BBA 46270

BIOGENESIS OF CHLOROPLAST MEMBRANES

IX. DEVELOPMENT OF PHOTOPHOSPHORYLATION AND PROTON PUMP ACTIVITIES IN GREENING *CHLAMYDOMONAS REINHARDI* y-1 AS MEASURED WITH AN OPEN-CELL PREPARATION

D. WALLACH, S. BAR-NUN AND I. OHAD
Department of Biological Chemistry, The Hebrew University of Jerusalem (Israel)
(Received November 12th, 1971)

SUMMARY

Chlamydomonas reinhardi y-1 cells are rendered permeable to substrates and cofactors for photosynthetic electron transfer and photophosphorylation by applying low shearing forces in a hypertonic viscous medium. The chloroplast integrity is well preserved in such open-cell preparations which exhibit high and stable photophosphorylation rates for both photosystems and proton pump activity. These activities were measured during greening of dark-grown y-1 cells using open-cell preparations. It was found that photophosphorylation and light-dependent proton uptake are absent in preparations obtained from cells grown in the dark for 5–6 generations which still contained 1–2 μg chlorophyll per 10^7 cells. The activities became measurable following exposure of the cells to the light and increased rapidly reaching a maximal rate per chlorophyll unit 3–4 times higher than that exhibited by light-grown cells after 2–3 h of illumination when the chlorophyll content of the cells has increased only slightly. As chlorophyll continues to accumulate the activity per chlorophyll unit declined and became equal to that usually found in light grown cells. Photosynthetic ATP formation and pH rise do not develop in cells greening in the presence of chloramphenicol which specifically inhibits the synthesis of chloroplast made proteins. Chloroplast membranes formed in the presence of chloramphenicol which are enriched in proteins of cytoplasmic origin resume the light-dependent proton pump activity and photophosphorylation by both photosystems if the cells are further incubated in absence of chloramphenicol. The repair of the inactive membranes requires synthesis of proteins within the chloroplast and does not require light and additional synthesis of chlorophyll or proteins of cytoplasmic origin.

INTRODUCTION

The y-1 mutant of the unicellular alga *Chlamydomonas reinhardi* does not synthesize chlorophyll in the dark. The decline in chlorophyll content of the cells

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS, phenazine methosulphate.

after division in the dark is paralleled by a decline in the quantity of the chlorophyll-containing membranes[1]. Thus, after several generations in the dark, only few vesicular remnants are left from the numerous chloroplast discs which fill up the chloroplast of the light-grown cells.

Transfer of the dark-grown cells to light causes a rather rapid replenishment of the membraneous content of the chloroplast which is completed within a few hours[2]. The ultrastructural changes during this process as well as changes in the level of the pigments, specific lipids and proteins were characterized[2–7]. Based on the effects of specific inhibitors for protein and RNA synthesis on the greening process, regulatory mechanisms for the coordinated production of these components were suggested[4–9]. To acquire further knowledge on the mechanisms of the membrane assembly, a sensitive indicator for differences between membranes synthesized under different conditions seemed necessary.

Photosynthetic activity during the greening process such as O_2 evolution, photooxidation of cytochrome *f*, photoreduction of $NADP^+$, light-dependent pH rise* and phosphorylation have been measured using whole cells or cell homogenates[1,2,7]. Comparison of photosynthetic activities exhibited by whole cells is of limited use because of our inability to affect the internal environment of the cell or chloroplast and thus measure activities of isolated portions of the photosynthetic reaction chain. On the other hand, the procedure used in order to obtain active cell or chloroplast fragments from *C. reinhardi* y-1 cells have been rather severe and have resulted in preparations exhibiting low and unstable activities[7]. This renders comparison of activity of cells at different stages of the greening difficult.

In this work, a mild technique for breaking efficiently the diffusion barriers between the chloroplast discs and the medium (cell wall, plasma membrane and chloroplast envelope) without significantly affecting the chloroplast structure and the thylakoids' integrity, is described. The use of this preparation for the sensitive determination of the ability of chloroplasts to function and for determination of different stages in the chloroplasts development are demonstrated.

MATERIALS AND METHODS

Chlamydomonas reinhardi y-1 cells were grown on a mineral medium containing acetate as a carbon source[1]. For greening experiments, the cells were washed by centrifugation (3000 × *g* for 5 min, 4 °C) and resuspension in the growth medium and then exposed to white fluorescent light as described before[2,4]. Cell concentration was determined by counting in a hemocytometer. In order to obtain photosynthetically active preparations the cells were washed as above and resuspended in one of the following media: (1) 20 mM sodium tricine (*N*-tris(hydroxymethyl)-methylglycine), pH 8.0, 250 mM sucrose, 20 mM KCl, 20 mM sodium ascorbate (pH 8.0) and 1 mg/ml bovine serum albumin. This medium will be further denoted as TSK medium; (2) 20 mM sodium phosphate buffer (pH 8.0), 500 mM mannitol, 20 mM KCl and NaCl each, 2 mM $MgCl_2$, 1 mM $MnCl_2$, 0.1 mg/ml bovine serum albumin, 5% Dextran T40, 2.5% Ficoll; this medium which was modified from the one originally described by Sager and Ishida[12] will be further referred to as Wp

* Light-dependent pH rise measured in whole cells is considered to be an indication of photosynthetic CO_2 uptake[10,11] rather than proton uptake as reported earlier[7].

