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[Original]

Physiological Events Occurring during the Conversion from Heterotrophy to Autotrophy in *Euglena gracilis*

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When oxygen was supplied to cells of *Euglena* containing lipids accumulated by stationary organic culture, proplastids did not develop although oxidative degradation of lipids produced energy and materials for growth. When the lipids-accumulated cells were transferred to an inorganic medium in darkness and aerated, oxidative degradation of accumulated lipids occurred and the chlorophyll synthesis ability increased. This increase in the synthetic ability was suppressed by the addition of an organic carbon source, and inhibitors of nucleic acid and protein synthesis also exhibited a similar effect. These findings are summarized as follows. 1. It is plausible that, in *Euglena* cells transferred to inorganic medium, energy produced by the oxidative degeneration of lipids and degraded products are used for proplastid. 2. Removal of organic carbon sources from the medium induces disinhibition of the early step of the proplastid development, and nucleic acid transcription occurs regardless of the presence of light.

Key words: *Euglena*, heterotrophy, autotrophy, conversion, chlorophyll

Introduction

The growth of microorganisms requires carbon and energy sources for synthesis of cellular components. Microorganisms are divided into several groups based on their mode of acquisition of the major source of nutrients and auxotrophy. Microorganisms that utilize energy released by the oxidization of inorganic compounds and photoenergy for growth are defined as autotrophs. Microorganisms that require organic compounds are defined as heterotrophs, and most pathogenic bacteria, protozoa, and fungi are included in this type. The objective of this study was to analyze the mechanism of conversion from heterotrophy to autotrophy using a unicellular alga, *Euglena glacilis*.

When *Euglena glacilis* is transferred to a medium containing an organic carbon source and cultured in a dark place, it heterotrophically proliferates and shows animal-type metabolism, but when the organic carbon source is removed from the medium, and the alga is irradiated with light in an inorganic medium, autotrophic proliferation occurs, and it develops a plant-type state. This phenomenon is reversible, and is not seen in higher biologics, raising a big issue in taxonomy[1,2]. Schiff's group[3,4] have actively reported studies of the greening of *Euglena gracilis* var. *bacillaris*. They traced chloroplast development by a photoinduction method using cell division-arrested cells, and found that paramylum accumulated in cells simultaneously with protein and nucleic acid metabolism plays an important role. Rosenberg *et al.*[5] analyzed lipids (mainly wax ester) markedly accumulated

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in the cytoplasm in a stationary culture of *Euglena*, and found that the lipids were consumed upon light irradiation.

The lipids accumulated to 500–700 pg/cell within 5 days of stationary culture, and cell proliferation stopped. When these cells were transferred to an inorganic medium and cultured with shaking, the lipids rapidly decreased, and greening partially progressed even in a dark place.[6,7] Lipids started to increase on 2 days of stationary culture in dark in a medium containing an organic carbon source, and the content reached about 700 pg/cell within 5 days. However, the lipids were degraded by oxidization in a subsequent shaking culture, and not accumulated (70 pg/cell). When these cells containing accumulated lipids were transferred to an organic carbon source-removed inorganic medium, and aerated with 1% CO_2, the lipid content decreased for 24 hours, and then stayed at a constant level (150 pg/cell), during which time chlorophyll synthesizing ability develops (preparatory step for chloroplast formation). When changes in respiratory activity in this step (measured by the oxygen electrode method) were investigated, respiratory ability reduced as the lipid level decreased, and the curves of the 2 parameters were similar[8]. At this point, no decrease in the paramylum content was noted, suggesting that accumulated lipids play an important role in the progression of greening in the dark in the presence of inorganic nutrients. To analyze the mechanism of conversion from heterotrophy to autotrophy based on the above findings, we investigated the inhibitory effect of an organic carbon source as a factor that controls the early process of light-independent greening as the first step. To elucidate the inhibition mechanism, we performed the experiments using inhibitors of protein and nucleic acid synthesis and of respiration.

