Effects of illumination and inhibitors on the retention of intracellular glycerol in *Dunaliella tertiolecta*

Shuhei FUJII, Shin NAKAYAMA
Shiro MANTANI, Ryoichi YAMAMOTO

ABSTRACT

When *Dunaliella tertiolecta* cells were incubated in a 0.5 M NaCl medium at 25℃ in the dark, the cell volume gradually decreased and the intracellular glycerol leaked out from cells. The application of 10 μM of 3 – (3,4 – dichlorophenyl) – 1,1, – dimethylurea (DCMU) did not affect the retention of intracellular glycerol, although the application severely inhibited activities of photosynthesis and respiration in *D. tertiolecta*. N,N' – dicyclohexylcarbodiimide (DCCD) at 10 μM or vanadate at 100 μM severely inhibited activities of photosynthesis and respiration in *D. tertiolecta* as well as DCMU or sodium azide at 1 mM. However, different from DCMU or sodium azide, the application of DCCD or vanadate caused the leakage of intracellular glycerol. These results imply that activities of membrane–bound ATPases play an important role for the retention of intracellular glycerol, and also that the incubation in the dark for a few days might cause some changes in integrity of plasma membrane or interfere of membrane–bound ATPase activities, resulting in the leakage of intracellular glycerol.

Key words : *Dunaliella tertiolecta* ; glycerol ; illumination ; DCCD ; vanadate ; retention

INTRODUCTION

It is well known that glycerol acts as the osmoregulative substance in *Dunaliella*, which is micro –green alga belonging to Volvocales. To understand the osmoregulation of *Dunaliella*, mechanisms on the retention of intracellular glycerol should be also understood. Gimmler et al. (1988) reported that the plasma membrane of *Dunaliella* has low permeability for glycerol different from that of higher plant and other algae, although the reason why its membrane is low permeable for glycerol has not been well understood. So far, the intracellular glycerol has been reported to leak temporarily from the cells when *Dunaliella* cells are subjected to down shock (Gilmour et al. 1984, Fujii et al. 1985, Zidan et al. 1987), raised temperature (Wegmann et al. 1983) or low concentration of external Ca^{2+} (Fujii 1993).

In this report, we investigated effects of illumination and inhibitors on the retention of intracellular glycerol in *Dunaliella tertiolecta*. The obtained results indicate that illumination keeps low permeability for glycerol. The role of ATPase in the retention of intracellular glycerol was discussed.

MATERIALS AND METHODS

a) Growth condition

Dunaliella tertiolecta was axenically precultured in a 200 ml culture flask with 100 ml of a 0.5 M NaCl medium under continuous illumination with fluorescent lamps (20 w/m^2 at the surface of the flask) at 25℃ bubbled with 3 % CO_2 enriched air. Cells at the stage of late logarithmic phase were harvested by centrifugation (1,500 × g, 10 min). The pellet was resuspended in a 0.5 M NaCl medium, and then incubated at 25℃ by standing in a Erlenmeyer flask in the light with fluorescent lamps (20 w/m^2 at the surface of the flask) or in the dark.

b) Measurements of glycerol, starch and cell volume.

Glycerol content was determined enzymatically as previously described (Fujii et al. 1984). In brief, the sample solution of 20 μl was added to 3 ml of enzyme solution, and after incubation of the mixture at 37℃ for 15 min, the absorbance was measured at 545 nm and the glycerol content was estimated as μg per 10^6 cells. Starch content was also determined enzymatically by use of F–kit starch (Boehringer Mannheim Yamanouchi, Tokyo). About 100 mg of the lyophilized cells was put into a 100 ml Erlenmeyer flask and mixed with a mixture solvent of 20 ml dimethyl sulfoxide and 5 ml hydrochloric acid (8 M). Then, the suspension was warmed in a water bath at 60℃ for 30 min to solubilize starch. After about 50 ml of deionized water was added, the solution was adjusted to pH 4 ~5 with 5 M NaOH. The solution was filled up to 100 ml by adding deionized water. The solution of 0.1 ml was mixed step by step with an enzyme solution including either one of amyloglucosidase, hexokinase or G–6–P dehydrogenase, and incubated at 55℃ for 15 min for amyloglucosidase, at 25℃ for 3 min for hexokinase and at 25℃ for 15 min for G–6–P dehydrogenase. The absorbance of the solution was measured at 340 nm. The starch content was expressed as μg per 10^6 cells. To measure the cell volume of *D. tertiolecta*, cells were photographed through a microscope. Then, the mean cell volume was calculated on the assumption that the cell shape is ellipsoidal.