medium. For measurements of photosynthetic pH rise the cells were washed and resuspended in a medium of the same composition as Wp medium from which the phosphate was omitted and the pH was adjusted to pH 6.5 with NaOH or HCl (Wh medium). The cell suspension was passed through a French Press chilled in ice and operated at pressures as indicated. Throughout the whole procedure, the temperature of all solutions was kept at 4 °C. Photophosphorylation activity was measured in a medium containing 10 mM sodium tricine (pH 8.0), 10 mM NaCl, 7.5 mM potassium phosphate buffer (pH 8.0) containing 33 μCi/mmole [^{32}P]orthophosphate, 2.5 mM of both sodium ADP and sodium AMP (pH 7.0), 5 mM glucose, 10 μg/ml hexokinase, 2 mM $MgCl_2$, 3 mg/ml bovine serum albumin and 0.1 ml of cell homogenate containing up to 30 μg chlorophyll. The final volume was 2 ml. The reaction mixture was preincubated in the dark for 3–5 min at 25 °C in 25-ml erlenmeyer flask in a photosynthetic Warburg apparatus fitted with appropriate holders. The reaction was started by onset of illumination using 100-W Sylvania lamps providing $5 \cdot 10^5$ ergs$\cdot$cm$^{-2}\cdot$sec^{-1}. Unless otherwise specified, the gas phase was air. The reaction was stopped after 4 min by addition of 0.2 ml 30 % trichloroacetic acid. The amount of esterified ^{32}P formed was measured after extraction of the inorganic phosphate as phosphomolybdate complex according to the method of Avron[13]. ^{32}P radioactivity was measured in Bray[14] solution with a Packard Tricarb liquid scintillation spectrometer Model 3320. The esterified ^{32}P of a dark-incubated control sample was always measured and the results substracted from the value of the light-incubated samples. Photosynthetic pH rise was measured as described by Schuldiner and Ohad[7] in a medium containing 100 Mm KCl adjusted to pH 6.3 and with addition of pyocyanine ($5 \cdot 10^{-5}$ M) as cofactor. The electrode tip was shielded from the light source by a piece of aluminum foil. Ferricyanide photoreduction was measured as described by Avron and Shavit[15]. Chlorophyll concentration was measured as described by Arnon[16].

Preparation of samples for observation by electron microscopy

Samples of open-cell preparations were washed in 0.1 M phosphate buffer (pH 7.4) by centrifugation (5000 $\times$ *g* for 2 min) and resuspension 3 times. The pellet was fixed in 2 % OsO_4 in 0.1 m phosphate buffer (pH 7.4) overnight at 4 °C. The fixed material was dehydrated and embedded in epon as described by Luft[17]. Thin sections obtained with an LKB ultrome III, were stained with lead citrate and uranyl acetate according to the method of Reynolds[18] and examined in a Philips EM 300 electron microscope. For freeze etching, open-cell samples were washed by centrifugation (5000 $\times$ *g* for 10 min) in Wp medium containing 10 % glycerol and resuspended in the same medium at a final concentration of 10^9 cells per ml. Droplets of the cell suspension mounted on gold holders were quickly frozen in liquid Freon. The frozen material was fractured and replica of the fracture planes prepared as described by Moor *et al.*[19] using a Balzers freeze etching apparatus.

All reagents used throughout this work were of analytical grade. Hexokinase (Boehringer, Mannheim, Germany) was dialyzed at 4 °C overnight against three exchanges of 100 vol. of 1 % glucose solution. Bovine serum albumin (Calbiochem Co., Los Angeles, U.S.A.) was freed from lipids as described by Chen[20]. Chloramphenicol, 99 % pure was a gift from Abic Ltd, Tel Aviv and cycloheximide was purchased from Sigma Chem. Co., St. Louis, U.S.A. 3-(3,4-Dichlorophenyl)-1,1-

dimethylurea (DCMU) was a gift from DuPont De Nemours U.S.A. Dextran T40 and Ficoll were obtained from Pharmacia, Uppsala, Sweden. [^{32}P]Orthophosphate free of pyrophosphate was prepared and purified for us by Nuclear Research Center, Negev, Israel. Phenazine methosulphate (PMS) was obtained from Sigma Chem. Co., St. Louis, U.S.A. Diquat was obtained from Plant Protection Ltd, Yalding U.S.A. Ferricyanide was purchased from Johnson of Hendon Ltd, London, England, and pyocyanine perchlorate was obtained from Mann Research Labs, N.Y., U.S.A.

RESULTS

Due to its tough cell wall and the size and shape of its plastid it is not possible to obtain intact chloroplasts from *Chlamydomonas* cells in good yield. Homogenization techniques such as grinding with sand[21] or pressuration by French Press[7] have to be used in order to break open these cells. Cells suspended in hypotonic or isotonic media can be efficiently broken by passing through a French Press operated at high hydrostatic pressure. In such preparations, the chloroplasts are heavily fragmented to pieces of varying size and only a very small percentage of the resulting particles retain the similarity to an intact chloroplast. Such preparations, however, exhibit photosynthetic electron transfer activities[1,2] and low and variable photophosphorylation[7]. Cell homogenates prepared in TSK medium at 2300 lb/inch^2 show phosphorylation rate of 10–20 μmoles ATP per mg chlorophyll per h with ferricyanide and diquat and 20–60 μmoles ATP per mg chlorophyll per h with pyocyanine as cofactors. The stability of such a preparation is rather poor. The homogenate does not lose activity if stored in the cold for up to 1 h in TSK medium containing 20 mM ascorbate but the activity declines rapidly when the homogenate is incubated in the phosphorylation medium (Fig. 1, compare with Fig. 6). Additions of different concentrations of $MgCl_2$, bovine serum albumin or carrying out the whole procedure of preparation and assay under anaerobic conditions did not significantly improve the activity of such homogenates or stabilize them.