Materials and Methods

1) Algal strain: The test strain was *Euglena gracilis* Klebs var. *bacillaris* Cori cultured in the dark[9]. This strain has been subcultured in the dark since 1964, and was kindly provided by Professor J.A. Schiff (Institute for Photobiology, Brandeis University, MA 02254, USA).

2) Media: Organic medium: Vitamin B_{12}-supplemented Hutner medium[10]. Inorganic medium: Modified Cramer & Myers medium[11].

3) Conversion from heterotrophy to autotrophy: Culture fluid (50 m*l*) of about 2.0×10^6 cells/m*l* cultured with shaking was transferred to a 500-m*l* Erlerumeyer flask containing 400 m*l* of organic medium and kept at 28°C in the dark for 5 days. These heterotrophically grown and lipid-accumulated *Euglena* cells were washed twice with inorganic medium to remove the organic carbon source, transferred to inorganic medium at a cell density of about 0.6×10^6 cells/m*l*, and cultured in the dark with aeration of 2% CO_2. The influence of the organic carbon source on the chlorophyll synthesis ability in *Euglena* cells was investigated with the inorganic medium (control), 1.5% glucose-inorganic medium, 1.5% galactose-inorganic medium, or Hutner's organic medium[10] and cultured in the dark for 19 hours. Proteins of the inorganic culture were sampled at appropriate intervals. *Euglena* cells thus harvested were used for cell counts and for measurements of chlorophyll, chlorophyllide, lipid and respiration.

4) Cell counting: After adding a few drops of formalin solution to the sample cell suspension, the cells were counted under a microscope at 100 times magnification using a Fucks–Rosenthal counting chamber. The number of cells in 1 m*l* of the original cell suspension was calculated by multiplying the count by the constant (3.1×10^2 cells/ml).

5) Measurement of absorbance of chlorophyll[12]: Chlorophyll-synthesizing capacity of

Euglena cells was assayed as follows. Lipid-accumulated cells transferred to inorganic medium and aerated in the dark as described above were sampled every 3 hours, and irradiated with 3,000–4,000 lux of fluorescent light (daylight color) at 25°C for 30 minutes, and the amount of chlorophyll synthesized was regarded as the latent synthetic ability. To measure the chlorophyll amount, cell suspension was centrifuged at 3,000 rpm for 30 minutes at 4°C. An appropriate volume of 80% acetone was added to the precipitate and mixed, and the mixture was kept at 4°C for 30–60 minutes to extract chlorophyll. The mixture was then centrifuged at 3,000 rpm for 30 minutes at 4°C, and the absorption spectrum of the supernatant was measured using a spectrophotometer, UV 210A (Shimadzu Seisakusho Co.). The wavelength range used was 420–700 nm. The chlorophyll content (pg) was calculated by the equation below:

Chlorophyll content (pg/cell)[12]
$=[(OD663\times 8.05)+(OD645\times 20.3)]$
$\times$ total volume of acetone/
[volume of cell suspension $\times$ (cells/m*l*)
$\times 10^{-6}$]

6) Measurement of chlorophyll fluorescence[13]: Chlorophyll was extracted from the cell suspension as described above, except that the volumes of cell suspension (1.0×10^6 cells/m*l*) and 80% acetone for extraction were 50 m*l* and 5 m*l*, respectively. The fluorescence spectrum of the supernatant was measured using a fluorescence spectrophotometer at 470 nm excitation. The range of wavelengths measured was 610–730 nm.

7) Quantification of lipids[2, 14]: Cell suspension was centrifuged at 3,000 rpm for 30 minutes at 4°C, and the precipitate was washed with cold distilled water and centrifuged. The precipitate was then mixed with 20 m*l* of a 2 : 1 mixture of chloroform and methyl alcohol and kept at 4°C for 1 hour (stirred several times) to extract lipids. The mixture was centrifuged at 3,000 rpm for 30 minutes at 4°C, and the supernatant was dried under N_2 gas flow in a water bath controlled at 35°C. The dried residue was dissolved with a small volume of chloroform/methyl alcohol mixture, combined with 0.88% KCl solution to adjust the final volume to 1/5, and stirred well. The solution was centrifuged at 500 rpm for 10 minutes, and the upper layer of 2 layers separated by centrifugation was discarded. The lower layer was washed with an equivalent volume of a 3 : 48 : 47 mixture of chloroform/methyl alcohol/1.76% KCl and centrifuged, and the upper layer of 2 layers separated by centrifugation was discarded. This procedure was repeated until the white turbid material produced in the boundary of the 2 layers disappeared, and the lower layer was finally dried at 50°C and weighed.

