity. Starch is abundant, but not as massive as when grown in glycerol at the same light intensity. The inset is an enlarged section of the chloroplast lamellae. Fig. 4 $\times 23,000$; inset $\times 96,000$. Fig. 5. Section of a cell grown on DV medium supplemented with glycerol in "relative darkness" for 7 days. Thylakoid widths are greater under these conditions than in the light, as can be seen by comparison with the above inset of Fig. 4. The presence of starch and an eyespot were characteristic features. ES = eyespot. $\times 23,000$; inset $\times 96,000$.

cyanin content (Fig. 7B), the intrathylakoidal width was 200 Å. These results are the same as those obtained with cells grown at high and low intensity light, where the intrathylakoidal width was also greatest in cells with the highest phycocyanin content (Fig. 2, 3, 7B and Table 1).

Eyespots were most commonly found in RD-G cells (Fig. 5) as well as in cells grown at the lowest light intensity (100 μw/cm²). It should be noted that when RD-G cells after 7 days growth were exposed to light several events occurred: the eyespots tended to disappear, the average width of the chloroplast lamellae was reduced from 300 Å to 200 Å, the chloroplast area increased, and starch accumulated. Furthermore, in some chloroplast regions there was a type of vesiculation which can be interpreted as representing new lamellar growth.

DISCUSSION

Cryptophyte algae are a group of organisms which normally grow very slowly (2,4). Chroomonas sp. exhibited the best growth at the highest light intensity tested, and in this respect is similar to Cryptomonas ovata as reported by Brown & Richardson (4). At this point not enough species have been tested to draw any definite conclusions, but it seems that the cryptophytes are disappointing experimental laboratory organisms because of their slow growth.

A previous report by Cheng & Antia (5) has shown that other cryptophytes, namely, Hemiselmis virescens and Rhodomonas lens were also incapable of heterotrophic growth. Our data presented here show that Chroomonas sp. cannot grow heterotrophically on any one of a number of energy sources tested, whether grown in light or dark. It seems that glycerol can be taken up in light, as indicated by the increased starch accumulation, although there was neither an increase in growth, nor pigment synthesis above that of cells grown on a minimal medium. Antia et al. (2) reported heterotrophic growth in the dark on glycerol of the marine photosynthetic cryptophyte C. salina. This species contains phycoerythrin rather than phycocyanin, but appears to be closely related to Chroomonas sp. Although we followed their culture conditions (medium excepted) in growing Chroomonas sp. we obtained different results. It may be that their use of enriched seawater, rather than use of a defined seawater medium (DV), is significant and responsible for the different results. While our initial observations with glycerol in "relative darkness" had been encouraging, and in line with their results, we found that in total darkness (as defined in Methods) growth of Chroomonas sp. was not maintained. From the results obtained by our study we have concluded that Chroomonas sp. is an obligate phototroph. Whereas a dedifferentiation or significant reduction of the chloroplast was not attained, the reduction of the photosynthetic

pigments was reflected in the cell morphology especially in cells grown in "relative darkness." Antia et al. (2) have also found a reduction of photosynthetic pigments but not a complete absence, because after 16 transfers the dark grown cells were still pigmented. Since their work involved only light microscopy, a comparison of ultrastructural changes with pigment variation is not possible.

Chroomonas sp. showed variation in pigment content when grown under different light intensities as has been shown in other algal groups (4). Generally the pigment content increases as the light intensity decreases, as also happened in this species. This is particularly apparent in the increase of the photosynthetic accessory pigments. It has long been known that in some red and blue-green algae (9) the phycobiliprotein-to-chlorophyll ratio varies inversely with light intensity. A similar adaptation appears to occur in Chroomonas sp. as shown by the increase of the PC:Chl ratio. Chlorophyll c which is generally regarded as an accessory pigment, did not behave in a similar manner. The Chl a:c ratio remained essentially unchanged as has been found in 2 marine chrysomonads (12).

Recently it was proposed that the phycobiliproteins in cryptophyte algae are located in the intrathylakoidal lumens (7). The present data, which show a positive correlation between higher PC content and wider intrathylakoidal widths, support this hypothesis. Although Chroomonas sp. is not an ideal experimental organism, our data indicate that the photosynthetic apparatus of cryptophyte algae adapt similarly to changes in light intensity as do other algae and higher plants.

ADDENDUM

Since submission of this manuscript a paper by Antia et al. (J. Protozool. 20:377, 1973) has appeared dealing with the ultrastructure of Chroomonas salina. The emphasis of their and our studies was different, therefore, a comparison of thylakoid widths and pigment content is not possible. However, it appears that the two species differ in their response to photoheterotrophic conditions because Chroomonas salina lost its phycobiliprotein content, whereas Chroomonas sp. did not.

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A CONTINUOUS CULTURE STUDY OF PHOSPHATE UPTAKE, GROWTH RATE AND POLYPHOSPHATE IN *SCENEDESMUS* SP.[1]

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SUMMARY

The kinetics of phosphate uptake and growth in Scenedesmus *sp. have been studied in continuous culture with particular reference to the shifts in the cellular P compounds as a function of growth rate.*

Uptake velocity is a function of both internal and external substrate concentrations and can be described by the kinetics of noncompetitive enzyme inhibition. The concentrations of polyphosphates (alkali-extractable or 7-min) can be substituted as inhibitors in the kinetic equation. The apparent half-saturation constant of uptake, K_m, *is 0.6 μM. The apparent half-saturation concentration for growth is less than* K_m *by 1 order of magnitude. Growth is a function of cellular P concentrations, and the polyphosphates (alkali-extractable or 7-min) appear to regulate growth rate directly or indirectly. To understand P limitation, therefore, it is necessary to measure both external P and internal polyphos-* *phate levels. Evidence indicates that alkali-extractable polyphosphates, which can be quantitatively determined by a simple method of measuring surplus P, are involved in cell division process and that a maintenance concentration of functional phosphate exists in the form of polyphosphates. Alkaline phosphatase activity has an inversely linear relationship to growth rate and to the reciprocals of both polyphosphates and surplus P. Changes in lipid P, RNA P, and presumably all other forms except DNA are related to changes in growth rate.*

INTRODUCTION

In previous batch culture studies on the phosphate limitation of *Scenedesmus* sp. (44), I found that the exponential phase of the algal growth continues even after phosphate is exhausted from the medium. This observation is not consistent with the conventional assumptions about the relationship of