The use of a lower hydrostatic pressure during the homogenization in TSK medium seems to improve the phosphorylation activity. Lowering the pressure from 4000 lb/inch^2 to 500 lb/inch^2 increased the activity from about 4 to 45 μmoles ATP per mg chlorophyll per h, in a given preparation. However, at 500 lb/ich^2 only about 40 % of the cells were broken as estimated by counting in the phase contrast microscope the cells which have lysed upon dilution in distilled water.

To find proper conditions for pressuration such as to cause a minimal damage to the chloroplast, and affect the majority of the cells, a method was required in order to assess the efficiency of pressuration and degree of damage to the cells. For this, advantage was taken of the fact that the light-induced pH rise as exhibited by intact cells differs from that of open cells or chloroplast fragments preparation. In intact cells, the response to illumination is not immediate; it is enhanced by repeated cycles of illumination (conditioning) and completely inhibited by 100 mM KCl[7]. On the other hand, in open cells, the response is immediate (Fig. 2) and maximal on the first illumination, and it is stabilized by KCl. Thus, pressuration conditions were sought such as to obtain an immediate light-induced pH rise in the presence of KCl. It was found that cells pressurised at low pressure (100 lb/inch^2) in our isotonic medium (0.25 M mannitol) did not exhibit a light-induced proton

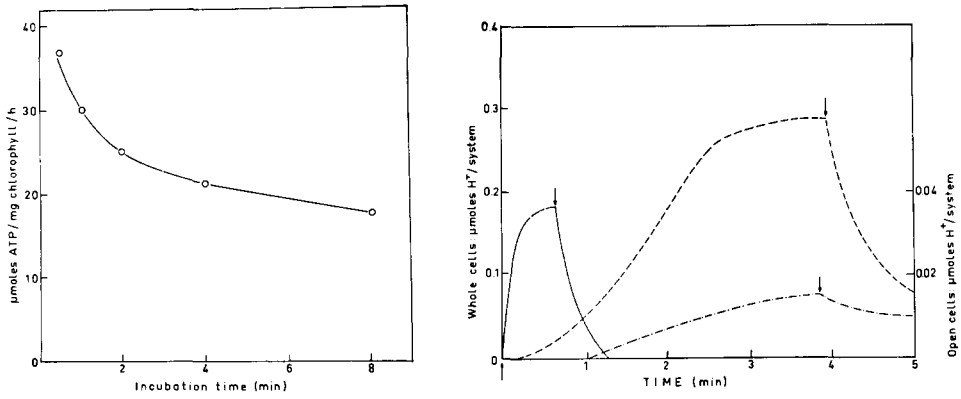

Fig. 1. Decline of the photophosphorylation activity of homogenates prepared in TSK medium as a function of incubation time in the hypotonic reaction mixture. Light-grown cells were suspended in TSK medium at a final concentration of 10^8 cells per ml and were broken open by passing through a French Press operated at 2300 lb/inch^2. Samples of 0.1 ml pressurate containing 27 μg chlorophyll were added to the reaction mixture as described in Materials and Methods and exposed to the light without preincubation. Pyocyanine ($5 \cdot 10^{-5}$ M) with addition of ascorbate ($2 \cdot 10^{-2}$ M) were used as cofactors.

Fig. 2. Kinetics of light-dependent pH rise exhibited by whole cells and open-cell preparations. Light-grown cells were washed and suspended in distilled water as described by Schuldiner and Ohad[7]. Open cells were prepared from the same culture in Wh medium at 700 lb/inch^2 and resuspended in 0.1 M KCl with addition of $5 \cdot 10^{-5}$ M pyocyanine. The initial pH was adjusted to pH 6.3. Chlorophyll content of each system was 60 μg. —·—·—, whole cells, first illumination cycle; -------, whole cells, fourth illumination cycle; ———, open-cell preparation, first illumination cycle; ↑, light on; ↓, light off.

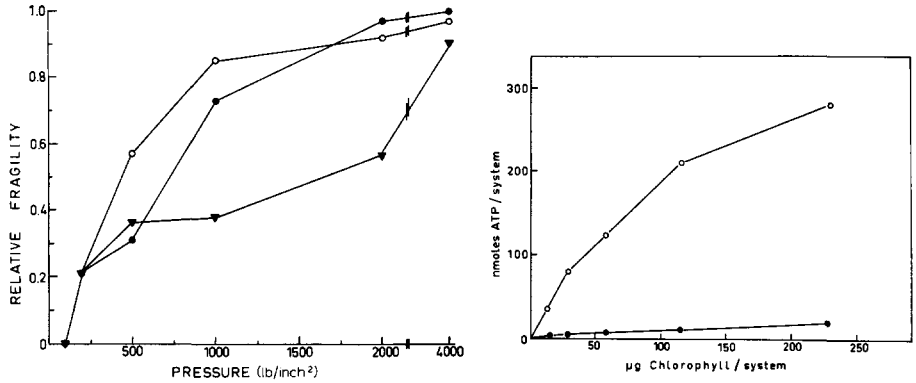

Fig. 3. Osmotic fragility of cells passed through a French Press at different pressures in the presence of mannitol, Dextran and Ficoll. Light-grown cells containing 30 μg chlorophyll per 10^7 cells were suspended in a medium of the same composition as Wp medium or Wp medium from which the Dextran and Ficoll were omitted and mannitol concentration was 0.25 M or 0.50 M. After pressuration, the cells were diluted 1:50 in distilled water and the relative fragility of lysed cells counted in a hemocytometer. A relative fragility of 1.0 indicates 100% lysis. ○—○, 0.250 M mannitol; ●—●, 0.50 M mannitol; ▼—▼, 0.50 M mannitol with addition of 5% Dextran and 2.5% Ficoll.

