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Studies of Developing Proplastids in Dark-Grown Cells of *Euglena gracilis* Z: A Role of Lipid (wax)

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Euglena gracilis Z cells were cultured in Hutner's sugar-containing medium without agitation in darkness for 4–5 days. The cells accumulate large amount of lipid (wax). When these cells were transferred to an inorganic medium and aerated with 2% CO_2 in air in darkness, the lipid markedly decreased with a concomitant decrease in the oxygen-uptake activity of the cells. The dark-grown wax-rich cells contained extremely degenerate proplastids with the girdle thylakoid located close to the envelope. These proplastids expanded, and prolamellar bodies and some prothylakoids were formed within 6 days after transfer to the inorganic medium in the dark with aeration. Fluoroacetate, malonic acid, antimycin A and 2,4-dinitrophenol suppressed the expansion of proplastids and the concomitant development of structure. It was inferred that oxidative metabolism of lipid provides materials and energy for the dark development of proplastids in dark-grown *E. gracilis* Z cells transferred to the inorganic medium.

Introduction

Previous study[1] has shown that when *Euglena* cells are grown in Hutner's sugar-containing medium in the dark without agitation, they accumulate large amounts of wax, in addition to paramylum, and their proplastids contain no internal structure except for a single prothylakoid close to the envelope. When these wax-rich cells are transferred to an inorganic medium containing an ammonium salt with aeration in darkness, the proplastids expand, and a prolamellar body and some additional prothylakoids are formed in about one or two days, concurrent with the disappearance of wax.[2] It was inferred that the wax accumulated in the semi-anaerobic dark-grown *Euglena* cells is oxidatively metabolized under subsequent aerobic conditions to provide energy and substrates for the dark development of proplastids. This inference was supported with the *bacillaris* strain and the W_3BUL mutant.[3]

In the present work, the details of the dark processes of proplastid development were examined in wax-consuming *Euglena gracilis* Z cells which were grown under aerobic culture conditions in the dark in an inorganic medium after 4 days preincubation in a sugar-containing medium in the dark without agitation.

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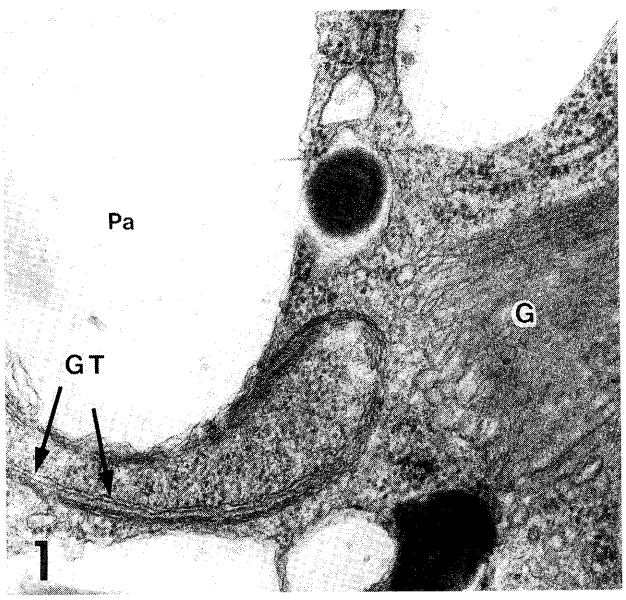
Material and Methods

Euglena gracilis Klebs strain Z Pringsheim was obtained from the Department of Microbiology, Tokyo Medical College, and has been maintained in Department of Life Sciences, Nippon Sport Science University.