8) Measurement of respiratory activity[8]: The respiratory activity of each sample cell suspension was measured using a Yanagimoto recording respiratory oxygen measurement system (PO-100A). The respiratory level was represented as the oxygen amount consumed by μmol O_2/ cell/ hour[8].

9) Influences of carbon sources[16]: Lipid-accumulated cells grown for 5 days were harvested and transferred into the inorganic medium (control), 1.5% glucose-inorganic medium, 1.5% galactose-inorganic medium and Hutner organic medium. After 19 hours at 28°C in the dark cells were harvested and their chlorophyll-synthesizing ability was assayed by the fluorescent wethod as described above.

10) Influences of metabolic inhibitors: Cells containing accumulated lipids were transferred to inorganic medium, followed by addition of 6-methylpurine (6MP), Cycloheximide (CHI), or Lincomycin (LIN) and cultured in the dark. The drug was removed after 15 hours, the cells were irradiated with light for 30 minutes, and the rate of chlorophyll synthesis during this period was

measured, as mentioned above. The concentrations of the drugs are shown in Figs. 7–9.

11) Transmission electron microscopy[15]: *Euglena* cells taken from the culture were fixed with 2.5% glutaraldehyde (TAAB) for 1 hour at 4°C and then post fixed with 1% osmium tetroxide (EM Science, USA) for 1hour at 4°C. The suspension was then centrifuged and the pellet was embedded in 2% agarose (Nacalai Co.) followed by dehydration in a 50–100% graded ethanol series followed by 100% acetone (Wako Pure Chem, Co.). The samples were embedded in Spurr's resin (EM Science, USA) and subjected to serial sectioning on a Leica UCT microtome. Sections were stained with 3% uranyl acetate (Merck, Germany) at room temperature followed by lead citrate, after which they were examined with a JEOL CX-100 electron microscope in EM room of Nippon Sport Science Univ.[17]

Results

Electron microscopic features of *Euglena* cells

When *Euglena* cells were grown in the organic medium at 28°C in the dark, the lipid content increases by stationary culture and the lipid has been shown to be wax ester by Rosenberg *et al.*[5, 18] In this study, lipids started to rapidly increase at 24 hours. The content reached 0.7 ng on day 5 and remained at this level thereafter, and at the same time, cell division stopped. The lipid content did not increase in the control cells on shaking culture, indicating that wax ester was oxidatively degraded by shaking.

Conversion from heterotrophy to autotrophy was analyzed using cells on day 5 of stationary culture whereby lipid accumulation had reached a maximum. In proplastids on day 5 of stationary culture, the prolamellar body was absent, no membrane structure was noted in the stroma, and lipids had accumulated in the cytoplasm (needle-like structure) as shown in Fig. 1. These cells were washed twice with inorganic medium to remove the organic carbon source, transferred to inorganic medium, and dark cultured with aeration of 2% CO_2 for 72 hours. A part of a section of the cells is shown in Fig. 2.