Fig. 4. Phosphorylation activity of an open-cell preparation as a function of chlorophyll concentration in the assay systems. Open cells were prepared from light-grown cells. Samples containing different amounts of chlorophyll were added to the reaction mixture using pyocyamine ($5 \cdot 10^{-5}$ M) as cofactor. The gas phase was argon. The reaction was started immediately after addition of the sample without pre-incubation. ○ and ●, phosphorylation in the light and dark, respectively.

uptake in the presence of 100 mM KCl. However, a proton uptake characteristic for chloroplast was obtained if the osmolarity of the pressuration medium was raised by addition of 0.5 M mannitol. For measurement of the efficiency of pressuration use was made of the observation that when pressuration was carried out in the presence of mannitol the cells preserved their integrity and high refractility typical of plasmolyzed cells as seen in the phase-contrast microscope. However, when pressurized cells were transferred to the hypotonic medium used for the assay of proton pump activity they became swollen, lost their refractility and lysed. Addition of Dextran and Ficoll to the pressuration medium protected the pressurized cells against osmotic lysis. The protective effect of mannitol, Dextran and Ficoll against osmotic lysis of cells pressurized at different pressures is shown in Fig. 3. It was found that cells pressurized at 500–1000 lb/inch^2 in 0.5 M mannitol with addition of Dextran and Ficoll exhibited easily measurable light-dependent pH rise typical of chloroplast preparations (Fig. 2) as well as photophosphorylation. When the osmolarity of the pressuration medium was due to addition of mannitol rather than sucrose and phosphate was substituted for tricine, a significant increase in the phosphorylation rate could be obtained. The activity was highest at Dextran, Ficoll and $MgCl_2$ concentrations as used in the Wp medium. Preparations obtained by pressuration of cells in the Wp or Wh medium at hydrostatic pressures of 700 lb/inch^2 will be further referred to as open-cell preparations.

Characterization of photophosphorylation activity of open-cell preparations

Open-cell preparations obtained from light as well as dark-grown y-1 cells exhibited phosphorylation activity in the absence of light. The rate of the dark reactions varied in different cell preparations between 0.4–0.6 μmole ATP per 10^7 cells per h, in both dark- or light-grown cells. The activity was proportional to the cell concentration at least up to $5 \cdot 10^7$ cells per ml of the incubation system (Fig. 4) and was enhanced by addition of ascorbate in the presence of PMS (Fig. 5)

TABLE I

EFFECT OF ASCORBATE ON THE INHIBITION BY DCMU OF PHOTOPHOSPHORYLATION WITH PYOCYANINE OR PMS AS COFACTORS

Open cells were prepared in Wp medium from light-grown cells as described in Materials and Methods. Samples containing 17 μg chlorophyll in 0.1 ml were added to the reaction mixture which was preincubated in the dark for 3 min and then illuminated for 3 min at 25 °C. The concentration of cofactors and DCMU were as follows: PMS and pyocyanine, $3 \cdot 10^{-5}$ M; ascorbate, $1 \cdot 10^{-2}$ M; and DCMU, $5 \cdot 10^{-6}$ M.

Cofactor	*ATP (μmoles/mg chlorophyll/h)*	*% Inhibition by DCMU*
Pyocyanine	44	—
Pyocyanine + DCMU	7	84
Pyocyanine + ascorbate	47	—
Pyocyanine + ascorbate + DCMU	4	91
PMS	64	—
PMS + DCMU	20	68
PMS + ascorbate	247	—
PMS + ascorbate + DCMU	203	17

and inhibited by high concentration of ferricyanide. The dark reaction with PMS and ascorbate as cofactor was only about 50 % inhibited by KCN (*cf.* ref. 22) and only about 10 % with ferricyanide as cofactor. In absence of hexokinase and glucose in the incubation medium the open cells preparation also exhibited a low light independent ATP–P_i exchange. Most of the dark phosphorylation activities can thus be ascribed to the presence of mitochondria in the cell preparations. The light-dependent phosphorylation of preparations obtained from light-grown cells was about 50–100 μmoles ATP per mg chlorophyll per h with pyocyanine, ferricyanide and diquat and about 150–200 μmoles ATP per mg chlorophyll per h with ascorbate and PMS as cofactors. Pyocyanine-dependent photophosphorylation either in the presence or absence of ascorbate as well as photophosphorylation with ferricyanide or diquat were inhibited by DCMU (Table I). The activity with PMS as cofactor was also DCMU sensitive. However, addition of ascorbate released the activity from DCMU inhibition (Table I). The light-dependent reaction with PMS showed an optimal activity at a concentration of 20 mM ascorbate (Fig. 5). Both activities of Systems I and II in preparations obtained from light grown cells were saturated by light at intensities below $5 \cdot 10^5$ erg·cm^{-2}·sec^{-1} which was the usual light intensity used in all experiments. As shown in Fig. 6, open-cell preparations from both green