c) Measurement of photosynthetic and respiration rate

D. tertiolecta cells incubated in a 0.5 M NaCl medium were spun down by centrifugation (1,500 x g, 10 min). The cell pellet was resuspended in a 0.5 M NaCl medium with either of inhibitors such as vanadate (100 μM), DCCD (10 μM), DCMU (10 μM) and sodium azide (1 mM), and incubated for 1 hr at 25℃ in the light with fluorescent lamps (20 w/m^2 at the surface of the flask). Then, the respiration and photosynthesis in *D. tertiolecta* cells were measured by use of an O_2 electrode (Model 5300, Yellow Spring Inst., OH, USA) at 25℃ in the presence of 40 mM $NaHCO_3$. Cell numbers

were determined by counting formaldehyde–fixed cells in a haemocytometer. The rate of respiration and photosynthesis was expressed as μmol O_2 per 10^6 cells.

RESULTS AND DISCUSSION

It is well known that *Dunaliella* cells are able to retain glycerol against high concentration gradients ($>10^4$) between the intracellular space and the medium (Wegmann, 1986). However, effect of illumination on the retention of intracellular glycerol is not known. It was examined whether or not *Dunaliella* cells is able to retain intracellular glycerol even in the dark as well as in the light. Figure 1 showed changes in the content of intracellular glycerol and starch, and the mean cell volume when *D. tertiolecta* cells were incubated in the dark for 2 hr. During the incubation for 2 hr in the dark, the content of intracellular glycerol was almost constant. As well, the content of extracellular glycerol was constant (data not shown). On the other hand, the content of starch gradually decreased, probably due to the consumption as respiratory substrate. During the incubation, the average cell volume was almost constant (Fig. 1). These results suggest that *Dunaliella* cells can retain glycerol for a few hours even in the dark. Next, we examined changes in the content of glycerol and starch, and the mean cell volume when the cells were incubated for 3 days in the dark.

As seen in Fig. 2, the content of intracellular glycerol gradually decreased while the extracellular content of glycerol increased. Moreover, the content of starch showed faster decrease than that of the intracellular glycerol. Starch seemed to be consumed as the respiratory substance. The mean cell volume appeared to decrease in parallel with the decrease in the content of intracellular glycerol, implying that the intracellular concentration of glycerol was almost kept to be constant. In addition, judging from the results that the total content of glycerol was almost constant. It was initially, 12.5 ± 0.5 ; 1 day after the incubation

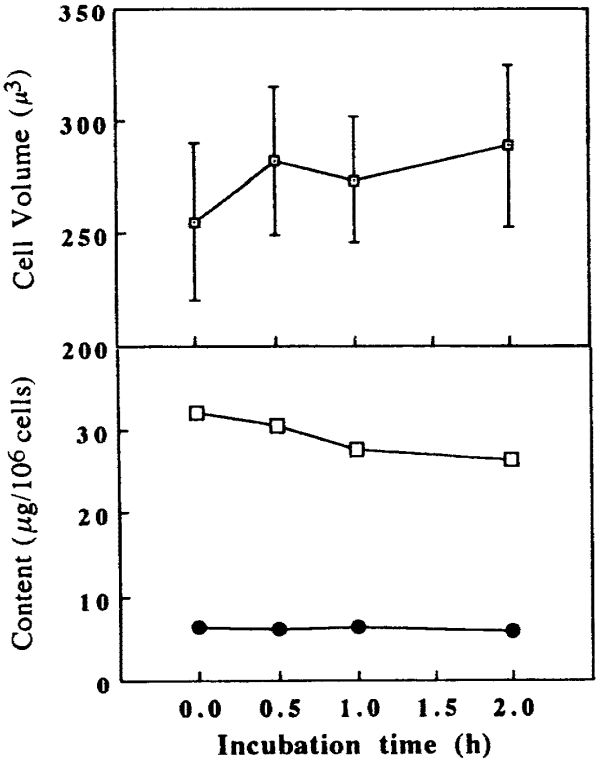

Fig. 1 Effects of the dark on cell volume, content of intracellular glycerol (●) and starch (□) in *D. tertiolecta*. Cells were incubated in a medium with 0.5 M NaCl in the dark by standing at 25℃. Glycerol and starch were measured enzymatically as described in Materials and Methods. Data represent means of three experiments (±S.D.).