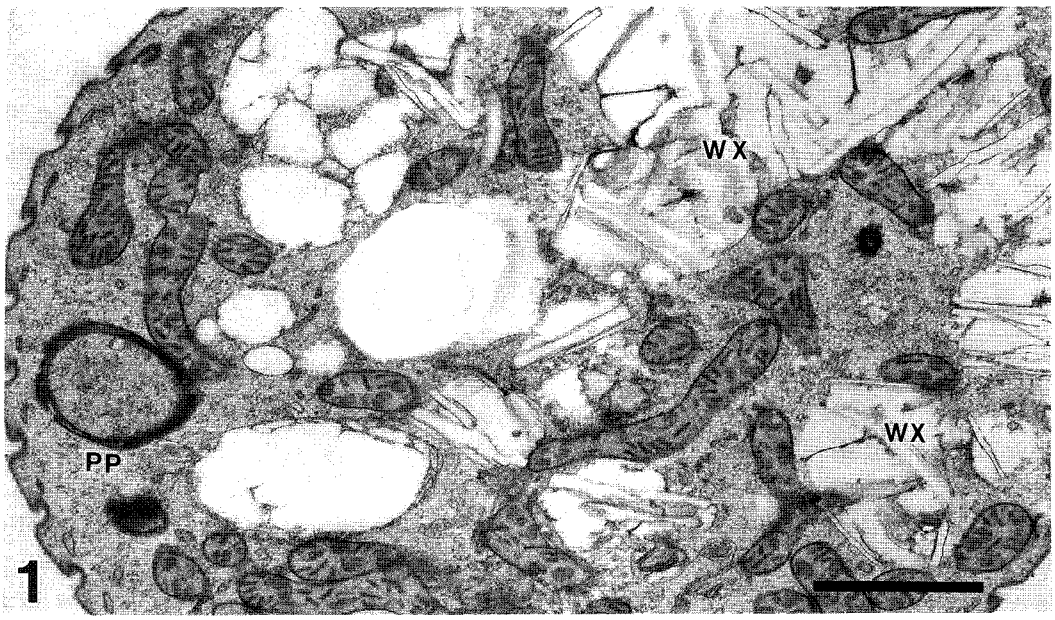

Fig. 1. A section of the dark-grown wax-rich cells of *Euglena*. Note the presence of many scars of wax ester (WX) in cytoplasm. Cell contained proplastid (PP) showing no internal structure. Bar: 1 μm

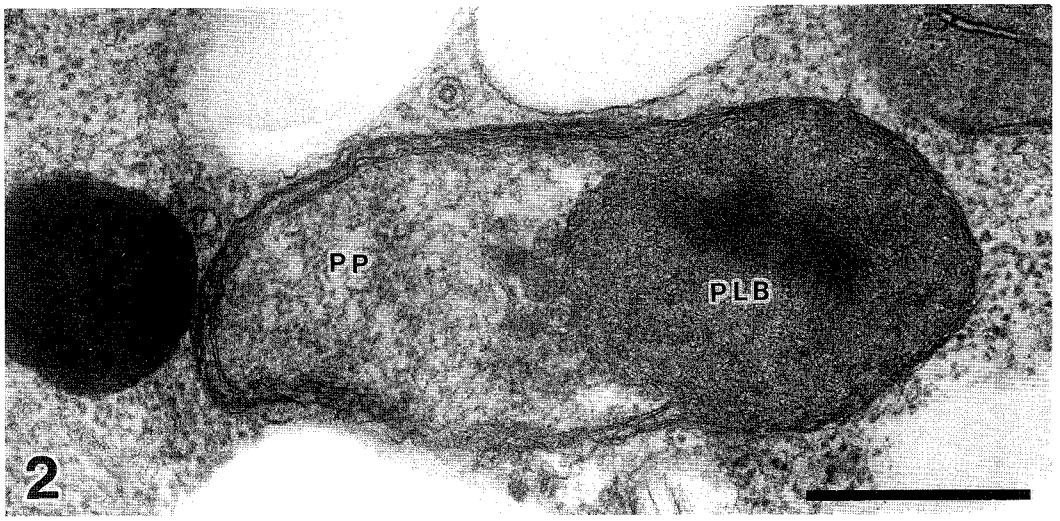

Fig. 2. A section of developing proplastid (PP) in *Euglena* containing a prolamellar body (PLB). Cells of *Euglena* were washed twice with inorganic medium to remove the organic carbon source, transferred to inorganic medium, and dark cultured with aeration for 72 hours. Bar: 0.5 μm

Prolamellar bodies were formed in the stroma in proplastids, and prothylakoids also appeared (Fig. 2).