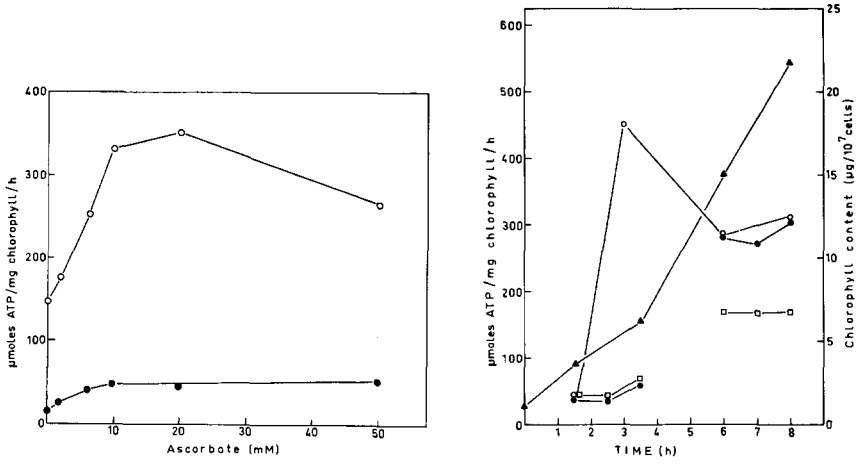

Fig. 5. Effect of ascorbate concentration on photophosphorylation activity with PMS as cofactor. Same conditions as in Fig. 4. The gas phase was air. Samples containing 28 μg chlorophyll in 0.1 ml were added to the reaction mixture. PMS concentration was $3 \cdot 10^{-5}$ M and the samples were preincubated in the dark for 5 min and then illuminated for 4 min. ○ and ●, phosphorylation in the light and dark, respectively.

Fig. 6. Stability of photophosphorylation activity of open-cell preparations during storage under different conditions. Samples of greening cells were taken after 1.5 h, 3 h, 6 h, and 8 h of greening and the photophosphorylation activity of open-cell preparations measured immediately as described in Materials and Methods (○—○). Open cells prepared from samples taken at 1.5 h and 6 h were kept in Wp medium (●—●) or diluted 1:20 in the same medium as used for measurement of phosphorylation activity from which ADP, AMP, ^{32}P and hexokinase were omitted (□—□). The photophosphorylation activity of these samples was measured after storage in ice for 1 h and 2 h. Prior to the measurement, the diluted samples were concentrated by centrifugation and resuspension in a small volume of the storage medium. Photophosphorylation was measured with PMS ($5 \cdot 10^{-5}$ M) with addition of ascorbate ($2.5 \cdot 10^{-2}$ M) in the presence of DCMU ($1 \cdot 10^{-5}$ M) using between 20 and 50 μg chlorophyll for each measurement. ▲—▲ chlorophyll content.

and dark-grown cells were stable after washing and prolonged incubation in hypertonic as well as hypotonic media. The lower activity per chlorophyll unit of open cells stored in hypotonic medium after dilution shown in Fig. 5 might be only apparent since part of the cells might have been lost during the last centrifugation stage and thus the activity measured was divided by a higher calculated value for the chlorophyll content.

Electron microscopical examination of thin sections of fixed material or of surface fracture replicas obtained from open cell preparations revealed that the general structure of the chloroplast was well preserved while the plasma membrane