Culture conditions

Cells of *Euglena gracilis* Z, maintained in darkness for many generations were used in the present work. They were cultured in darkness at 28℃ without agitation in Hutner's sugar-containing medium,[4] containing 5 μg per liter vitamin B_{12}, unless otherwise mentioned.[2] Aseptic conditions were maintained throughout all procedures and all manipulations were done under a green safelight.[5] The cell number was counted with a Fuchs-Rosenthal plate after a drop of 50% formalin was added to a cell suspension.[2] At 5 day intervals about 50 ml of a 5 day culture containing $1-2\times10^6$ cells·ml^{-1} was added to 350 ml of fresh medium in a 500 ml Erlenmeyer's flask. The cell multiplication under these conditions ceased after about 4 days and the cells harvested on the fifth day showed the highest lipid content, as was the case with *Euglena gracilis* Z.[1] These cells were washed twice with the inorganic medium (complete inorganic medium or resting medium) used in the subsequent experiments, and resuspended in the same medium at a concentration of $2-4\times10^5$ cells·ml^{-1}. The cell suspension was placed in a flat oblong vessel, and aerated with air containing 2% CO_2 at 26℃ in darkness. When the culture was illuminated, a bank of 20 W cool-white fluorescent tubes (Fishlux; Toshiba, Tokyo) was used, and the light intensity was 3 μE·m^{-2}·S^{-1} (about 0.2 Wm^{-2}) at the surface of the culture vessel. Other culture conditions



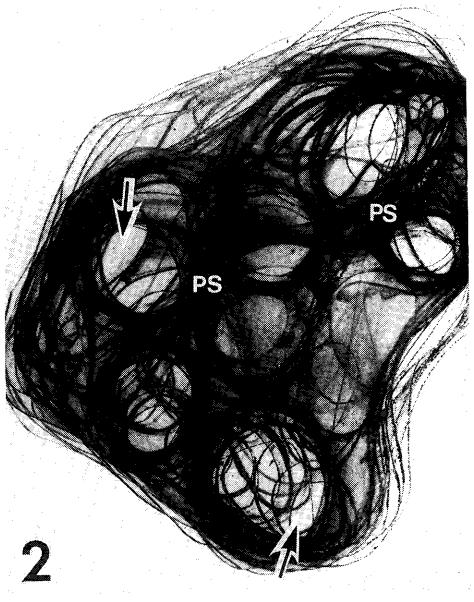


Fig. 1. A section of the proplastid in dark-grown cells (×51000). The arrows show girdle thylakoid. GT: girdle thylakoid. Note ribosomes in the proplastid, which are smaller than those in the cytoplasm.

Fig. 2. Three-dimensional model of the proplastid with several cavities. PS: proplastid stroma. The arrows show cavity.