in the dark, 11.2 ± 0.6 ; 2 days after the incubation in the dark, 11.4 ± 0.5 ; 3 days after the incubation in the dark ; 11.8 ± 0.4. The decrease in the content of intracellular glycerol was concluded to be due to the leakage into the medium, which caused the decrease in the cell volume. It is not likely that glycerol is utilized as the substance for reparation in the dark. It has been reported that *Dunaliella* cells can produce glycerol by degradation of starch to osmoregulate even in the dark when the cells are subjected to the hypertonic shock (Borowitzka et al. 1977 ; Gimmler et a. 1981). When *D. tertiolecta* cells was incubated under an isotonic condition in the dark, glycerol formation from starch did not appear to compensate the lost glycerol (Fig. 2).

The extracellular glycerol in *Dunaliella* has been observed to occur under normal conditions, probably due to leak from aging and dead cells (Hellebust, 1965). Moreover, there are some reports about the leakage of intracellular glycerol when the cells are subjected to rapid changes in growth conditions such as increased temperature (Wegmann et al., 1980), down shock (Gilmour et al. 1984, Fujii et al. 1985, Zidan et al. 1987), or low concentration of external Ca^{2+} (Fujii 1993). Recently,

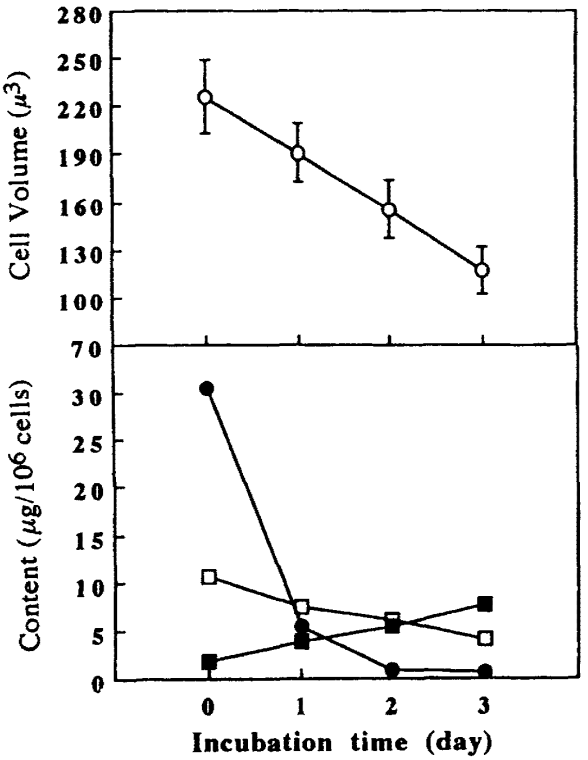

Fig. 2 Effects of the dark on cell volume, content of intracellular glycerol (□), extracellular glycerol (■) and starch (●) in *D. tertiolecta*. Cells were incubated in a medium with 0.5 M NaCl in the dark by standing at 25℃. Glycerol and starch were measured enzymatically as described in Materials and Methods. Data represent means of three experiments (±S.D.).