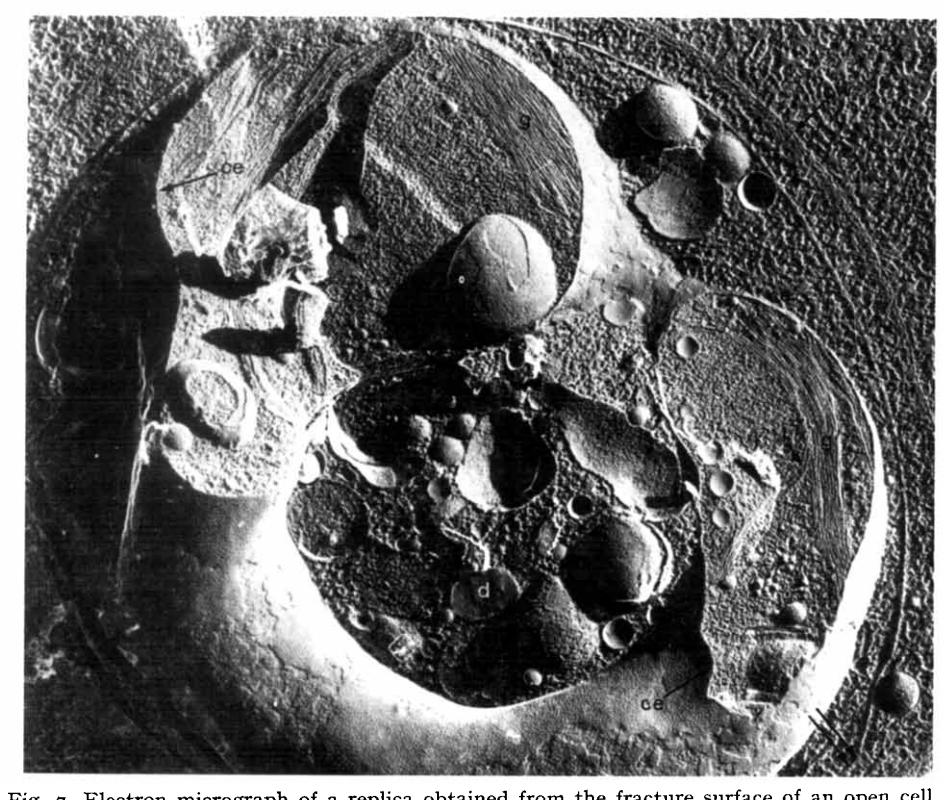

Fig. 7. Electron micrograph of a replica obtained from the fracture surface of an open cell by the freeze etching technique. The open-cell preparation was obtained by pressuration in Wp medium at 700 lb/inch^2 of greening cells exposed to the light for 8 h and containing 16 μg chlorophyll per 10^7 cells. Notice the preservation of the chloroplast cup-shaped form. The outer chloroplast envelope (ce) shows numerous closely packed particles on its fractured surface. In several places (arrows) the envelope has been superficially removed and discloses the matrix of the chloroplast underneath. A deep fracture breaks through the distal chloroplast lobes exposing the dense chloroplast matrix and arrays of photosynthetic lamellae (l) organized into grana (g). The surface of a thylakoid is seen at t. The cup formed by the chloroplast encloses numerous vesicles probably originating from a dictyosome (d). Notice the coarse granularity of the material found within the matrix of the cytoplasm which has the same appearance as the ice surrounding the cell. This might indicate penetration of the medium in which the cells have been suspended within the cytoplasmic space. The plasma membrane (pm) is open (arrows) and remote from the cell wall (cw). Micrographs obtained from sections prepared from similar open-cell preparations after fixation in osmium and Epon embedding (see Materials and Methods) show also discontinuities in the plasma membrane of the cells.

seemed to be broken in places and the cytoplasmic matrix showed vacuoles and numerous vesicles probably derived from the dictyosomes or mitochondria.

Changes in photosynthetic activities during greening of y-1 cells as measured with the open-cell preparations

Since the open-cell preparations exhibited stable and reproducible activities when obtained from both light as well as dark-grown cells, it was possible to use them in order to measure photosynthetic activity of the y-1 cells at different stages of the greening.

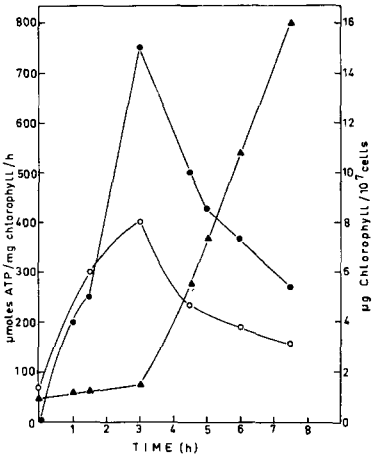

Fig. 8. Development of photophosphorylation activity during greening of *C. reinhardi* y-1 cells. Dark-grown cells were washed by centrifugation, resuspended in fresh growth medium and exposed to light as described in Materials and Methods. At different times, samples were taken for preparation of open cells in Wp medium at 700 lb/inch². Photophosphorylation was measured with ferricyanide ($2 \cdot 10^{-3}$ M) (○—○), or PMS ($5 \cdot 10^{-5}$ M) and ascorbate ($2.5 \cdot 10^{-2}$ M) with addition of 10^{-5} M DCMU (●—●); ▲—▲, chlorophyll content.

The photophosphorylation rate and proton pump activity of cells grown for five generations in the dark was negligible. When such cells were exposed to the light, a rapid increase in the photophosphorylation and proton pump activity ensued. This rise in activity occurred before the phase of constant rate chlorophyll synthesis was attained (Figs 8 and 9) while only small amounts of chlorophyll were synthesized. The specific activity (µmoles ATP per mg chlorophyll per h) of both photosystems reached a peak after about 2–3 h of illumination which was 3–4 times higher than the activity measured in preparations obtained from light-grown cells. As chlorophyll continued to be synthesized and accumulated the specific activity of photophosphorylation decreased to the level usually obtained with light-grown cells (Fig. 8). Addition of chloramphenicol to the medium at the onset of illumination (150 µg/ml) prevented only partially the synthesis and accumulation of chlorophyll while inhibiting completely the development of the photosynthetic activities (Figs 9 and 10). When chloramphenicol was removed from the greening medium both photophosphorylation and proton pump activity recovered. The reactivation of photosynthetic activities can occur in the dark