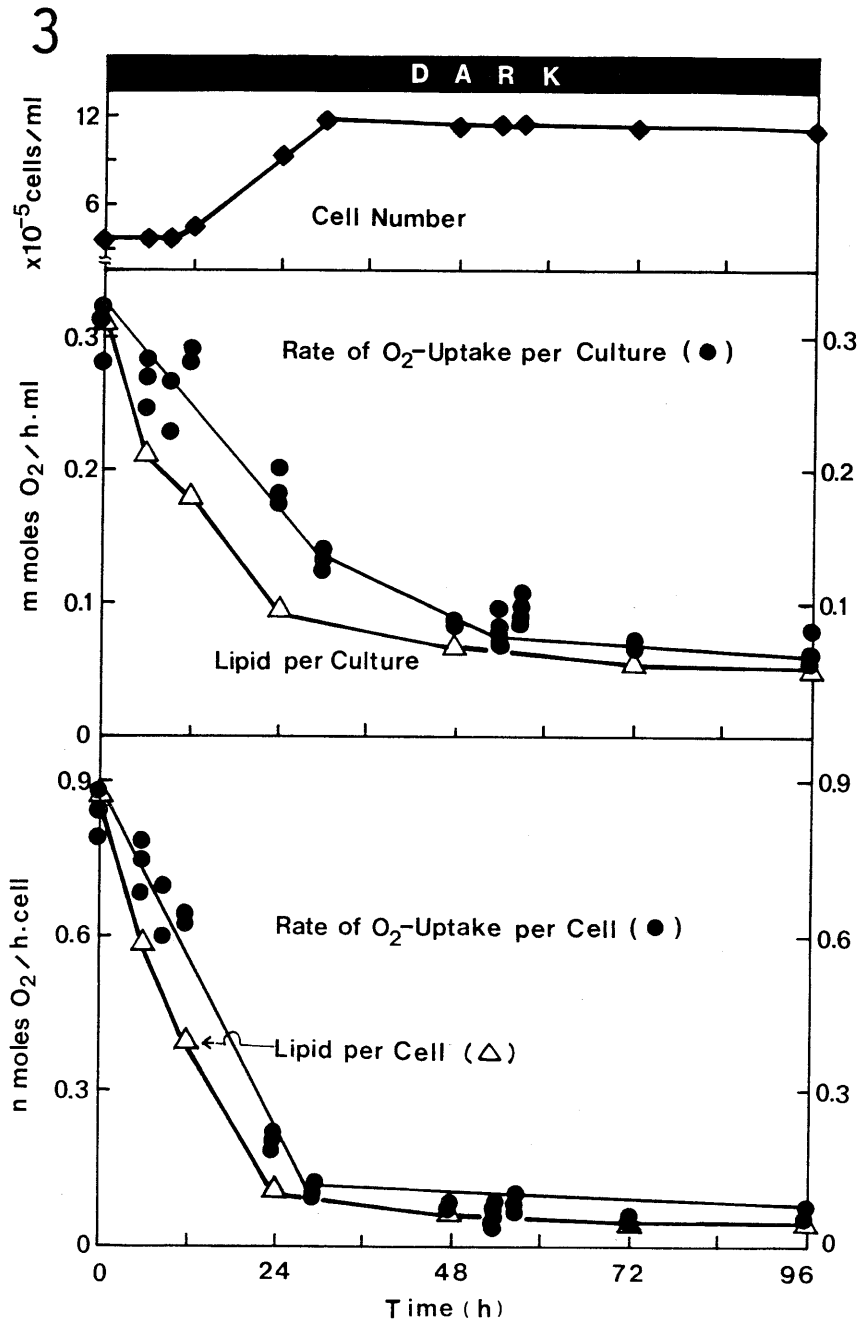
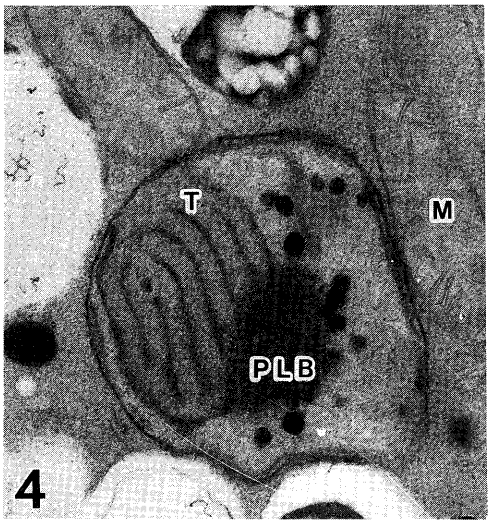