Changes in chlorophyll-synthesizing ability during the conversion

Changes in chlorophyll synthesis ability with the progression of this process were investigated. The maximum abilities to synthesize chlorophyll per cell and per culture fluid were noted at 18 hours, at which time cells had divided 1–2 times (Fig. 3).

The results of light irradiation for 5, 15, and 30 minutes measured by the fluorometric method in similar samples are shown in Fig. 4. Chlorophyll precursor decreased and chlorophyll increased with time, showing that light irradiation rapidly converted the precursor to chlorophyll. The maximum value was detected at 21 hours in all samples. Therefore, the period of maximum chlorophyll synthesis ability exists at about 18–21 hours.

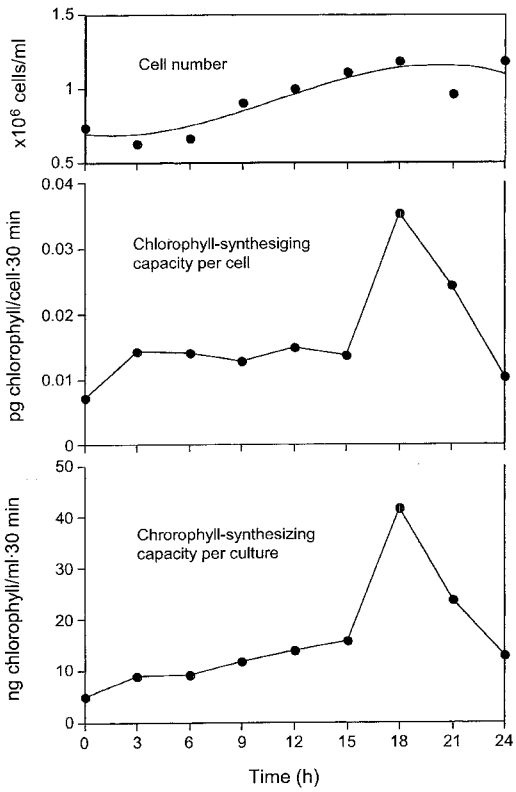

Fig. 3. Changes of the cell number of *Euglena*, and chlorophyll synthesis ability under aeration in the dark culture condition.

Lipid degradation and respiration

To examine an association of development of

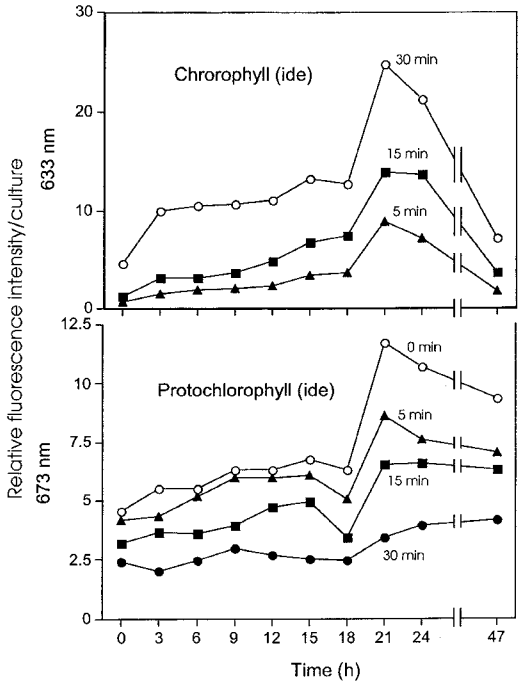

Fig. 4. Changes of protochlorophyll and chlorophyll in *Euglena*. Chlorophyll precursor decreased and chlorophyll increased with time, showing that light irradiation rapidly converted the precursor to chlorophyll.