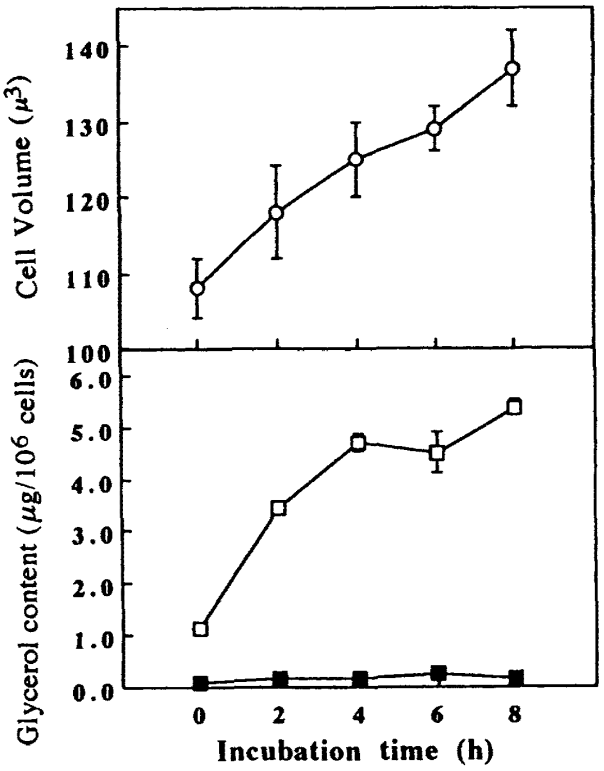

Fig. 3 Effects of the light on cell volume, content of intracellular glycerol (□) and extracellular glycerol (■) in *D. tertiolecta*. Cells, which was incubated in a medium with 0.5 M NaCl in the dark by standing for 4 days, were transferred to the light with fluorescent lamps (20 w/m^2 at the surface of the flask). Glycerol was measured enzymatically as described in Materials and Methods. Data represent means of three experiments (±S.D.).

Shariati & Lilley (1994) reported that about 10 % of intracellular glycerol in *D. tertiolecta* was released to the external medium by the treatment of electroporation at constant osmotic pressure.

The release of glycerol caused by the incubation of *Dunaliella* cells in darkness mainly seems to be due to not the rupture of cells but any change in the integrity of plasma membrane involved in keeping the low permeability for glycerol. Almost of *Dunaliella* cells incubated in the dark for 4 days was motile by microscopic observation although its motion was very slow compared with cells in the light (data not shown). Moreover, as shown in Fig. 3, *Dunaliella* cells started to synthesize glycerol when exposed to the light. In spite of the rapid formation of glycerol in the light, the leakage of glycerol was not observed at all (Fig. 3). The mean cell volume also appeared to recover in parallel with the glycerol formation when cells were transferred to the light (Fig. 3). The characteristic of plasma membrane in *Dunaliella* which has a low permeability for the intracellular glycerol as pointed out by Gimmler et al (1988), is considered to be the case in the light. Thus, illumination seems to be effective for the retention of intracellular glycerol in *D. tertiolecta*.

Next, we examined effects of various inhibitors on the retention of intracellular glycerol in *D. tertiolecta*. The application of 10 μM DCMU did not influence the retention of intracellular glycerol (Fig. 4), although the application severely inhibited activities of photosynthesis and respiration in *D. tertiolecta* (Fig. 5). The application of 1 mM sodium azide also did not affect the retention of intracellular glycerol (data not shown). On the other hand, the application of 10 μM DCCD or 100 μM vanadate caused the leakage of intracellular glycerol (Fig. 4), although the application

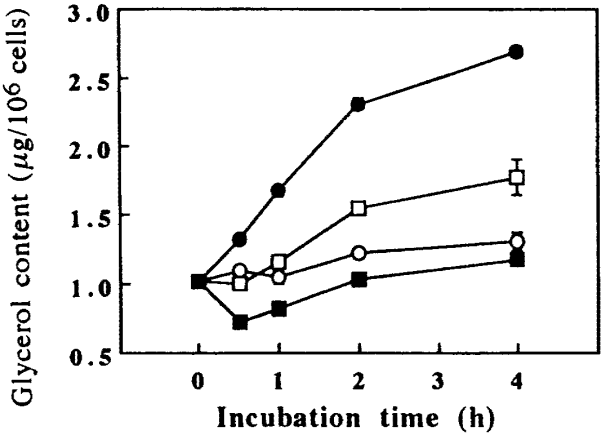

Fig. 4 Effects of various inhibitors on the content of extracellular glycerol in *D. tertiolecta*. Cells were incubated in the light with or without either one of inhibitors in the light by standing at 25℃. (○) Control ; (●) 100 μM Vanadate ; (□) 10 μM DCCD ; (■) 10 μM DCMU. Glycerol was measured enzymatically as described in Materials and Methods. Data represent means of three experiments (±S.D.).