Fig. 3. Time courses of cell division and decrease in total lipid after dark-grown wax-rich cells were transferred to the complete inorganic medium with aeration, respectively. Closed square shows cell number. The circles show the rate of oxygen uptake activity represent per culture or cell, and triangles represent amount of lipid per culture or cell in the dark.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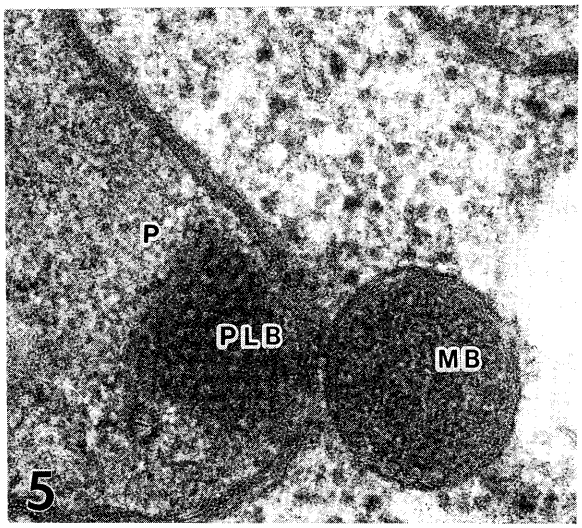
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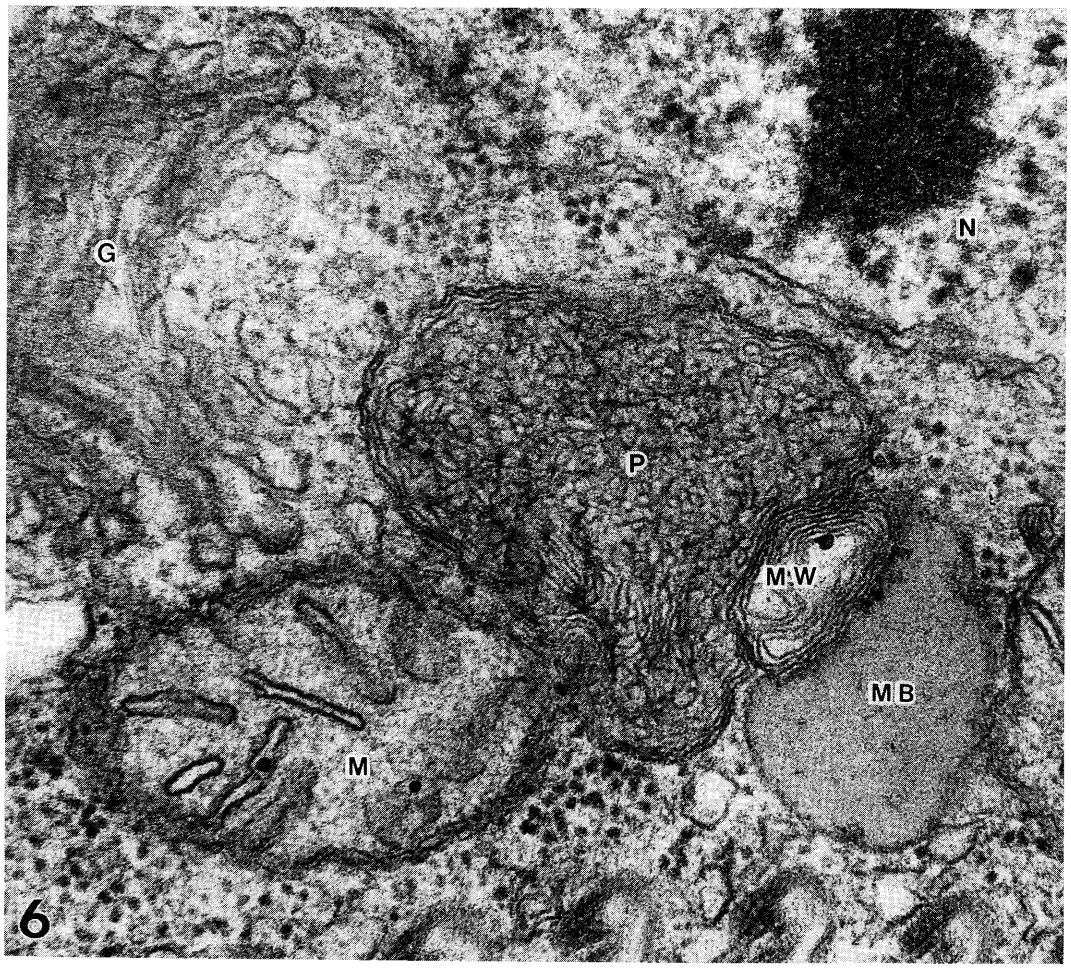
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were as described previously.[1] The compositions of complete inorganic medium and resting medium used as the inorganic media were as follows. Complete inorganic medium:[2] 1 g KH_2PO_4, 1 g $(NH_4)_2HPO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.026 g $CaCl_2 \cdot 2H_2O$, 3.3 mg $FeSO_4 \cdot 7H_2O$, 0.1 mg vitamin B_1, 0.5 μg vitamin B_{12} and 1 m*l* trace element solution per liter, pH 6.8.