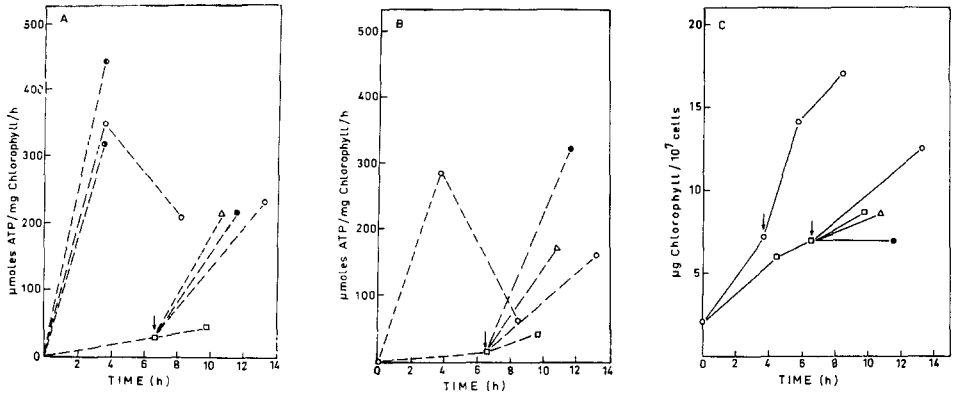

Fig. 9. Effect of alternate inhibition of protein synthesis within the chloroplast by chloramphenicol and within the cytoplasm by cycloheximide on the development of photophosphorylation activity during greening of dark-grown y-1 cells. Dark-grown cells were washed and resuspended at a final concentration of 10^7 cells per ml in fresh growth medium with or without the addition of chloramphenicol (150 µg/ml). The cell suspensions were exposed to the light as described in Materials and Methods and samples were taken at different times for measurement of photophosphorylation (A and B) and chlorophyll content (C). At the times indicated by arrows the cells were washed by centrifugation, suspended in fresh growth medium and the greening was continued as above. The cells which initially were greened in the presence of chloramphenicol were divided into four parts; one part was further incubated in the dark while the other three were exposed to the light with or without addition of chloramphenicol (150 µg/ml) or cycloheximide (0.5 µg/ml). Cells incubated in absence of inhibitors: activity measured with (A) diquat (◑---◑), pyocyanine (◐---◐), PMS–ascorbate (○---○) and (B) ferricyanide (○—○); cells incubated in medium containing chloramphenicol: activity measured with (A) PMS–ascorbate (□---□) and (B) ferricyanide (□---□); cells transferred from chloramphenicol to in fresh growth medium containing cycloheximide and incubated in the light: activity measured with (A) PMS–ascorbate (△---△) and (B) ferricyanide (△---△); transferred from chloramphenicol to fresh growth medium and incubated in the dark: activity measured with (A) PMS–ascorbate (●—●) and (B) ferricyanide (●—●). Photophosphorylation was measured with 0.1 ml samples of open cells containing between 5 and 19 µg chlorophyll prepared in Wp medium at 700 lb/inch². Cofactor concentration was as follows: pyocyanine, $5 \cdot 10^{-5}$ M; diquat, 10^{-5} M; PMS, $5 \cdot 10^{-5}$ M with addition of $1.5 \cdot 10^{-2}$ M ascorbate; ferricyanide, 10^{-4} M; DCMU did not inhibit photophosphorylation with PMS in the presence of ascorbate.

and it is not inhibited by addition of cycloheximide in the light indicating that synthesis of chlorophyll and cytoplasmic proteins are not required for the process of reactivation (Figs 9 and 10).

DISCUSSION

Unicellular green algae have been widely used for the investigation of photosynthetic activity under a variety of growth or assay conditions. Most of the work was done so far with whole cells or homogenates due to the fact that isolation of intact chloroplast from these organisms encounters great difficulties. The need for the quantitative isolation of pure chloroplasts was circumvented in cases where activities could be measured in whole cells or chloroplast fragments[1,2,7,21]. Chloroplast preparations have been obtained from algae whose cell wall can be easily broken under mild conditions such as *Euglena*[23], *Phormidium*[24] or *Bumilleriopsis*[25]. Preparations of chloroplast have been reported also for *Chlamydomonas*[25] but the low yields and the fact that the procedure requires time-consuming sedimentation

on density gradients makes the method unsuitable for use when kinetic analysis of chloroplast development is attempted. In addition, such preparations were not reported to exhibit photophosphorylation or proton pump activities. In order to analyze the process of chloroplast membrane formation, it is not sufficient to gain information on the electron transfer system only, since at least part of the phenomenon associated with the assembly of membrane components into functionally

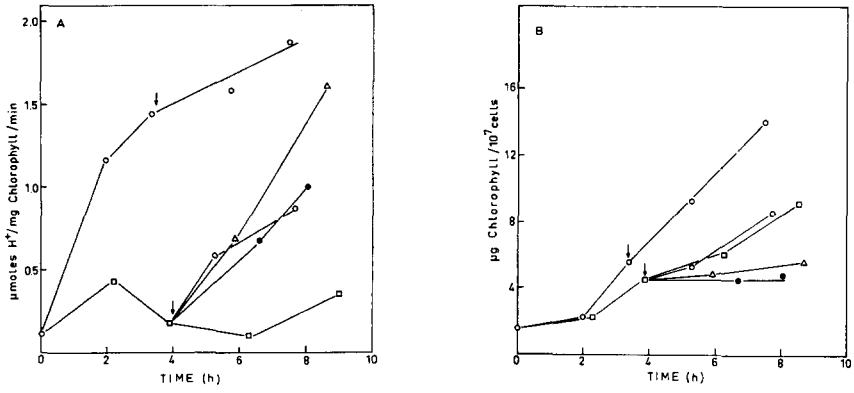

Fig. 10. Effect of alternate inhibition of protein synthesis within the chloroplast by chloramphenicol and within the cytoplasm by cycloheximide on the development of proton pump activity during greening of dark-grown cells. Same experimental conditions as in Fig. 9. Open cells were prepared in Wp medium at 700 lb/inch^2 and the proton pump activity was measured as described in Materials and Methods using samples containing 50–150 μg chlorophyll. Activity was linear with the concentration of open cells added to the assay system. Measurements were done in duplicate. Proton pump activity. (B) Chlorophyll content. Cells incubated in the light without (○—○) or with (□—□) addition of chloramphenicol; cells incubated in the light with addition of cycloheximide (△—△); cells incubated in the dark (●—●). Chloramphenicol and cycloheximide concentrations were 150 and 0.5 μg/ml, respectively. Arrows indicate the time at which the cells were washed by centrifugation and resuspended in fresh growth medium without or with addition of protein synthesis inhibitors.

normal membranes might be expressed at the level of energy coupling reactions. Thus, it was felt that analysis of photophosphorylation and proton pump activity during the formation of chloroplast membranes will contribute to our knowledge on the process of membrane assembly and degree of organization during their growth. Measurement of photophosphorylation activity was reported for *Chlamydomonas* using preparations obtained by grinding a cell paste with sand[21] or passing a cell suspension at high hydrostatic pressure through a French Press[7]. Both methods have inconveniences resulting from the need for great cell quantities in the first case and the instability and variability of the activity of the homogenate in the second case.