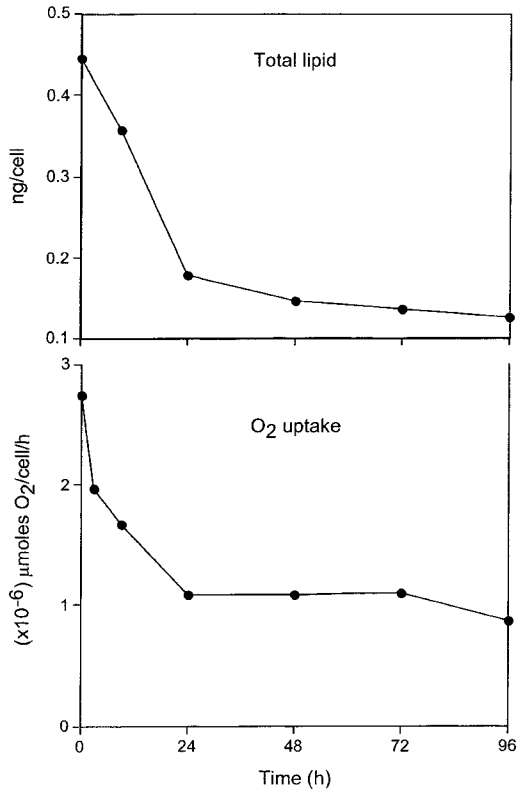

Fig. 5. Changes in the lipid content and respiratory activity in cells of *Euglena* cultured in inorganic medium with aeration in darkness. The lipid content decreased to 1/2 or less and stabilized by 24 hours, and oxygen absorption showed a similar curve.

the chlorophyll synthesis ability and oxidative degradation of lipids, changes in the lipid content and the respiratory activity in *Euglena* cells transferred into the inorganic medium in the dark were measured.

As shown in Fig. 5, the lipid content decreased to 1/2 or less and stabilized by 24 hours, and oxygen absorption showed a similar curve, showing that oxygen was markedly consumed for 24 hours, and the consumption stabilized thereafter. These findings indicated that lipids (wax ester) were rapidly decreased by oxidation. A similar experiment was performed using aeration with nitrogen instead of oxygen. The total lipid content did not decrease, and the latent chlorophyll synthesis ability did not increase, demonstrating the above findings. Regarding the reduction of lipids in cells with accumulated lipids by culture in inorganic medium with aeration in the dark, changes in lipid content in the presence of an organic carbon source were investigated (data not shown). The presence of the organic carbon source did not make a difference in the reduction of the lipid content.

Influences of carbon sources and metabolic inhibitors on chlorophyll synthesis

The influence of the organic carbon source on the chlorophyll synthesis ability was investigated by tracing the relative chlorophyll level by fluorometry. The maximum level was noted at 12 hours in cells cultured in glucose-containing medium, and 19 hours in the other cells including the control, but the levels were

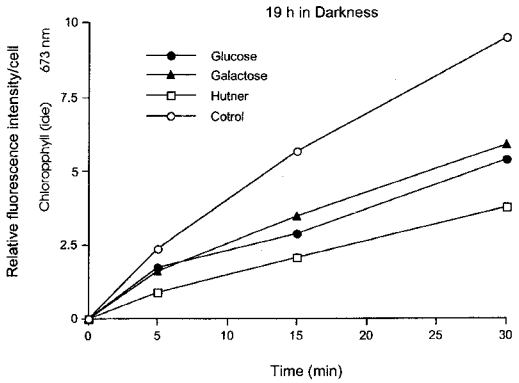

Fig. 6. The influence of the organic carbon source on the chlorophyll synthesis ability in *Euglena* was investigated by tracing the relative chlorophyll level by fluorometry. Lipid-accumulated cells were transferred to the inorganic medium (control), 1.5% glucose-inorganic medium, 1.5% galactose-inorganic medium, or Hutner's organic medium and cultured in the dark for 19 hours. Cells harvested these cultures were subjected to assay the chlorophyll synthesis ability by illumination of light.