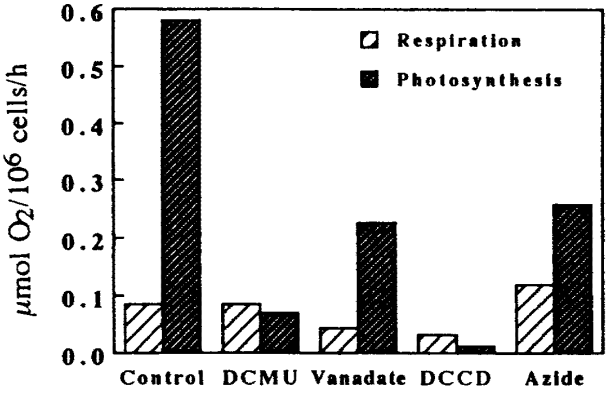

Fig. 5 Effects of various inhibitors on the rate of respiration and photosynthesis in *D. tertiolecta*. Cells were preincubated in the light with or without one of either inhibitor in the light by standing at 25℃ for 1 h. Then, the rate of respiration and photosynthesis was measured by use of an O_2 electrode at 25℃.

of DCCD or vanadate inhibited the activities of respiration and photosynthesis as well as DCMU or azide (Fig. 5). Thus, it is likely that the leakage of glycerol caused by the application of DCCD or vanadate is not related with inhibitions of respiration and photosynthesis, but any inhibition of activities in membrane-bound ATPases. These results imply that activities of membrane-bound ATPases play an important role for the retention of intracellular glycerol.

It is unclear how darkness causes changes in the permeability of plasma membrane for glycerol in *D. tertiolecta*. However, it is considered that the incubation in the dark for a few days provoke some changes in integrity of plasma membrane or interfere of membrane-bound ATPase activities, which resulted in release of intracellular glycerol to the external medium.

Acknowledgment

This research was partially supported by a grant from Tezukayama Academy.

References

Borowitzka L.J., Kessley D.S. & Brown A.D. (1977) The salt relations of *Dunaliella*. Further observations on glycerol production and its regulation. Archives Microbiology 113 : 131–138.

Fujii S., Yamamoto R. & Takada H. (1984) Contribution of glycerol to osmoregulation in *Dunaliella tertiolecta* under magnesium hypertonicity. The Japanese Journal of Phycology 32 : 300–306.

Fujii S. & Hellebust J.A. (1992) Release of intracellular glycerol and pore formation in *Dunaliella tertiolecta* exposed to hypotonic stress. Canadian Journal of Botany 70 : 1313–1318.

Fujii S. (1994) Effect of Ca^{2+} on the retention of glycerol and amino acids in *Dunaliella tertiolecta*. Plant, Cell and Environment 17 : 227–230.

Gilmour D.J., Hipkins M.F. & Boney A.D. (1984) The effect of decreasing the external salinity on the primary processes of photosynthesis in *Dunaliella tertiolecta*. Journal of Experimental Botany 35 : 28–35.

Gimmler H., Wiedemann C. & Moller E.M. (1981) The metabolic response of the halotolerant green alga *Dunaliella parva* to hypertonic shock. Berichte der Deutschen Botanischen Gesellschaft 94 : 631–636.

Gimmler H. & Hartung W. (1988) Low permeability of the plasma membrane of *Dunaliella parva*. Journal of Plant Physiology 133 : 165–172.

Goyal A. (1989) Intracellular glycerol in *Dunaliella* is depleted by intracellular metabolism in response to hypoosmtoic stress by dilution. FEMS Microbiology Letters 61 : 145–148.

Hellebust J.A. (1965) Excretion of organic compounds by marine phytoplankton. Limnology and Oceanography 10 : 192–206.

Shariati M. & Lilley R.McC. (1994) Loss of intracellular glycerol from *Dunaliella* by electrophoration at constant osmotic pressure : sebsequent restoration of glycerol content and associated volume changes. Plant, Cell and Environment 17 : 1295–1304.

Wegmann K., Ben-Amotz A. & Avron M. (1980) Effect of temperature on glycerol retention in the halotolerant algae *Dunaliella* and *Asteromona*. Plant Physiology 66 : 1196–1197.

Wegmann K. (1986) Osmoregulation in eukaryotic algae. FEMS Microbiology Reviews 39 : 37–43.

Zidan M.A., Hipkins M.F. & Boney A.D. (1987) Loss of intracellular glycerol from *Dunaliella tertiolecta* after decreasing the external salinity. Journal of Plant Physiology 127 : 461–469.