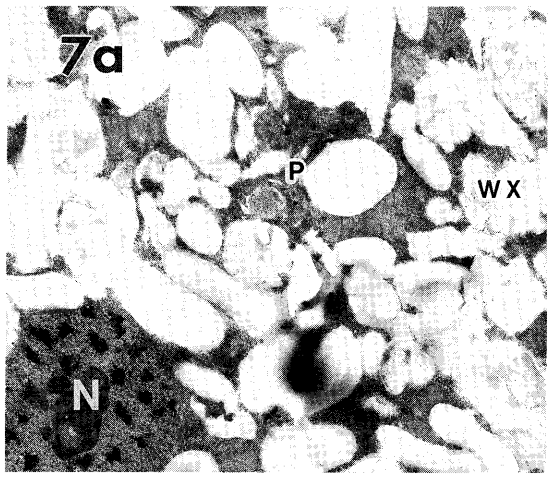
Lipid was extracted with chloroform-methanol (2 : 1, v/v), and after evaporating to dryness, weighed as described previously.[6] The rate of oxygen uptake by cells was determined with a Clark type oxygen electrode (Rank Brothers, London).[8] All the above measurements were repeated at least three times, and similar results were obtained.

The methods of electron microscopy were the same as those described previously.[2]

Results and Discussion

Euglena gracilis Z cells are harvested in the stationary phase of dark-organotrophic growth in a culture grown without agitation in Hutner's sugar-containing medium, they contained rich lipid and proplastids showing no internal structure except for a thylakoid close to the envelope, called a girdle thylakoid (the arrows show in Fig. 1). Figure 2 shows a part of the model of a proplastid constructed by superinposition of twenty-three consective sections, through an entire proplastid. The arrows show the cavity of proplastid in the dark (Fig. 2). Another consequence of these findings is that the proplastid number per cell may be smaller than assumed. This is similar to the findings regarding mitochondria in *Euglena* (see Osafune *et al.*).[7] The many single mitochondria seen in a given section were found to be part of one or two giant mitochondria. The definate number of proplastids per cell was not established. It can, however, be definitely stated that there are more than one. These morphologies are virtually the same as those observed previously with *bacillaris* cells cultured with shaking culture conditions in the dark.[2] When these cells are transferred, after washing, to the complete inorganic medium in the dark with aeration of 2% CO_2 (v/v) in air, the lipid content of cells rapidly decreased to a low level as shown in Fig. 3. When the same dark-grown wax-rich cells were transferred to the resting medium lacking utilizable carbon and nitrogen, the rate of decrease of the lipid was as rapid as in the complete inorganic medium during an initial period of about six hours, but became significantly slower, and the stationary level reached after about three days was twofold higher than in the complete inorganic medium (data not shown). The cell division occurred more actively in the complete inorganic medium than in the resting medium, but the cells ceased to divide about 36 h after the transfer in both cases (Fig. 3). In the dark for a period of 24–48 h, the proplastid expanded, and prolamellar bodies and some peripheral prothylakoids were formed. Figures 4–6 are electron micrographs showing developing proplastids and other organelles in the dark-grown wax-rich cells subsequently incubated in complete inorganic medium in darkness with aeration for 144 h. The rudimentary pyrenoids (called propyrenoids), whose dense matrices were distinguishable from the neighboring stroma, developed in association with the prolamellar body localized in the peripheral region of the proplastid, as seen in Fig. 4. The incidence of occurence of propyrenoids varied greatly with