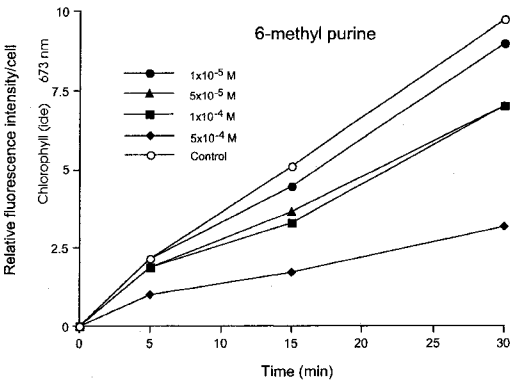

Fig. 7. The influences of 6-methylpurine on the development of chlorophyll synthesis ability were investigated by fluorometry. Lipid-accumulated cells were transferred to the inorganic medium containing 6MP at various concentrations and cultured for 15 houres in the dark. Cells harvested these cultures were subjected to assay the chlorophyll synthesis ability by illumination of light.

lower than that in the control. The rate of chlorophyll synthesis at 19 hours was investigated (Fig. 6). Compared to the control, the rate was

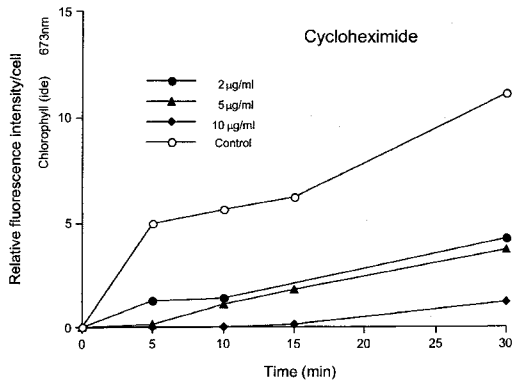

Fig. 8. The influences of cycloheximide on the development of chlorophyll synthesis ability were investigated by fluorometry. Culture conditions and assay of the chlorophyll synthesis ability are the same as mentioned in Fig. 7.

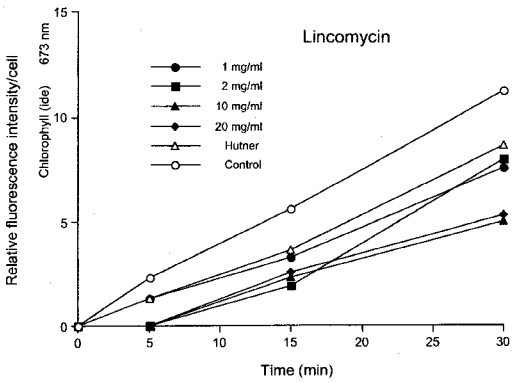

Fig. 9. The influences of lincomycin on the development of chlorophyll synthesis ability were investigated by fluorometry. Culture conditions and assay of the chlorophyll synthesis ability are the same as mentioned in Fig. 7.

markedly inhibited, particularly in cells cultured in the organic medium, clarifying that the carbon sources did not inhibit consumption of accumulated lipids, but inhibited the development of chlorophyll synthesis ability.

Thus, the influences of various metabolic inhibitors on the development of chlorophyll synthesis ability were investigated by fluorometry. Lipid-accumulated cells were cultured in the inorganic medium for 15 hours in the presence of 6MP, CHI or LIN at various concentrations and

the chlorophyll synthesis ability was assayed. The influence of 6MP is shown in Fig. 7. The inhibition increased with an increase in the concentration, and the synthesis was inhibited to 1/4 of the control at 5×10^{-4} M. Similarly, CHI (inhibitor of protein synthesis on 80S ribosomes) exhibited a strong inhibitory effect in a concentration-dependent manner, as shown in Fig. 8, and synthesis was delayed by more than 15 minutes at 10 $\mu g/ml$. Lincomycin (inhibitor of protein synthesis on 70S ribosomes) delayed the synthesis by 5 minutes at 2, 10, and 20 mg/m*l*, as shown in Fig. 9, and the synthesis was inhibited to about half of the control level at 10 and 20 mg/m*l* even though the cells were irradiated with light for 30 minutes. Thus, it was suggested that the development of chlorophyll synthesis ability is mostly due to cell nuclei-derived cytoplasmic protein synthesis.