The results obtained in this work demonstrate that the use of hypertonic solution renders the cell membrane vulnerable to low shearing forces. The addition of Dextran and Ficoll have a protective effect resulting in a better preservation of the chloroplast integrity. This is demonstrated at the structural level, as seen in the electron microscope, by the preservation of the general shape of the chloroplast including the envelope and the presence of parallel arrays of thylakoids often still fused into grana, for both, cells having low and high chlorophyll content. However,

these preparations were rendered permeable to substrates and cofactors as indicated by the ADP-dependent incorporation of ^{32}P into ATP and by the instant rise in the pH of the medium upon exposure of open cell preparations to illumination in the presence of 0.1 M KCl. The homogeneity and reproducibility of the preparation as well as the stability of the photosynthetic activity exhibited by preparations obtained from greening cells irrespective of their chlorophyll content and degree of chloroplast development made the open cell preparation suitable for the measurement of changes in the photosynthetic activity during greening. Thus, the fact that photophosphorylation and proton pump activity of dark-grown cells was barely measurable, can be regarded as an intrinsic property of the photosynthetic membrane remnants after division of the cells in the dark. In the same way, the dramatic increase in the activity over the first 2–3 h of illumination indicates that a reorganization of the membrane remnants occurs before a significant increase in the chlorophyll and total membrane content of the chloroplast takes place. These results validate similar conclusions based on measurements of cytochrome *f* photooxidation and light-dependent pH rise activity obtained previously with homogenates or intact cells[7] and are in agreement with the observation that membrane remnants after growth in the dark are highly fluorescent, the fluorescence decreasing sharply during the first 2–3 h of illumination[27]. Preliminary data show a parallelism between the rise in photosynthetic electron transfer activity and photophosphorylation as measured with ferricyanide in the early phase of greening indicating that the degree of coupling between the two activities is not affected during the greening. Thus, it seems that the observed changes in the specific activity (ATP or H^+ per mg chlorophyll per unit time) during the greening are due to changes in the degree of organization of other photosynthetic chain components.

The fact that changes in the activity of existing membranes occur during the initial phase of the greening and that membranes can be formed in an incomplete, or inactive form and be repaired afterwards indicate that the assembly of the membrane components can proceed stepwise within certain limits. This conclusion is further supported by results obtained in our Laboratory showing gradual changes in the buoyant density of newly formed chloroplast membranes, *i.e. C. reinhardii* y-1 cells greening in the absence or presence of chloramphenicol[27]. The lack of activity of membranes synthesized in the presence of chloramphenicol and the concomitant reactivation of both electron transfer and energy transduction activity after removal of the inhibitor demonstrate that some of the components responsible for the reactivation process are synthesized in the chloroplast and can be added to already present membranes. Whether the reactivation is linked with the synthesis and addition of a single key component or several components cannot be decided at the present time.

REFERENCES

1 I. Ohad, P. Siekevitz and G. E. Palade, *J. Cell Biol.*, 35 (1967) 521.
2 I. Ohad, P. Siekevitz and G. E. Palade, *J. Cell Biol.*, 35 (1967) 553.
3 I. Goldberg and I. Ohad, *J. Cell Biol.*, 44 (1970) 563.
4 G. Eytan and I. Ohad, *J. Biol. Chem.*, 245 (1970) 4297.
5 J. K. Hoober, *J. Biol. Chem.*, 245 (1970) 4327.
6 B. De-Petrocellis, P. Siekevitz and G. E. Palade, *J. Cell Biol.*, 44 (1970) 618.
7 S. Schuldiner and I. Ohad, *Biochim. Biophys. Acta*, 180 (1969) 165.

8 G. Eytan and I. Ohad, *J. Biol. Chem.*, 247 (1972) 122.
9 I. Goldberg and I. Ohad, *Israel J. Chem.*, 8 (1970) 127p.
10 A. Atkins and D. Graham, *Biochim. Biophys. Acta*, 226 (1971) 481.
11 J. Neumann and R. P. Levine, *Plant Physiol.*, 47 (1971) 700.
12 R. Sager and M. R. Ishida, *Proc. Natl. Acad. Sci. U.S.*, 50 (1963) 725.
13 M. Avron, *Anal. Biochem.*, 2 (1961) 535.
14 G. A. Bray, *Anal. Biochem.*, 1 (1960) 279.
15 M. Avron and N. Shavit, *Anal. Biochem.*, 6 (1963) 549.
16 D. I. Arnon, *Plant Physiol.*, 24 (1949) 1.
17 J. H. Luft, *J. Biophys. Biochem. Cytol.*, 9 (1967) 409.
18 E. S. Reynolds, *J. Cell Biol.*, 17 (1963) 208.
19 H. Moor, K. Muhlethaler, H. Waldner and A. Frey-Wyssling, *J. Biophys. Biochem. Cytol.*, 10 (1967) 1.
20 R. F. Chen, *J. Biol. Chem.*, 242 (1967) 173.
21 D. S. Gorman and R. P. Levine, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 1665.
22 B. T. Storey, *Plant Physiol.*, 45 (1970) 455.
23 B. A. Melandri, A. Baccarini-Melandri and A. SanPietro, *Arch. Biochem. Biophys.*, 138 (1970) 598.
24 J. Biggins, *Plant Physiol.*, 42 (1967) 1447.
25 P. Z. Boger, *Pflanzen Physiol.*, 61 (1969) 85.
26 S. J. Surzycki, *Proc. Natl. Acad. Sci. U.S.*, 63 (1969) 1327.
27 G. Eytan and I. Ohad, *J. Biol. Chem.*, 247 (1972) 112.