Figs. 4–6. Electron micrographs of the proplastids in cells obtained 144 h after the transfer of dark-grown wax-rich cells to complete inorganic medium in darkness with aeration. ×50000, ×65000, ×70000
See text for further explanations of these electron micrographs.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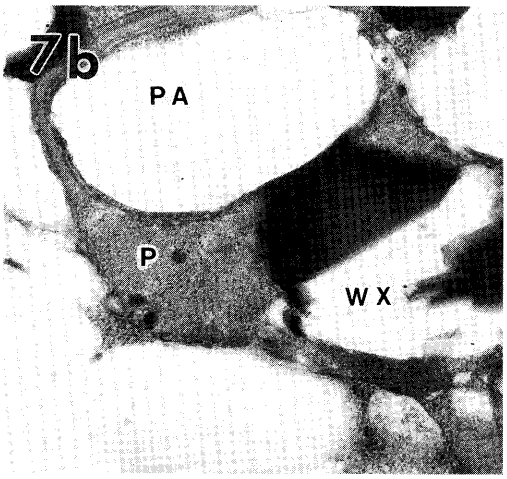
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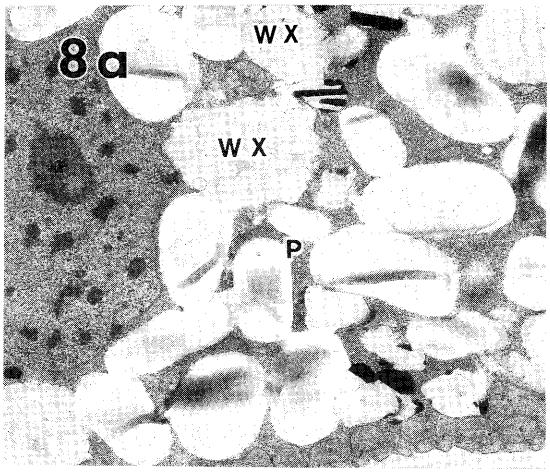
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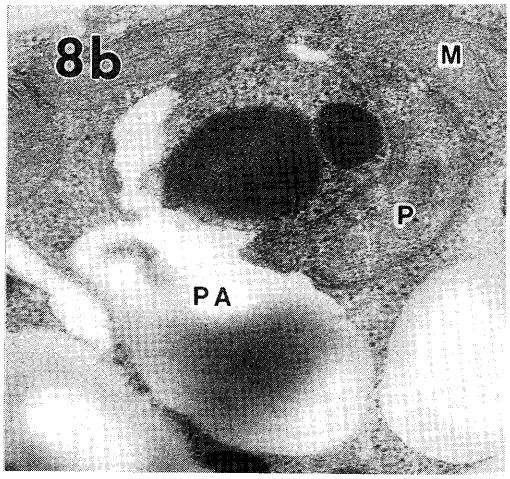
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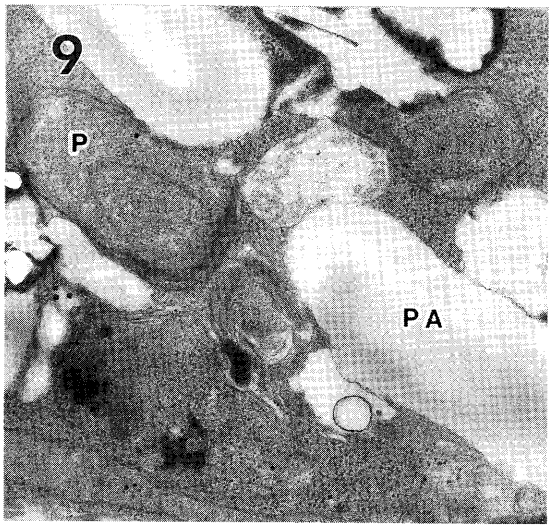
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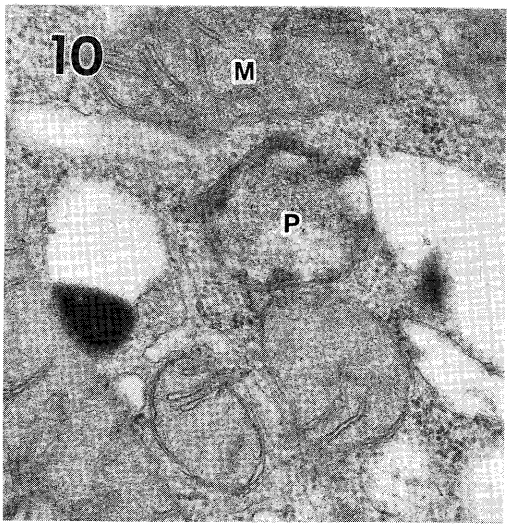
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different batches of cells and among different proplastids in the same cell. As maybe seen from Fig. 4, the propyrenoid contained prothylakoids of different quantities and orientations. Some prothylakoids showed a parallel pattern, while others were convoluted thylakoids (data not shown). A large part of the prothylakoids close to the plastid envelope was composed of a single membrane, but in a region near the propyrenoid and prolamellar body this became paired as seen in Fig. 4. It is further seen (in Fig. 6) that in parallel with the paired prothylakoids, small single prothylakoids were present adjacent to the plastid envelope. The small single prothylakoid appeared to join the pre-existing single thylakoid to form the paired portion. The peripheral prothylakoid present in the vicinity of the prolamellar body showed a U-turn at the surface of the prolamellar body (Fig. 4). Figure 6 shows that a mitochondrion, microbody-like entity and membrane whorl were in close contact with the proplastid, in which a prolamellar body was developed. The microbody-like entity was also frequently in contact with the developing proplastid as seen from Fig. 5 and 6. As seen from Fig. 6, the prolamellar body in some cells contained membrane whorls. The most striking difference in proplastid development between the wax-rich cells transferred to complete inorganic medium and the cells cultured with shaking culture conditions (containing little wax) and transferred to the resting medium was the formation of rudimentary pyrenoids in the former cells. In the cells grown in darkness with shaking culture conditions and transferred to resting medium with carry over of culture medium no rudimentary pyrenoid was formed in darkness; instead the plastid structures once developed started to degenerate on long incubation of cells in resting medium, as reported previously.[8] This appears to be due to depletion of carbon and/or nitrogen source. As suggested above, the oxidation products from wax would be metabolized, at least in part, through the TCA cycle with collaboration of the glyoxylic acid cycle and changed into organic acids (yielding amino acids) and glucose derivatives via gluconeogenesis.