Discussion

When *Euglena* is cultured with organic nutrients in the dark, chloroplasts degenerate and become proplastids lacking prolamellar bodies[21,22]. When these dark cultured cells are transferred to an inorganic medium in the dark, chloroplasts are partially formed, and the latent chlorophyll synthesis ability increases. These facts demonstrate that the autotrophic process structurally and physiologically to some extent even in the dark when the organic carbon source is removed. Ten, what was the energy source driving these phenomena? Freyssinet[19] and Schwartbach *et al.*[20] reported that paramylum was not degraded in dark, and light was essential for its utilization. Thus, we focused on lipids, and investigated changes in the lipid content and respiratory activity in cells cultured in inorganic medium with aeration in darkness.

It is very interesting that the proplastid development in the dark is dependent on the degradation of wax ester stored in the cytoplasm, reported by Rosenberg *et al.*[5] in 1964, unlike the light irradiation-induced proplastid development, which is dependent on the degeneration of paramylum (β-1,3-glucan). The curve of lipid reduction in inorganic medium in the dark can be regarded as due to the degradation of wax ester, and the oxygen activity curve was similar to this curve, indicating that the degradation was oxidative (Fig. 5). No lipid degradation was noted in nitrogen, nor was there an increase in chlorophyll synthesis ability, further supporting the oxidative degradation hypothesis.

When the organic carbon source was added to the inorganic medium, and changes in lipids were followed, lipids markedly decreased in all cell groups, showing that the energy required for proplastid development in the dark was supplied by oxidative degradation of wax ester, even when an organic carbon source was present. However, the organic carbon source inhibited chlorophyll synthesis (Fig. 6). Hase's group has already reported that an organic carbon source inhibited chloroplast development in *Chlorella protothecoides*[23]. This phenomenon may be called the glucose effect, but other organic carbon sources that may be used as the substrate of respiration also exhibit a similar effect [24-26]. This phenomenon is very similar to that in *C. protothecoides*, and seen in not only photosynthetic biologics but also widely in microorganisms[27,28].

The site affected by this inhibitory effect of the organic carbon source was investigated using a nucleic acid synthesis inhibitor, 6MP[29], and two protein synthesis inhibitors, CIH that inhibits protein synthesis on 80S ribosomes and LIN that inhibits protein synthesis on 70S ribosomes. All agents inhibited chlorophyll synthesis (Figs. 7–9), suggesting that the organic carbon source had inhibited the nucleic acid synthesis step because if this step in the chlorophyll synthesis process were active, chlorophyll synthesis would have progressed after removal of the organic carbon source, and treatment with

an inhibitor of this process (6MP) should not have affected the progression of chlorophyll synthesis. Therefore, it was demonstrated that nucleic acid and protein synthesis progressed even in the dark when the cells were transferred from the organic to inorganic medium. Further analyses using different carbon sources and inhibitors are required to resolve details of mechanisms to induce the conversion of heterotrophy-to-autotrophy in *Euglena*.

Although oxidative degradation of lipids produces energy and substrates for growth by exposing *Euglena* cells to oxygen, proplastids do not developed when an organic carbon source is present. This inhibition is most likely due to inhibition in steps of synthses of nucleic acids and proteins, as mentioned above. Therefore, removal of the organic carbon source induces disinhibition of the nucleic acid transcription step, and promotes conversion from heterotrophy to autotrophy even in the dark.

Conversion of *Euglena* from heterotrophy to autotrophy has previously been considered to be induced by light irradiation, but it was demonstrated that the conversion progresses regardless of light when adequate preculture conditions are selected[30]. When *Euglena* was transferred to medium containing an organic carbon source and cultured in the dark, chloroplasts degenerated and became proplastids lacking prolamellar bodies[31–33]. As these cells were subjected to stationary culture, lipids (wax ester) markedly accumulated in the cytoplasm, and the latent chlorophyll synthesis ability (presented as the relative amount of chlorophyll synthesized when placed under a light source) was low. When the cells were transferred to inorganic medium in darkness and aerated, the chlorophyll synthesis ability increased, and oxidative degradation of accumulated lipids occurred. This synthetic ability was inhibited by the addition of an organic carbon source, and nucleic acid and protein synthesis inhibitors also inhibited it.

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