Figures 7–10 show severe suppression of the development of proplastid internal structures caused by the addition of fluoroacetate (Fig. 7), malonic acid (Fig. 8), antimycin A and 2,4-dinitrophenol (Fig. 10). Virtually no wax was detected in sections of the cells treated with antimycin A and 2,4-dinitrophenol (Figs. 9–10), suggesting that the oxidative degradation of wax was not suppressed by these inhibitors. However, antimycin A (10 μg/ml) was found to show about 40% inhibition of the oxygen-uptake by 0 h-cells and about 70% inhibition with 72 h-cells (data not shown). It is inferred that products of oxidative degradation of wax enter TCA cycle and the respiratory chain producing ATP. Figures 7a–b indicate that a fluoroacetate-treated cell contains

Figs. 7–10. Inhibitory effects of fluoroacetate, malonic acid, antimycin A and 2,4-dinitrophenol on the development of the proplastids in dark-grown wax-rich *Euglena* cells were transferred to the complete inorganic medium in darkness. The inhibitor was added immediately after the transfer, and after 144 hours, cells were taken out for electron microscopic observations. Fig. 7a. The effect of fluoroacetate. (×7500). Wax was not suppressed by these inhibitors, although the prolamellar body was absent. Wax was crystallized during the preparation of cell samples for electron microscopy, and was mostly removed by organic solvents leaving behind scars of the crystals. Fig. 7b. A higher magnification of the proplastid in fluoroacetate. (×40000). Fig. 8. Effect of malonic acid (×7500). Fig. 8b. A higher magnification of the proplastid in malonic acid. (×40900). Fig. 9 shows effect of antimycin A. Note that any amount of wax remaining and undeveloped proplastid in Figs. 9–10. (×40000). Fig. 10. Effect of 2,4-dinitrophenol. (×40000). N: nucleus.

a great deal of undegraded wax. Fluoroacetate suppressed the decrease of the lipid. These results support the inference that wax is utilized as a source of carbon and energy for the early development of proplastids in dark-grown *Euglena gracilis* Z cells transferred to the complete inorganic medium with aeration in darkness. This suggests that products of lipid degradation enter TCA cycle. Figure 3 shows the rate of oxygen-uptake of dark-grown cells after the transfer to the complete inorganic medium with aeration. This is consistent with the observation that the rapid decrease in rate of oxygen-uptake was parallel with the rate of lipid breakdown during the initial period of several hours, as may be seen from Fig. 3.

The degradation of wax is induced by oxygen, probably the alpha-oxidation of fatty acids being involved as a limiting step in an initial phase. The oxidation products of lipid (wax) and their derivatives are used, as such or after being converted into nitrogenous compounds, for the dark development of proplastids.

Acknowledgments

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